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GLUCOSAMINE AND CHONDROITIN SULFATE INFLUENCE CATABOLIC RESPONSES TO RECOMBINANT EQUINE INTERLEUKIN-1 IN EQUINE CHONDROCYTES.

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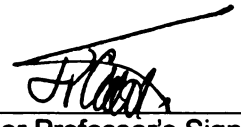

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By

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ABSTRACT

GLUCOSAMINE AND CHONDROITIN SULFATE INFLUENCE CATABOLIC RESPONSES TO RECOMBINANT EQUINE INTERLEUKIN-1 IN EQUINE CHONDROCYTES

By

Kirsten Maree Neil

Joint disease, and in particular osteoarthritis, is a significant cause of lameness and poor performance in horses. Currently, a number of pharmacological agents are available for the treatment of osteoarthritis, including glucosamine and chondroitin sulfate. Although a number of *in vitro* studies have been conducted using these compounds, concentrations used have exceeded those that are obtained by oral administration. A subsaturating dose of recombinant equine interleukin-1 (500 ρg/ml) was established that resulted in approximately half maximal induction of expression of mediators of osteoarthritis. The effect of glucosamine and chondroitin sulfate on gene expression of these mediators of osteoarthritis was investigated using equine chondrocytes in pellet culture stimulated with recombinant equine interleukin-1. Glucosamine at 10.0 ρg/ml significantly reduced mRNA expression of matrix metalloproteinase 13, aggrecanase 1 and c-Jun-N-terminal kinase. Further, a trend for reduction in interleukin-1 induced expression of inducible notric oxide synthase and cyclo-oxygenase 2 was observed. Chondroitin sulfate had no effect at the concentrations tested.

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KEY TO ABBREVIATIONS

ADAMTS	A disintegrin-like and metalloprotease (reprolysin type) with thrombosponding type 1 motifs
ADME	Absorption, Distribution, metabolism, excretion
AP-1	Activator Protein-1
AUC	Area under curve
BID	Twice daily
Cmax	Maximum plasma concentration
COX	cyclo-oxygenase
CS	Chondroitin sulfate
DJD	Degenerative Joint Disease
DS	Dermatan sulfate
ECM	Extracellular Matrix
ECSIT	evolutionary conserved signaling intermediate in Toll pathways
ERK	Extracellular signal-related kinase
GAG	Glycosaminoglycan
GlcAT-1	UDP-glucuronyl transferase 1
GHCl	Glucosamine hydrochloride
GLN	Glucosamine
GS	Glucosamine sulfate
HA	Hyaluronic acid
HPLC	High Performance Liquid Chromatography
IAP	Intra-articular pressure
ICE	interleukin converting enzyme
IGD	Interglobular domain
IκB	Inhibitor of κB
IKK	Inhibitor of κB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL1Ra	Interleukin-1 receptor anatagonist
IL1RacP	Interleukin-1 receptor accessory protein
IM	Intramuscular
IRAK	IL-1 receptor associated kinase
IV	Intravenous
JNK	c-Jun N Terminal Kinase
KS	Keratan sulfate
LIF	Leukemia inhibitory factor
LMWCS	Low molecular weight chondroitin sulfate
LOQ	Level of Quantification
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MAP2K	Mitogen activated protein kinase kinase
MAP3K	Mitogen activated protein kinase kinase kinase
MIC	Minimum inhibitory concentration

MMP	Matrix metalloproteinase
MyD88	Myeloid differentiation protein
NAG	N-acetyl-glucosamine
NFκB	Nuclear factor kappa B
NIK	NFκB inducing kinase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OCD	Osteochondrosis
PO	oral
PG	Proteoglycan
PGE_2	Prostaglandin E_2
PSGAG	Polysulfated glycosaminoglycan
reIL-1	recombinant equine interleukin-1
rhIL-1	recombinant human interleukin-1
SID	Once daily
Spl	Specificity Protein
TAB	Transforming-growth-factor-β-activated kinase binding protein
TACE	TNFα converting enzyme
TAK1	Transforming-growth-factor-β-activated kinase 1
TID	Three times daily
TIMP	Tissue inhibitor of metalloproteinase
TIR	Toll-IL-1R domain
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor receptor
Tollip	Toll interacting protein
TRAF	Tumor necrosis factor receptor associated factor
UDP	Uridine diphosphate
WOMAC	Western Ontario and McMaster University Osteoarthritis index

INTRODUCTION

Joint disease, and in particular osteoarthritis (OA), is a significant cause of lameness and loss of function in horses. In this species, OA occurs in previously normal joints that have been damaged by one of a variety of insults; including trauma, septic arthritis, and developmental disorders such as osteochondrosis (OCD) and angular limb deformities. In racing horses, OA is frequently a consequence of sudden trauma or chronic loading (Pool *et al* 1990).

The hallmark of OA is the progressive and permanent degeneration of articular cartilage, accompanied by changes in the underlying subchondral bone and soft tissue structures of joints. Chondrocytes, the cellular component of articular cartilage, are responsible for the synthesis and maintenance of the extracellular matrix (ECM) in which they are embedded. Homeostasis of cartilage involves an equilibrium between synthesis and degradation of ECM components, including collagen and proteoglycans (PG). Degradative enzymes, such as matrix metalloproteinases (MMPs), play a central role in the degradation of articular cartilage. MMPs are present in normal cartilage, their expression in part controlled by the relatively higher concentration of their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).

Other biological factors have also been implicated in the pathogenesis of OA, including nitric oxide (NO) and prostaglandins. Stimulation of their release, and that of degradative enzymes, is mediated by the production of cytokines, in particular interleukin-1 (IL-1).

Cytokines are produced by synoviocytes early in the disease process; later chondrocytes are an important source of these mediators (Martel-Pelletier 1998).

Currently, a number of pharmacological agents are available for the treatment of OA. Among them are glucosamine and chondroitin sulfate, so called nutraceuticals or slow acting disease modifying agents. These nutraceuticals have been the focus of a number of *in vitro* studies. Possible beneficial effects of these compounds include the reduction of proteoglycan and collagen degradation, and the inhibition of MMP, NO and prostaglandin synthesis.

The purpose of the study presented here was to elucidate the influence of glucosamine and chondroitin sulfate on catabolic responses of equine cartilage to recombinant equine IL-1β (reIL-1β) *in vitro*. The hypothesis tested was that preventative use of glucosamine and chondroitin sulfate regulates the gene expression of proteins involved in chondrocyte catabolism. The specific aims were to 1) determine the ideal dose of reIL-1β to result in half-maximal stimulation of the genes of interest; 2) determine if the reIL-1β induction of some or all of the genes of interest could be reduced/prevented by glucosamine or chondroitin sulfate; and 3) determine the lowest effective dose of these compounds that could reduce/inhibit reIL-1β induction of susceptible genes. Chapter 1 represents a review of current literature, and chapters 2-4 results of experiments.

CHAPTER 1: LITERATURE REVIEW

Synovial Joint:

Structure – Function Relationships

Diarthrodial (synovial) joints are designed to facilitate smooth, frictionless motion and to distribute and transfer weight-bearing loads, thereby absorbing concussion. Ligaments, joint capsule, osteocartilaginous contour and periarticular soft tissue structures restrict translational and rotational planes of motion. Mechanisms of structural support and stability vary with joint location. Muscle masses surrounding proximal limb joints control rotational motion and contribute to mechanical stability, whereas distal limb joints are more reliant on ligaments, joint capsule integrity and bony contour (Todhunter 1996). Menisci, located in the femorotibial and temporomandibular joints, contribute to rotational stability and disperse the load transmitted by adjacent articular cartilage.

Synovial Fluid and Synovium

The synovial membrane lining the joint capsule is modified mesenchyme, composed of an intimal layer overlying the connective tissues of the lamina propria. The intima is incomplete, 1-4 synoviocytes thick and lacks a basement membrane. Synoviocytes have both secretory and phagocytic functions, and are responsible for the synthesis of a number of products including hyaluronan and the glycoprotein lubricin.

Synovial fluid is an ultrafiltrate of plasma. Solute exchange is facilitated by the absence of a basement membrane and the close proximity of capillaries to the intimal surface.

Normal equine synovial fluid contains less than 500 nucleated cells/μl, with mononuclear cells predominant. Starling's forces govern fluid exchange; however, long-term balance is also dependent upon motion driven lymphatic flow and joint angle, with intermediate angles favoring filtration. Small molecules, less than 10 kDa, move by diffusion, which, for glucose is facilitated. Nutrition of articular cartilage is derived from, and ultimately dependent upon, the synovial fluid.

Hyaluronan, a non-sulfated glycosaminoglycan (GAG), is present in high concentration in equine synovial fluid (0.5mg/ml) (Saari 1989), and imparts viscosity to the fluid. Hyaluronan synthase catalyzes its synthesis, with hyaluronan chains elongated by the alternate addition of uridine diphosphate (UDP) hyaluronan to UDP-N-acetylglucosamine and UDP-glucuronic acid. Synovial fluid can support transient shear stresses and has an energy absorbance capacity attributable to its viscosity. Lubricin adhers to the surfaces of the synovial membrane and articular cartilage, reducing synovial fluid surface tension - a phenomenon known as boundary lubrication (Palmer *et al* 1996).

Articular Cartilage

Articular cartilage is a highly specialized tissue maintained by a relatively sparse population of chondrocytes. Cartilage has a high water content, approximately 70% by weight in mature cartilage and 80% in neonatal cartilage. On a dry weight basis, articular cartilage is composed of approximately 50% collagen and 35% proteoglycans, with the remainder comprised principally of glycoproteins. Articular cartilage is heterogeneous,

with variation between weight bearing and non-weight bearing areas, between joints and between age groups.

Histologically, cartilage may be defined into 4 zones (Mankin *et al* 1997). The delineation between unmineralized and calcified cartilage is referred to as the tidemark. The highest cell density occurs superficially (zone 1), with chondrocytes oriented parallel to the surface. Cells are larger and rounded in the transitional zone (zone 2), with larger perpendicularly oriented cells in the radiate zone (zone 3). Zone 4 consists of calcified cartilage, with its lower boundary constituting the cement line that is formed during endochondral ossification. Cell arrangement is complementary to the organization of collagen fibrils. In the superficial zone, fibrils have a tangential orientation; in the middle zone a three dimensional network is apparent with some fibrils perpendicular to the surface, while larger perpendicularly orientated fibrils are present in zones 3 and 4. Hydroxyapatite crystals impregnate collagen fibrils in the calcified zone. Fibril orientation contributes to the functional characteristics of each layer, specifically providing resistance to tension in superficial layers and to compressive loading in middle and deep zones. Proteoglycan content also differs between zones, the concentration increasing with increasing depth (Aydelotte *et al* 1988a).

Chondrocytes are responsible for the synthesis and maintenance of the ECM, including proteoglycans and collagens. Proteoglycan turnover is fairly rapid, whereas collagen turnover is minimal. Both biological and mechanical factors influence chondrocyte

metabolism. Normally a balance exists between ECM synthesis and degradation, and it is the disruption of this equilibrium that is a key feature of OA.

The collagen of articular cartilage is predominantly Type II collagen (85-90%), with small amounts of Types VI, IX, XI, XII and XIV. Type II collagen is arranged in fibrils and is responsible for the tensile strength of articular cartilage. Type XI collagen is arranged in the center of these fibrils with the smaller Type IX collagen, containing a glycosaminoglycan side chain of chondroitin sulfate, located on the surface of the fibril, extending into the extracellular matrix. Type VI collagen has a pericellular location. Type IX collagen provides an interface between the type II collagen fibrils and the proteoglycan domain, while type XI collagen plays a role in organization of the ECM by regulating type II collagen.

Type II collagen is a homodimer of three identical amino acid chains arranged in a triple helix [$(\alpha 1)_3$]. These chains consist of glycine-X-Y repeats, with X/Y usually proline or hydroxyproline. Hydrogen bonds stabilize the triple helix, with intramolecular and intermolecular cross-linking responsible for the high tensile strength of type II collagen (Schmidt 1987). Type II collagen has a higher proportion of hydroxylysine residues and more glycosylation than type I collagen [$(\alpha 1)_2$,$(\alpha 2)$] (Todhunter *et al* 1994b). Type II collagen is produced by chondrocytes, with post-translational modification including hydroxylation of proline and lysine residues. The enzymes responsible for this catalytic process are maintained in their active state by ascorbic acid, with an additional requirement for ferrous ion and oxygen. Procollagen is secreted into the ECM where it is

enzymatically processed by the cleavage of N- and C- terminal propeptides into mature collagen molecules which then self-assemble into fibrils and are cross linked. In addition, lysyl oxidase mediates cross-linking of type II collagen and type IX collagen (van der Rest *et al* 1988). During growth and development, marked degradation and resynthesis occurs, however collagen turnover in adults is limited (Caron *et al* 1999).

Proteoglycans consist of a central protein core to which are attached one or more GAG side chains. As GAGs are negatively charged, they attract water (Poole 1986) and thereby impart resistance to compression and load dissipation, properties characteristic of articular cartilage. Aggrecan is the largest and most predominant proteoglycan of articular cartilage. The GAGs of aggrecan are principally chondroitin sulfate (CS) and keratan sulfate (KS). These GAGs are attached to the core protein during post-translational modification. Once synthesized by the chondrocyte, proteoglycan monomers are secreted into the ECM where they aggregate with hyaluronan. A separate globular protein termed link protein assists this binding. The N-terminal region of the protein core contains two globular domains, G1 and G2, separated by an interglobular domain. G1 is the site at which proteoglycan attaches to hyaluronic acid (HA), thereby anchoring the aggrecan molecule. GAG side chains are attached between G2 and G3, with a KS rich portion immediately adjacent to the G2 domain, while CS is attached throughout the rest of the region.

Non-aggregating small proteoglycans are also present in articular cartilage. Decorin, present in superficial layers (Poole *et al* 1986), consists of a core protein substituted with

a single GAG side chain that may be either CS or dermatan sulfate (DS). Decorin, through interaction with collagen, may regulate fibrillogenesis of Type II collagen (Scott 1988). Biglycan consists of two GAG chains (CS or DS) attached to a protein core and has structural similarities to decorin, but is unable to bind collagen. Fibromodulin is variably glycosylated in its central domain with KS, and, like decorin, can bind to collagen fibrils and inhibit fibril formation.

GAGs are composed of linear chains of repeating disaccharide units of hexosamine (glucosamine or galactosamine) alternating with another residue of glucuronic acid, iduronic acid or galactose. GAG proportions vary with the type and age of cartilage (Bayliss *et al* 1999). Chondroitin sulfate consists of repeating disaccharide subunits of glucuronic acid and N-acetylgalactosamine, and keratan sulfate galactose and N-acetylglucosamine. Substitution of a sulfate group can occur at the C4 or C6 position of chondroitin sulfate to form chondroitin-4-sulfate (C-4-S) and chondroitin-6-sulfate (C-6-S) respectively. In immature cartilage, C-4-S predominates.

Topographical (Aydelotte *et al* 1988a,b) and age related (Wells *et al* 2003) variation exists in chondrocyte morphology, ECM composition, mechanical properties, and ECM synthesis. Biochemical and morphological alterations in articular cartilage are apparent with ageing. This includes reduced chondrocyte numbers (Bobacz *et al* 2003), alterations in proteoglycans (Wells *et al* 2003), C-4-S:C-6-S ratios (Lauder *et al* 2001), aggrecan and link protein (Bayliss *et al* 2000), and increased hyaluronan concentration (Platt *et al* 1998). ECM collagen remains unchanged from maturity (Brama *et al* 1999b). In addition,

chondrocyte mitotic and synthetic activity decreases, and the response to anabolic mechanical stimuli and cytokines decline. In particular, the response of equine cartilage to stimulation with IL-1 declines with increasing age (MacDonald *et al* 1992). Chondrocytes also lose the ability to maintain and repair the ECM coincident with an accumulation of senescent chondrocytes (Martin *et al* 2003). Further, decreasing MMP activity occurs in equine synovial fluid until maturity, after which time MMP activity is not related to age (Brama *et al* 1998d).

Subchondral Bone

Subchondral bone provides structural support for the overlying articular cartilage. It is a mixture of trabecular and osteonic bone, whereas epiphyseal bone beneath it is trabecular. Osteons are arranged parallel to the joint rather than to the long axis of bone (Mankin *et al* 1997). Bone remodeling occurs in response to loading, with subchondral bone structure varying with joint location. The stiffness of subchondral bone enables it to support relatively high loads without substantial deformation, and thereby attenuate force.

Joint Biomechanics

Hyaluronan imparts the property of thixotropy to articular cartilage. With rapid movement (high shear rates), synovial fluid viscosity decreases. In the unloaded joint, opposing articular surfaces are not completely congruent. With loading, deformation of cartilage increases contact area and joint conformity, providing additional stability (Todhunter 1996). Cartilage surrounding weight-bearing areas is subjected to transverse tensile strains, which tends to redistribute fluid away from the compressed region.

Boundary lubrication is important under high load/low speed situations to resist shear stress; however it becomes less effective under high-speed situations where fluid-film lubrication provides the majority of load support (Palmer *et al* 1997).

At low loads, hydrodynamic lubrication contributes to frictionless movement, with fluid translocation from loaded to unloaded areas. A wedge of fluid is created that separates cartilage surfaces, increasing surface area while retaining fluid lubrication between surfaces. The relatively low permeability and high water content of cartilage means that loads are first borne by the fluid compartment with subsequent transmission to cartilage and subchondral bone after fluid has been extruded out of the compressed area into the synovial space. Initially, load application causes instant deformation of cartilage with slower creep deformation until equilibrium is reached between compressive stress within the cartilage and the external stress applied. With axial loading of cartilage, lateral expansion and axial compression occur due to fluid exudation and pore collapse. At low loads, fluid is extruded into the synovial space; however, as the load and therefore contact area increases, less fluid is extruded in an attempt to support the applied load.

Loading and the perceived loading, or intra-articular pressure (IAP), depend on joint position and location within the joint. Normal intrasynovial pressure is sub atmospheric (–2 to –6 cm H_2O). Minor elevations in fluid volume or joint angle increase IAP. When IAP exceeds atmospheric pressure, the joint capsule becomes more compliant, and the synovial membrane and synovial fluid compartments expand in an attempt to dissipate pressure. With further increases in IAP (>20 cm H_2O), the slope of the pressure-volume

curve changes rapidly with decreasing compliance and greater pressure increases with small increases in volume.

Normal synovial membrane is elastic at low to moderate pressures, dissipating energy during the load/unload cycle (hysteresis). Compressive stiffness of articular cartilage is related to proteoglycan content and quality. Because the load born by different areas of cartilage varies, mechanical properties and morphology vary topographically (Murray *et al* 1999). Highly congruent joints generally have thinner cartilage, while lower congruency centers the applied load over a smaller surface area.

The effect of loading on articular cartilage depends on the duration and magnitude of the applied load. Moderate exercise stimulates proteoglycan synthesis (Palmer *et al* 1995a), increases GAG concentration (Brama *et al* 1999a), and enhances mechanical stiffness, whereas strenuous exercise induces deleterious effects. Increasing exercise intensity increases cartilage water content and reduces collagen cross-linking (Brama *et al* 1999c). Subchondral bone remodeling and thickening of the calcified layer of articular cartilage alter joint biomechanical properties (Muller-Gerbl *et al* 1987). The response of articular cartilage to loading depends on both the location within the joint and exercise intensity level. Under load, mean pressure and contact area increase on the dorsal aspect of the radial facet of the equine third carpal bone, corresponding to the area subjected to the most trauma in racing horses (Palmer *et al* 1994). Consequently, the structure and hence function of cartilage overlying the dorsal aspect of the radial facet is altered, with reduced stiffness and proteoglycan content (Palmer *et al* 1995). Dorsal sites in the carpus are

subjected to intermittent high loading, and, with high intensity exercise, collagen and proteoglycan content decrease. Collagen content is greater at dorsal compared to palmar sites, and GAG content higher at palmar sites that are subjected to lower loads (Murray *et al* 2001). Both exercise intensity and cartilage location within a joint (due to different load distributions) influence composition and mechanical properties of articular cartilage, leading to an inherent predisposition for injury and disease. The density of subchondral bone also influences joint biomechanics. Subchondral bone remodeling and thickening of the calcified layer of articular cartilage alter joint biomechanical properties (Muller-Gerbl *et al* 1987).

In disease states, inflammation alters the synovial membrane, articular cartilage, and synovial fluid content and volume. As the severity of clinically evident joint disease increases, biomechanical properties are compromised. For instance, the range of motion in flexion decreases. With disease, stiffness of the soft tissue of the dorsal pouch of metacarpophalangeal joints increases along with IAP, with changes in OA less severe than with synovitis (Strand *et al* 1999).

Osteoarthritis

Joint disease is a well-known and expensive problem for the equine athlete that causes substantial morbidity in the form of lameness in affected animals and may seriously curtail their quality of life in addition to their athletic serviceability. Indeed, OA and the resultant lameness is the major reason that horses become unserviceable for racing and estimates for the cost of this problem to the racing industry are staggering. A number of

studies have reported that lameness is the most significant cause of wastage both in racehorses (Bailey 1998, Jeffcott *et al* 1992, Rossdale *et al* 1983) and the general horse population (Kaneene *et al* 1997), with joint disease figuring prominently.

Initially, four main categories of OA, also referred to as degenerative joint disease (DJD), were described on the basis of joint characteristics and predisposing factors (McIlwraith 1982). This classification was modified to recognize three types of OA in the horse, namely:

1. Associated with synovitis and capsulitis. Common in both high motion joints (eg fetlock in racing Thoroughbreds) and low motion, high load joints (eg distal tarsal and distal interphalangeal joints).
2. DJD secondary to predisposing factors such as osteochondrosis, articular fractures, septic arthritis, subchondral bone injury and disease.
3. Incidental or nonprogressive idiopathic cartilage erosion.

Grossly, articular cartilage degeneration is apparent as fibrillation, erosion and wear lines (McIlwraith 1996), with subchondral sclerosis, osteophyte production and synovitis also features of OA. The progression of OA is divided into three stages. The initial stage involves proteolytic breakdown of ECM, then fibrillation and erosion of the cartilage surface occur, accompanied by the release of ECM degradation products into the synovial fluid. Finally, synovial inflammation occurs subsequent to the production of further degradative enzymes and proinflammatory cytokines (Pelletier *et al* 2000).

The hallmark of OA is the progressive permanent degeneration of articular cartilage. Normally a balance exists between the synthesis and degradation of the ECM. Biochemical and mechanical factors disrupt this balance, primarily by targeting chondrocytes with subsequent degeneration of the ECM (Goldring 2000). In early OA, chondrocytes transiently respond by increasing ECM synthesis in an attempt at repair. However, production of cytokines, inflammatory mediators and matrix degrading enzymes predominate, leading to PG loss, cleavage of type II collagen and degradation of aggrecan, events which are synonymous with OA.

Articular cartilage lesions are accompanied by biochemical alterations including changes in matrix composition, reflecting aberrant behavior of chondrocytes. Normally, a certain amount of mechanical loading is required for joint lubrication and the stimulation of proteoglycan synthesis, with detrimental effects apparent when an optimal load is exceeded. Three types of chondral and osteochondral injuries have been identified based on the type of tissue damage and repair mechanisms (Buckwalter 2002): damage to the joint surface without visible mechanical disruption of the articular surface, but with possible subchondral bone damage; mechanical disruption of the articular surface limited to the articular cartilage; and involvement of articular cartilage and subchondral bone. In most instances, the joint can repair damage that does not disrupt the articular surface provided it is protected from additional injury. Disruption of articular cartilage stimulates chondrocyte activity but rarely is complete repair possible. Damage to subchondral bone stimulates both chondrocyte and bony repair; however, the biological and mechanical properties of the articular surface are rarely restored to normal.

Clinical signs of OA include lameness, joint pain, reduced range of motion, and variable joint effusion. In high motion joints, OA manifests as synovitis, cartilage erosion, and subchondral bone sclerosis; whereas in low motion joints, synovitis is less a feature; however, full thickness cartilage necrosis with little erosion and subchondral bone lysis occurs. Joint predisposition to OA is apparent, with OA of the distal tarsal joints the most frequent cause of hock lameness in horses (McIlwraith 1996).

Synovitis and capsulitis are common initial changes in joints. Damage to joint structures such as articular cartilage can result in the release of cytokines and MMPs that may then influence not only the cartilage itself but also the synovium. Conversely, synoviocytes secrete MMPs (Martel-Pelletier 1986), PGE_2, free radicals and cytokines, with possible subsequent articular cartilage damage. Biological markers can be measured in both serum and synovial fluid to monitor joint metabolism, with CS epitopes (3B3, 7D4) released into synovial fluid in OA. Further, articular cartilage damage and synovial fluid levels of bone specific alkaline phosphatase and keratan sulfate epitopes are highly correlated in OA (Fuller *et al* 2001).

Irreversible degradation of type II collagen is an early critical event in OA. Cytokines such as IL–1 suppress synthesis of collagen type II and IX, and increase collagen type I and III (Goldring *et al* 1988). Thus in OA, collagen synthesis changes from cartilaginous to fibrous type, resulting in altered biomechanics and morphological changes such as joint capsule fibrosis. Denaturation of type II collagen usually originates in superficial

layers of cartilage, with a close correlation between sites of cleavage and the presence of collagenases (Wu *et al* 2002). Further, alterations in type VI collagen occur in OA, with loss of pericellular type VI collagen in superficial zones and net increase in synthesis in middle zones (Hambach *et al* 1998). The breakdown of ECM components is controlled by the activity of a number of degradative enzymes, including MMPs and aggrecanases; however, inflammatory mediators also play an important role in OA.

Degradative Enzymes and their Inhibitors

Matrix metalloproteinases

The matrix metalloproteinases are a group of zinc containing calcium dependent proteinases that are active at neutral pH. These enzymes are involved in normal physiological turnover of the ECM. In OA, MMPs play a central role in degradation of the ECM, in part due to the loss of the normal balance between MMPs and their inhibitors. The concentration of MMPs in OA cartilage exceeds that in normal cartilage (Dean 1991, Okada *et al* 1992), with production topographically related to lesion location (Martel-Pelletier *et al* 1994, Tetlow *et al* 2001).

MMPs are divided into groups based on their substrate specificity, sequence similarity and structure. Currently, four groups have been implicated in ECM degradation, namely the collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs). The collagenases (MMP 1, 8, 13) are unique in their ability to cleave the intact triple helix of collagen type I, II and III. The role of collagenases in maintaining ECM homeostasis is critical and is, in fact, the rate limiting step for fibrillar degradation. MMP 1 and 13

cleave type II collagen between glycine^{775}-isoleucine^{776}, producing a ¾ length TC_A N terminal fragment and a TC_B C terminal fragment. These fragments are then susceptible to further proteolysis by gelatinases (Mengshol *et al* 2002). MMP 13 is more efficient, has a broader substrate specificity and is also capable of cleaving collagen types IV, X and XIV as well as gelatin. MMP 8 is termed neutrophil collagenase; however, chondrocytes can also produce this MMP (Cole *et al* 1995).

The gelatinases (MMP 2, 9) cleave denatured collagen, aggrecan and link protein, and also activate other MMPs such as MMP 3 and 13. Stromelysins (MMP 3, 10, 11) degrade ECM components such as gelatin, aggrecan and fibronectin. The stromelysins exhibit similar substrate specificities; however, of the three, MMP 3 has a more efficient proteolytic capacity (Visse *et al* 2003). MMP 3 can also degrade link protein and denatured type II collagen, and activate latent/inactive MMP 1 and 9 (proMMP 1, 9), with its action on proMMP 1 thought to be critical for generation of the full activity of MMP 1 (Suzuki *et al* 1990). The fourth groups of MMPs, the membrane-type MMPs (MMP 14, 15, 16, 17, 24, 25), are transmembrane proteins capable of digesting ECM proteins such as aggrecan and fibronectin. MMP 14 also has collagenolytic activity, and the MT-MMPs are capable of activating proMMP 2.

Structurally MMPs contain a number of domains, ie discrete amino acid sequence regions, that impart a particular function. These include a prodomain, catalytic domain, hinge region and hemopexin domain. The prodomain and catalytic domains are common to all MMPs. All MMPs, except MMP 11, are synthesized as latent proenzymes and

secreted in the inactive form (proMMPs). A N-terminal sequence targets MMPs for secretion. Secreted MMPs contain a prodomain and are enzymatically inactive. Within the prodomain is a conserved sequence referred to as a cysteine switch motif. This motif is responsible for the latency of proMMPs as a complex forms between its cysteine residue and the zinc-binding motif of the catalytic domain, such that the complex lies over the substrate-binding cleft of the catalytic domain. Activation, one of the important steps in MMP regulation (Ohuchi *et al* 1997), requires detachment of the cysteine residue, achieved via detachment of the prodomain. Certain MMPs (MMP 11, 14-17) also contain a proprotein processing sequence at the C terminal end of the prodomain that also detaches.

In addition to the zinc-binding motif, the central catalytic domain also contains a unique conserved "Met-turn" structure that acts as a base to support the protein backbone that loops around the zinc residue. The catalytic domain is responsible for the proteolytic activity of MMPs, with zinc and calcium ions required for both stability and expression of activity. The C-terminal hemopexin-like domain has a number of functions. This domain is required for the collagenases to cleave the triple helix of collagen (Nagase *et al* 1999), for cell surface activation of MMP 9, and is also the site where TIMPs bind. Binding to cell surface receptors by MMP 2 and 9 is achieved via 3 repeats of fibronectin-type II domains within the catalytic domain.

ProMMPs can be activated by proteinases, such as tissue or plasma proteinases, and reactive oxygen species. In addition, serine proteases, such as plasma-derived urokinase-

type plasminogen activator, can activate MMP 1 and 3 (Milner *et al* 2001). Some of the serine proteases are active in OA joints, providing an *in vivo* mechanism for activation of latent MMPs (Kummer *et al* 1992).

In OA, both synoviocytes and chondrocytes produce MMPs (Zafarullah *et al* 1993), in particular MMP 1, 3, 8, 9, and 13 (Cole *et al* 1996, Shlopov *et al* 1997). Expression is usually restricted to sites of active lesions, and results in destruction of type II collagen and proteoglycans. Elevated synovial fluid concentrations of MMP 1, 2, 3 and 9 have been detected in equine OA joints (Clegg *et al* 1997, Trumble *et al* 2001, Brama *et al* 2004). Detection of increased MMP 3 levels in plasma of human patients with knee OA suggests that expression may not be localized to the affected joint (Naito *et al* 1999).

Control of MMP gene expression can occur at the transcriptional level. Many of the MMPs are inducible (eg MMP 1, 3, 9, 13) with enhanced expression under the influence of growth factors and cytokines such as TNF and IL-1. In contrast, MMP 2 is constitutively expressed, under minimal regulation and, as such, thought to have a lesser role in OA. MMP 1 and 3 are ubiquitously expressed, and MMP 3 is capable of activating MMP 1. Normally, MMP 13 expression is limited to sites of development; however, during OA, MMP 13 production increases (Billinghurst *et al* 1997, Reboul *et al* 1996). MMP 13 is thought to play a pivotal role in OA due to its efficient hydrolysis of type II collagen (Mengshol *et al* 2002), with expression localized to sites of collagen degradation (Mitchell *et al* 1996). MMP 9, also produced by peripheral blood monocytes and neutrophils, is thought to play a secondary role as collagen degradation occurs only after

MMP 1 and 13 have cleaved the triple helix (Nagase *et al* 1999). MMP 8 is only expressed by articular chondrocytes in small quantities, suggesting a less important role in OA (Stremme *et al* 2003).

MMP expression profiles in cartilage appear to differ depending on the stage of OA. Temporal induction of MMP expression in human chondrocytes by IL-1 has been demonstrated *in vitro* (Koshy *et al* 2002). Maximal induction of mRNA expression of MMP 1, 3 and 13 is observed early (maximal 8-12 hours), but MMP 8 expression is delayed, with maximum expression 48 hours post stimulation. Further, MMP expression profiles differ in early compared to late OA cartilage. MMP 2 and 13 are upregulated in late stage cartilage, whereas MMP 3 is upregulated early and down regulated later (Aigner *et al* 2001, Bau 2002).

Aggrecanases

Cleavage of aggrecan is an important and early stage event in ECM degradation, preceding the degradation of collagen (Aigner *et al* 2002). However, cleavage of collagen can then render proteoglycans susceptible to proteolysis. Catabolic factors such as IL-1 can induce degradation of aggrecan by both aggrecanases and MMPs (Sandy *et al* 1991, Flannery *et al* 1992, Lohmander *et al* 1993). Aggrecanases have an integral role in both the normal turnover of aggrecan in cartilage, as well as in OA (Lark *et al* 1997).

Aggrecanases are members of the ADAMTS family (A disintegrin-like and metalloproteinase domain with thrombospondin type I motifs) (Apte 2004). These

enzymes are a subgroup of the membrane bound ADAMs (a disintegrin and a metalloproteinase domain), but are not integral membrane proteins. Like MMPS, aggrecanases are also multi-domain enzymes containing a metalloprotease domain, the catalytic domain of which also contains a zinc binding motif, and a disintegrin-like domain along with a number of thrombospondin type-1 domains. Aggrecanases 1 and 2 (ADAMTS 4 and 5 respectively) are synthesized as inactive enzymes, with activation requiring removal of a prodomain and removal of a C-terminal spacer domain, removal of the latter possibly mediated via MMPs (Gao *et al* 2002).

Degradation of aggrecan involves proteolysis in the interglobular domain near the N-terminus of the core protein, resulting in release of core protein and GAG into synovial fluid with subsequent loss of compressive resistance. Two cleavage sites have been identified; at asparagine^{341}-phenylalanine^{342} and glutamic acid^{373}-alanine^{374} bonds. MMPs cleave at the first site and aggrecanases at the latter; however, aggrecanase-1 secondarily cleaves at the same site as MMPs (Westling *et al* 2002). The majority of aggrecan fragments found in OA synovial fluid are derived from cleavage due to aggrecanases (Sandy *et al* 2001). Although MMP 3 and 13 degrade the interglobular domain (IGD) of aggrecan, increased MMP expression is not correlated with GAG release into media of explant cultures (Little *et al* 1999), in contrast to a documented correlation with aggrecanase activity (Westling *et al* 2002). MMPs may play an important role in the later stages of aggrecan degradation through primary cleavage of the IGD and secondarily through action on aggrecanase generated metabolites (Little *et al* 2002).

ADAMTS 4 and 5 activity is detected in joint capsule, synovium and cartilage and may be upregulated in OA synovium either transcriptionally or post-translationally (Arner 2002). Results are conflicting as to the relative importance of each aggrecanase in OA. Whilst aggrecanase-2 expression, but not aggrecanase-1, increases in response to IL-1 stimulation of human chondrocytes (Koshy *et al* 2002), aggrecanase-1 expression predominated in another study (Bau 2002).

Tissue Inhibitors of Metalloproteinases

The tissue inhibitors of metalloproteinases (TIMPs) are the major endogenous regulators of the MMPs (Nagase *et al* 2003). In normal cartilage, TIMPs exist in slightly greater concentration than MMPs (Dean *et al* 1987), with aberrant MMP expression a feature of OA. TIMPs are constitutively expressed by synovial fibroblasts, and also produced by chondrocytes. Currently 4 isoforms have been identified, designated TIMP 1, 2, 3 and 4 (Brew *et al* 2000). TIMPs bind to MMPs in a 1:1 stoichiometry, and inhibit all MMPs except MMP 14. Equine synovial fluid contains both TIMP 1 and TIMP 2 with increased levels in OA joints (Clegg *et al* 1998a). In OA, both synovial and cartilage TIMP 1 and 3 mRNA levels are elevated (Su *et al* 1999). TIMPs bind to MMPs with varying degrees of affinity. TIMP 3 is a relatively weak inhibitor of MMP 3, with similar inhibition of MMP 1 and MMP 2 as TIMP 2 (Kashiwagi *et al* 2001). In addition, TIMP 3 binds to sulfated GAGs (Yu *et al* 2000), and inhibits aggrecanases, for which it has a stronger affinity than for MMP 1, 2 and 3 (Kashiwagi *et al* 2001). TIMP 3 binds to the active binding site of aggrecanases (Kashiwagi *et al* 2001), whilst TIMP 1 can inhibit activation of

aggrecanase-1 (Gao 2002). The ability of TIMP 3 to bind to the ECM is thought to be important for localized regulation of MMP activity.

Inflammatory Mediators

Nitric oxide

Nitric oxide is a cytotoxic free radical, the by-product of oxygenation of L-arginine. This conversion is catalyzed by nitric oxide synthase (NOS), of which there are 3 isoforms: NOS I and III are constitutively expressed whereas NOS II (iNOS) is inducible and transcriptionally regulated.

Chondrocytes, particularly in superficial cartilage layers (Hayashi *et al* 1997), are the major source of iNOS (Grabowski *et al* 1997), with spontaneous production of NO in OA and enhanced production with cytokine stimulation (Pelletier *et al* 1996). NO induces a number of pathophysiological events characteristic of OA including enhanced MMP synthesis (Murrell *et al* 1995), and reduced IL-1Ra (Pelletier *et al* 1996), PG (Oh *et al* 1998), and type II collagen synthesis (Cao *et al* 1997).

IL-1β is a potent inducer of iNOS activity in chondroytes (Frean *et al* 1997). Selective inhibition of iNOS in a canine model of OA decreased MMP, IL-1β and COX 2 expression (Pelletier *et al* 1999). In equine articular cartilage explants, NO mediated the suppressive effect of IL-1 on PG synthesis (Bird *et al* 2000), and inhibited aggrecan degradation, suggestive of a possible anti-catabolic effect of NO. Production of NO was increased in explants obtained from OA compared to normal equine cartilage. Further,

NO synthesis by equine articular cartilage is consistently higher than that of synovial membrane (von Rechenberg *et al* 2000), with similar results obtained in a canine cruciate ligament rupture model of OA (Spreng *et al* 2000)

IL-1 appears to mediate activation of iNOS via distinct signaling pathways. Stimulation with IL-1 induced a time and dose dependent increase in iNOS mRNA synthesis and activity, along with induction of NFκB and AP-1 (Mendes *et al* 2002). In addition, the degradation of the inhibitor of kappa B (IκBα) was inhibited as was NFκB DNA binding activity. This suggests an autoregulatory effect, consistent with previous findings that NO could modulate its own production by interfering with the interaction of NFκB with its binding site in the promoter region of the iNOS gene (Park *et al* 1997). Regulation may also be effected by reduction in iNOS catalytic activity (Colasanti *et al* 1995). Pathways appear to be diverse and complex with significantly decreased levels of both IL-1-converting enzyme (ICE) and IL-18 following selective inhibiton of iNOS (Boileau *et al* 2002). Further, additional reactive oxygen species such as superoxide react with NO to form peroxynitrite and may be required for IL-1 induced IκBα degeneration and consequently NFκB activation and iNOS expression (Del Carlo *et al* 2002, Mendes *et al* 2003).

Prostaglandins

Prostaglandins, especially PGE_2, are produced in inflamed joints, and increased synovial fluid PGE_2 concentration occurs in OA (Gibson *et al* 1996). Equine synovial membrane

PGE_2 concentration may exceed that of articular cartilage, with highest levels seen in moderate OA (von Rechenberg *et al* 2000). Spontaneous production of PGE_2 by OA cartilage has been related to up regulation of COX 2 activity (Amin *et al* 1997). Actions of PGE_2 in joints include vasodilation, cartilage proteoglycan depletion and enhanced pain perception. At least some elements of pain in joints may be mediated by substance P, a neurotransmitter found in increased concentration in equine OA synovial fluid (Caron *et al* 1992), and the concentrations of which are associated with PGE_2 levels (Kirker-Head *et al* 2000).

In equine monolayer cultures, exogenous PGE_2 had no effect on resting MMP and TIMP gene expression, but significantly decreased IL-1 induced gene expression of MMP 1, 3 and 13 as well as TIMP 1, suggesting a potential role for prostanoids in MMP regulation (Tung *et al* 2002a). In further support of this role, PGE_2 also inhibited rhIL-1β induced stimulation of MMP and TIMP 1 mRNA expression in human synovial fibroblasts (DiBattista *et al* 1995).

The Role of Cytokines

A number of studies have examined the effects of cytokines on cellular metabolism and their role in the pathophysiology of OA (Martel-Pelletier *et al* 1999). Cytokines appear to be first produced by cells of the synovial membrane (Pelletier *et al* 1995), and later by activated chondrocytes (Pelletier *et al* 1993). The predominant cytokines synthesized in OA are interleukins and tumor necrosis factor (TNFα), with IL-1 being the primary mediator of the characteristic degradative processes. IL-1 and TNFα can induce further

cytokine production by synoviocytes and chondrocytes, including enhanced expression of not only these cytokines but also IL-8, IL-6, leukemia inhibitory factor and proteinases.

TNF-α

Tumor necrosis factor exists as two forms, TNF-α and TNF-β. TNF-α induces synovial membrane inflammation and plays a role in degradation of the ECM. TNF-α, synthesized as a precursor protein, is activated via proteolytic cleavage by TNF-α converting enzyme (TACE) (Martel-Pelletier *et al* 1999). Levels of both TNF-α and TACE mRNA are increased in OA compared to normal cartilage (Fernandes *et al* 2002). Two types of TNF receptors are present on the cell membrane surface of most tissues (TNF-R55 and -75). In OA, chondrocytes and synovial fibroblasts have enhanced expression of TNF-R55. OA synovial fibroblasts and chondrocytes also spontaneously produce two soluble receptors, especially TNF-sR75 (Alaaeddine *et al* 1997).

Interleukin-1

Two forms of IL-1, IL-1α and IL-1β, have been identified. Of the two forms, IL-1β has received the most attention. It is synthesized as a precursor, and converted to its active form by the serine protease, IL-1β converting enzyme (ICE), prior to its release (Martel-Pelletier *et al* 1999). Both forms mediate their effects on cells through a cell membrane associated receptor (IL-1R). The two forms of this receptor are type I and type II (IL-1RI and IL-1RII), with the type I receptor having a higher affinity for IL-1β. The expression of IL-1 receptors, particularly type I (Sadouk *et al* 1995), by chondrocytes and synovial fibroblasts increases in OA, resulting in cells that are more sensitive to stimulation by IL-

1. Both types of IL-1R can be shed extracellularly in a soluble form (IL-1sR). Because ligand-binding regions are preserved, these soluble receptors are thought to function as receptor antagonists (Fernandes *et al* 2002). A receptor-associated protein (IL1-RAcP) is required as a co-receptor for IL-1 signaling (Radons *et al* 2002).

The response to IL-1 stimulation *in vitro* is multifactorial and includes synthesis of phospholipase A_2, COX 2, PGE_2, thromboxane A_2 and iNOS; and inhibition of collagen synthesis (type II, IX, and XI) (Cook *et al* 2001, Murrell *et al* 1995, Tung *et al* 2002b). IL-1 also increases the induction and secretion of MMPs (Caron *et al* 1996b, Reboul *et al* 1996, Richardson *et al* 2000), GAG release into media and aggrecanase activity (Cawston *et al* 1999). The effect on TIMPs appears to be minimal in some studies (Cawston *et al* 1999) with conflicting results in others, with both downregulation (Sadowski *et al* 2001) and upregulation reported (Tung *et al* 2002b). The effect on PG homeostasis appears two-fold, with dose dependent induction of PG degradation (Frean *et al* 2000) and inhibition of PG synthesis (Morris *et al* 1994, Frisbie *et al* 1997, MacDonald *et al* 1992, Platt *et al* 1994).

Elevated levels of IL-1-like biological activity in the synovial fluid of horses with clinical OA supports the role of IL-1 in degenerating equine joints (Morris *et al* 1990, Alwan *et al* 1991). Further, IL-1β has been detected in human OA synovial fluid (Kahle *et al* 1992). Pharmacological inhibition of the effects of IL-1 on equine cartilage, as a means to slow or arrest OA progression, illustrates its importance in OA (Caron *et al* 1996a, Frisbie *et al* 2000, Frean *et al* 2000).

Early *in vitro* studies utilized recombinant human interleukin-1 (rhIL-1), and demonstrated inhibition of ECM synthesis and induction of ECM depletion. In human chondrocyte cultures, rhIL-1β suppressed synthesis of type II collagen, associated with decreased α1(II) procollagen mRNA levels (Goldring *et al* 1998). Aggrecan mRNA also down regulated following exposure to IL-1 (Richardson *et al* 2000). Equine chondrocytes in explant and monolayer culture stimulated with rhIL-1 synthesize MMPs (Morris *et al* 1994, Caron *et al* 1996b) and the ECM of cartilage explants degrades as evidenced by decreased GAG and PG synthesis (MacDonald *et al* 1992, Frisbie *et al* 2000). Coincident with enhanced cartilage matrix MMP activity (including MMP 1, 13 and 3), are augmented concentrations of other inflammatory mediators such as PGE_2 and NO. Stimulation with rhIL-1α in equine explant cultures dose dependently decreased MMP 3, GAG and PG synthesis; with a compensatory increase in PG synthesis seen once stimulation was discontinued (Morris *et al* 1994).

The response to rhIL-1 stimulation varies, with an overall reduced response to IL-1 in cartilage obtained from older humans and horses (Ismaiel *et al* 1992, May *et al* 1992b, Morris *et al* 1994, MacDonald *et al* 1992). The effect of age was significant in terms of GAG synthesis, with greater change for a given increase in IL-1 concentration in cartilage obtained from younger horses, demonstrating a significant dose-by-age interaction (MacDonald *et al* 1992). Although PG synthesis was inhibited in all age groups, IL-1 did not influence degradation of PG in mature equine cartilage (Platt *et al* 1994).

The response to IL-1 varies between species and with the source of IL-1. A partially purified form of equine IL-1 obtained from mononuclear cells following LPS stimulation resulted in dissimilar and disproportionate stimulation of PGE_2 and MMP synthesis by chondrocytes and synoviocytes compared to rhIL-1 (May *et al* 1990). *In vitro,* activity of rhIL-1β is greater than that of rhIL-1α (May *et al* 1992a), attributable to differing affinity of IL-1 receptors on equine cells for the different forms of rhIL-1 (May *et al* 1992b).

Similarity between equine and human amino acid sequences of IL-1β is only 26% (Howard *et al* 1998). Given this lack of similarity, it is possible that the response of equine tissue to rhIL-1β may not be entirely representative of the *in vivo* situation. This finds support in research documenting that although only 5% of receptors need to be occupied for half maximal stimution of MMPs by rhIL-1β in human synoviocytes and chondrocytes (Martel-Pelletier *et al* 1992), 2-3 times this concentration is required for receptor saturation in other species (Chin *et al* 1990). Such variability and a need to assure species-specific effects prompted the development of a species- specific IL-1β (reIL-1β) (Tung *et al* 2002b). The nucleotide and deduced amino acid sequence homology of the species specific reIL with human and murine IL-1α was 71.6% and 60.2%, and 66.7% & 61.8% for IL-1β (Kato *et al* 1995). Purified reIL-1β in equine monolayers lead to dose saturable up regulation of MMP 1, 3, 13, TIMP1, and COX 2 gene expression (Tung *et al* 2002b), along with enhanced MMP activity and nitrite concentration in media.

In equine cartilage explants (Takafuji *et al* 2002) both reIL-1α and reIL-1β (0.1-100ηg/ml) dose dependently increased PGE_2 synthesis and PG release into media and

decreased PG synthesis, in agreement with previous studies utilizing rhIL-1 that had demonstrated near maximal response at concentrations greater than or equal to 0.1 ηg/ml (MacDonald *et al* 1992, Platt *et al* 1994, Morris *et al* 1994). However, concentrations of reIL-1 were 40-100 times lower than concentrations of rhIL-1 used in previous studies had substantial effects on PG degradation and synthesis.

The interleukin-1 receptor antagonist (IL-1Ra) is a competitive inhibitor of IL-1R. IL-1Ra is capable of inhibiting a number of pathological events in OA including synoviocyte PGE_2 synthesis, chondrocyte collagenase production, and degradation of ECM (Pelletier et al 1999). There are three forms of IL-1Ra: soluble IL-1Ra (IL-1sRa), and two intracellular forms (icIL-1RaI and icIL-1RaII) (Arend 1993). Both IL-1sRa and icIL-1Ra can bind to IL-1R, with the soluble form binding with greater affinity. A relative deficiency of IL-1Ra occurs in OA. In dogs with experimentally induced OA, intra-articular injection of IL-1Ra not only reduced MMP 1 mRNA expression, but also resulted in a dose dependent protective effect on osteophyte development along with a reduction in severity of cartilage lesions (Caron *et al* 1996a). The potential for using IL-1Ra as a clinically applicable treatment for OA is still being investigated.

Other Cytokines

In addition to TNF-α and IL-1β, a number of other cytokines may play a role in OA. Stimulation of chondrocytes with IL-1 induces IL-8 synthesis, which in turn stimulates the release of superoxide radicals and lysosomal enzymes from chondocytes (Platt 1996).

Although the exact role of IL-8 in ECM degradation is unknown, it is a potent chemotactic factor for neutrophils and as such may influence inflammatory cell migration.

IL-6 is constitutively produced in human chondrocytes, with a dose dependent increase following stimulation by IL-1, TNFα and LPS (Bunning *et al* 1990). IL-6 amplifies the effects of IL-1 on MMP synthesis and proteoglycan synthesis (Nietfield *et al* 1990); however its role in OA is as yet unclear, as IL-6 induced TIMP production (Lotz *et al* 1991). IL-17 also influences chondrocytes metabolism. Specifically, it up regulates IL-1β, TNFα, and IL-6 in a number of cell types, and increases NO production in chondrocytes (Attur *et al* 1997, Martel-Pelletier *et al* 1999).

Leukemia inhibitory factor (LIF) is a glycoprotein found in increased concentration in synovial fluid of OA patients (Dechanet *et al* 1994). LIF enhances IL-1β expression in chondrocytes and synovial fibroblasts (Villiger *et al* 1993), and stimulates the production of NO (Reid *et al* 1990). In contrast, IL-11 in articular chondrocytes and synovial fibroblasts has no effect on MMP production, but rather induces TIMP synthesis (Maier *et al* 1993) and decreases PGE_2 release by OA synovial fibroblasts (Alaaeddine *et al* 1999). IL-4, IL-10 and IL-13 have anti-inflammatory effects. IL-4 and IL-10 inhibits synthesis of IL-1 and TNF-α, whereas addition of IL-13 increases IL-1Ra production (Fernandes *et al* 2002).

Interleukin-1 Signaling Pathways

Following binding of IL-1 to its cell associated receptor, a number of phosphorylation dependent signaling pathways are initiated, leading to the production of transcription factors that subsequently regulate gene expression. The two main pathways by which this occurs involve the transcription factors AP-1 and NFκB.

The initial portions of their signaling pathways are shared. After IL-1 binds to its receptor (typically type I receptor for IL-1β), additional binding to the IL-1 receptor accessory protein (IL-1RacP) is required for signal transduction (Figure 1). Binding of IL-1 to its receptor induces conformational changes and recruitment of multiple receptor-bound proteins, including myeloid differentiation protein (MyD88). MyD88 functions act as an adaptor protein, coupling activation of the receptor to downstream signaling components (O'Neill *et al* 2003, Takeuchi *et al* 2002). MyD88 contains a Toll/Interleukin-1 receptor domain (TIR) through which it interacts with the IL-1R (Burns *et al* 1998). Subsequently, IL-1 receptor associated kinase (IRAK), a serine/threonine kinase, is recruited to the receptor complex by binding to MyD88. The Toll-interacting protein (Tollip) is involved in IRAK recruitment through association with IL-1RacP (Burns *et al* 2000). IRAK is autophosphorylated and then dissociates to interact with another adaptor, tumor necrosis factor receptor associated factor 6 (TRAF6), recruited to the receptor complex upon phosphorylation of IRAK (Jiang *et al* 2002). In turn, TRAF6 once ubiquitinated, activates pathways that ultimately results in the formation or activation of AP-1 or NFκB.

Activator Protein-1

Activator Protein-1 is a transcription factor composed of two proteins of the Jun and Fos families. Its most common form is the heterodimer c-Jun/c-Fos. AP-1 is one of the end products of the mitogen activated protein kinase (MAPK) signaling pathways. The MAPKs include the serine/threonine kinases such as c-Jun N terminal kinase (JNK), extracellular signal-related kinases (ERK), and p38, all of which are activated by IL-1 in chondrocytes (Geng et al 1996). The MAPKs are at the downstream end of a 3-tiered system that also contains mitogen activated protein kinase kinases (MAP2K) and mitogen activated protein kinase kinase kinases (MAP3K).

Following activation (Figure 1), TRAF6 associates with the TRAF associated protein evolutionary conserved signaling intermediate in Toll pathways (ECSIT) (Kopp *et al* 1999). ECSIT then interacts with mitogen activated protein kinase/extracellular signal-regulated kinase kinase kinase-1 (MEKK-1), a MAP3K, linking TRAF6 to the MAPK pathway. Subsequently, MEKK-1 activates a MAP2K that then activates the MAPK JNK (Ninomiya-Tsuji *et al* 1999). JNK phosphorylates c-jun at two N-terminal serines in its transactivation domain, providing the primary mode of c-jun regulation. Two JNK enzymes have been identified, JNK-1 and JNK-2 (also called MAPK9). JNK-2 through its higher binding affinity for c-jun is probably the more physiologically relevant form (Firestein *et al* 1999).

Following activation, c-jun translocates to the nucleus to interact with c-Fos, thereby forming AP-1. AP-1 then binds to the promoter region of a number of genes, including

MMPs, to influence their transcription. The role of transcription factors such as AP-1 in the response of synovium and chondrocytes to IL stimulation was originally investigated in models of rheumatoid arthritis. These studies established a key role of AP-1 and localized its components to synovium, with constitutive expression of JNK-2 by RA synoviocytes. Patients with rheumatoid arthritis have higher AP-1 binding activity compared to patients with OA (Asahara et al 1997). However, the pattern of MAPK activation in synoviocytes may differ to that in chondrocytes, and patterns differ depending on the initial stimulus. IL-1 stimulation of chondrocytes activates JNK-1 and -2, ERK and p38 in a time dependent manner. Conversely, in response to LPS stimulation only ERK is stimulated (Geng *et al* 1996).

Nuclear Factor-κB

NFκB is a ubiquitous protein that exists in the cytoplasm in inactive form, bound to its inhibitory subunit IκB (inhibitor of κB). The most prevalent activated form of NFκB is a heterodimer of p50 and p65 subunits that contain transactivation domains necessary for gene induction (Tak *et al* 2001).

Following activation of TRAF6 (Figure 1), TRAF6 dissociates and translocates to the cytoplasm to form a complex with transforming-growth-factor-β-activated kinase-1 (TAK1), and transforming growth factor β activated Kinase-1 binding proteins 1 and 2 (TAB1 and TAB2) (Qian *et al* 2001). TAK1, a MAP3K, plays a critical role in IL-1 mediated activation of the NFκB pathway by phosphorylating and hence activating NF-κB-inducing kinase (NIK) (Takaesu *et al* 2003). TAB2 is membrane bound, and

translocates to the cytoplasm when stimulated by IL-1 to function as an adaptor linking TRAF6 to TAK1 and TAB1 (Qian *et al* 2001).

In turn, NIK activates inhibitor of kappaB kinase (IKK) that is responsible for the phosphorylation of IκB. Phosphorylation of IκB leads to ubiquitination with subsequent degradation by proteosomes, thereby releasing NFκB. NFκB subsequently translocates to the nucleus where it binds to κB enhancer elements in the promoter region of a number of genes, thereby activating their transcription (Tak *et al* 2001).

IKK contains two subunits, α and β, as well as a regulatory subunit (IKKγ). IKKα is involved in transient NFκB activation while IKKβ is involved in sustained activation (Tak *et al* 2001). IKK activity increased ten fold within ten minutes of IL-1 stimulation of synovial fibroblasts, with increased IKK activity preceding NFκB translocation to the nucleus (Firestein *et al* 1999). IKK resides at a key convergence site for multiple signaling pathways that lead to NFκB activation. The primary pathway by which proinflammatory stimuli such as IL-1 and TNFα induce NFκB function is via activation of IKK-β rather than IKK-α (Aupperle *et al* 1999). Although both IKK-α and -β are constitutively expressed, production is enhanced by IL-1. NIK can also activate NFκB through association with another TRAF, TRAF2 (Firestein *et al* 1999).

NFκB binding sites are present on the promoter regions of a number of inflammatory genes, including IL-1, IL-2, IL-6, IL-8, TNFα, MMPs 1,3,9,13, iNOS, and COX 2 (Baldwin 2001, Baeuerle *et al* 1997, Mengshol *et al* 2000, Tak *et al* 2001). IL-1 induced

collagenase expression in synoviocytes is primarily activated by the p50 homodimer that binds to a critical NFκB-like binding site (Vincenti *et al* 1998). NFκB in synoviocytes has an antiapoptotic role and plays an important function in protection against cytotoxicity of TNFα (Miagkov *et al* 1998).

AP-1 and NFκB pathways appear to diverge at the level of IRAK/TRAF6 (Li *et al* 2001). However, these pathways are complex with cross-talk possible between signaling components. TAK1 can activate NIK and hence NFκB, as well as JNK (Takaesu *et al* 2003). More than one IRAK is involved in IL-1 signaling, and that alternative splicing of adaptor proteins such as MyD88, may influence the ultimate end product of these pathways. Myd88 acts as a bridging protein between IRAK-1 and IRAK-4, enabling IRAK-4 induced IRAK-1 phosphorylation (Burns *et al* 2003). The alternative splice form of Myd88 (MyD88s) may prevent NFκB activation, by preventing recruitment of IRAK-4 and hence phosporylation of IRAK1, while still allowing for phosphorylation and hence activation of JNK (Janssens *et al* 2003).

Role of AP-1 and NFκB in OA

The role of both AP-1 and NFκB in OA has been investigated, building largely on results obtained from *in vitro* studies of rheumatoid arthritis (RA) as well as other cell types. In both RA and OA, NFκB expression is increased concurrent with increased AP-1 components, c-Jun and c-Fos. DNA binding activities of both AP-1 and NFκB are greater in RA than OA, with elevated expression preceding the development of clinical signs and increased MMP levels (Han *et al* 1998).

Following IL-1 stimulation, the effects of COX 2, an enzyme known to have a crucial role in OA, are mediated by NFκB. COX 2 has two putative NFκB sites in its 5' promoter (Newton *et al* 1997). Other transcription factors, such as the p38 MAPK, have also been implicated in the regulation of COX 2 gene expression in chondrocytes (Thomas *et al* 2002).

NFκB and AP-1 are early response genes required for the transcription of MMPs following IL-1 stimulation (Vincenti *et al* 2001). The role of AP-1 in chondrocytes and synoviocytes is mediated via JNK (Mengshol *et al* 2000, Han *et al* 2001). The promoters of MMPs such as MMP 1 and 13 contain a core transcriptional unit (TAT box) at approximately –30 base pairs and an AP-1 binding site at –70 base pairs (Borghaei *et al* 1997). The latter is a TGAG/CTCA sequence that binds dimers of c-fos and c-Jun (Vincenti *et al* 2002). These binding sites correlate with inducible MMP expression (Borden et al 1997); however, several other AP-1 sites throughout MMP promoters may also contribute to gene expression (Benbow *et al* 1997). In murine models of inflammatory arthritis, activation of AP-1 and NFκB preceded clinical signs and MMP 1 and MMP 13 gene expression (Han *et al* 1998). The MMP13 promoter region contains a conserved AP-1 binding site that binds c-Jun and c-Fos (Tardif *et al* 1997), and transcription of AP-1 increases prior to the induction of MMP 13 gene expression (Goldring *et al* 1994).

Interestingly, IL-1 induction of MMP 13 in chondrocytes requires both NFκB and JNK (Mengshol *et al* 2001). These cells rely on p38, JNK and NFκB to activate MMP 13 (Mengshol *et al* 2000), suggesting that the MAPKs either directly or indirectly activate AP-1 subunits that then cooperate with NFκB to activate MMPs. AP-1 sites may interact with NFκB elements that are also present in MMP promoters (Barchowsky *et al* 2000, Vincenti *et al* 1998). Both AP-1 and NFκB binding sites are required for MMP 1, 3 and 9 expression (Bond *et al* 1998, Vincenti *et al* 1998); however, JNK or NFκB alone may be sufficient to increase MMP 1 expression (Bond *et al* 1999).

Drugs used in Osteoarthritis Treatment

Nonsteroidal anti-inflammatories

Nonsteroidal anti-inflammatories (NSAIDs) are frequently used to treat musculoskeletal disease, including OA, in horses. Commonly used NSAIDs include phenylbutazone and flunixin meglumine. NSAIDs are cyclooxygenase inhibitors, and thereby inhibit the production of prostaglandins. NSAIDs reduce the clinical signs of OA such as pain and synovitis, associated with reduced synovial fluid PGE_2 concentration (Owens *et al* 1996).

Concerns regarding the use of NSAIDs mainly relate to their potential side effects such as gastric ulceration, right dorsal colitis and renal papillary necrosis and medullary ischemia (Moses *et al* 2002). In addition, some NSAIDs have negative effects on both chondrocytes and bone, including inhibition of GAG synthesis (Dingle 1999), decreased mineral apposition rate in cortical bone and reduced healing rate of experimentally induced cortical defects (Rohde *et al* 2000). Clinical trials of the effect of oral

phenylbutazone (4.4mg/kg BID for 14 days) on cartilage metabolism showed a significant reduction in PG synthesis (Beluchi *et al* 2001).

A number of *in vitro* studies have been conducted to elucidate both efficacy and potential mechanisms of action of NSAID in OA. The reported effects of different NSAIDs on articular cartilage vary. In some studies, NSAIDs reduced PG catabolism and MMP activity induced by IL-1 (Pelletier *et al* 1999). Indomethacin inhibits MMP 3 and increases TIMP 1 expression (Yamada *et al* 1996), while phenylbutazone also inhibits MMP 3 synthesis, albeit at concentrations that exceed those achieved therapeutically (May *et al* 1988). Conversely, in another study, flunixin meglumine and phenylbutazone had no effect on MMP expression (Clegg *et al* 1998). NSAIDs increased PG synthesis in cartilage explants (Jolly *et al* 1995), and PGE_2 synthesis decreased in equine explants; with no effect on COX 2 or iNOS gene expression (Tung *et al* 2002c, d). Carprofen attenuated the LPS induced increase in IL-6 in equine chondrocytes and synoviocytes, but had no effect on release of IL-1 (Armstrong *et al* 2002). Similar to their effects in cartilage, NSAIDs suppressed PGE_2 production in equine synovial membrane explants without detrimental effects on synovial membrane viability and function (Moses *et al* 2001).

NSAIDs are capable of influencing some of the aforementioned IL-1 signaling pathways. Specifically, both aspirin and sodium salicylate block IKKβ, thereby preventing NFκB activation of genes including COX 2 (Yin *et al* 1998). The influence of phenylbutazone

and flunxin meglumine, the most commonly used NSAIDs in equine practice, remains to be determined.

Corticosteroids

Intra-articular administration of corticosteroids in horses with joint disease is widespread. Corticosteroids inhibit phospholipase A_2; however, their anti-inflammatory effects extend beyond inhibition of arachidonic acid metabolites. Corticosteroids diffuse into the cytoplasm to interact with steroid-specific receptors, and subsequently bind to promoter regions of glucocorticoid-responsive genes, thereby modulating their transcription (Trotter 1996).

In vitro, corticosteroids inhibit a variety of degradative enzymes, including MMP 13 (Caron *et al* 1996b), COX 2 and PGE_2 (Tung *et al* 2002d). MMP 1, 3, 13 and TIMP 1 are inhibited with lower doses than those required for down-regulation of type II collagen and aggrecan gene expression in equine chondrocytes (Richardson *et al* 2003). Dexamethasone treatment results in pretranslational regulation of iNOS expression *in vitro* (Tung et al 2002c). Dexamethasone decreases PG content more than that induced by IL-1 stimulation alone (Frisbie *et al* 1997, Stove *et al* 2002). *In vivo*, corticosteroids reduce the progression of experimentally induced OA lesions (Pelletier *et al* 1995).

The action of corticosteroids is due in part to their effects on IL-1 signaling pathways. The glucocorticoid-receptor complex binds to AP-1 (Krane 1993), and induction of the IκBα inhibitory protein leads to inhibition of NFκB (Auphan 1995, Kovalovsky *et al*

2000). The glucocorticoid-receptor complex can also repress NFκB through physical interaction with p65 subunits to inhibit NFκB transcription (Scheinmann *et al* 1995).

Hyaluronan

Sodium hyaluronan (HA) is frequently used in the treatment of equine joint disease (McIlwraith *et al* 1997). The specific mode of action is yet to be determined; however, HA improved mobility and reduced pain in OA joints (Ghosh 1994). HA may be administered intra-articularly or systemically. A number of clinical trials documented an improvement in lameness score (Gaustad *et al* 1995), as well as decreased synovial fluid total protein and PGE_2 levels (Kawcak *et al* 1997). Following intra-articular administration, the clearance of HA is fairly rapid (Hilbert *et al* 1995); however, a portion remains associated with synovial tissues. In humans, intra-articular HA is frequently used to treat pain associated with OA, with proven efficacy and safety (Altman *et al* 1998). In a rabbit instability model of OA, once weekly intra-articular injections of HA reduced disease progression and improved morphological appearance of articular cartilage (Amiel *et al* 2003).

In human and animal *in vitro* OA models, HA exerted effects on both synovium and cartilage, including stimulation of synoviocyte HA production (Smith *et al* 1987), prevention of PG and collagen degradation (Takahashi *et al* 1999, Morris *et al* 1992), stimulation of PG synthesis (Frean *et al* 1999), prevention of chondrocyte apoptosis (Takahashi *et al* 2000), and reduction of inflammatory mediators such as NO (Punzi 2001). HA also inhibits IL-1 induced PGE_2 production by chondrocytes (Akatsuka 1993),

and synoviocytes (Frean *et al* 2000). HA appears to exert no effect on COX 2, iNOS (Tung *et al* 2002a, b) or MMP expression (Clegg *et al* 1998, May *et al* 1988). Some favourable effects of HA may be in part due to inhibition of leukocyte migration and function (Howard *et al* 1996), as well as inhibition of arachidonic acid release from membrane phospholipids of synovial fibroblasts (Tobetto *et al* 1992).

Polysulfated Glycosaminoglycans

Polysulfated glycosaminoglycans (PSGAG) such as Adequan®, are semisynthetic heparinoids. The principal GAG in this putative "chondroprotective" agent is chondroitin sulfate. Intra-articular administration of PSGAG is thought to be of benefit in the treatment of lameness (Hamm *et al* 1984, Caron *et al* 1996c). It appears to be more effective when given intra-articularly rather than following intramuscular administration (Trotter *et al* 1989). Unfortunately, side effects of intra-articular administration of PSGAG have been documented including flare reactions (Todhunter *et al* 1994) and possible sepsis. PSGAG increased MIC of antibiotics required *in vitro* against *Staphylococcus aureus* and dramatically reduced the intra-articular inoculum of this bacterium to induce sepsis *in vivo* (Gustafson *et al* 1989a, b).

The precise mechanism of action of PSGAG is unknown. It has an affinity for proteoglycans and non-collagenous proteins, and stabilizes fibronectin-collagen complexes (Andrews *et al* 1985). PSGAG inhibits a variety of degradative enzymes, including MMP 3 (May *et al* 1988) and MMP 2 and 9; however, the latter effect is only recognized at concentrations exceeding those achieved clinically (Clegg *et al* 1998).

After experimental induction of OCD lesions in horses, PSGAG decreased type II collagen in articular cartilage (Todhunter *et al* 1993); however, in other studies, collagen and GAG synthesis were stimulated (Glade 1990). The reported effects of PSGAG on PG synthesis and degradation vary. A protective effect on IL-1 induced inhibition of PG synthesis was observed (Frean *et al* 2002). A surmised stimulatory effect on PG synthesis in normal and OA cartilage was not demonstratable, and minimal effects on PG degradation were observed in unstimulated cultures (Caron *et al* 1991 and 1993). PSGAG had no effect on synthesis and release of PGE_2 by reIL-1β stimulated equine chondrocytes or on COX 2 gene expression; however, iNOS expression decreased (Tung *et al* 2002a, b).

Pentosan Polysulfate

Pentosan Polysulfate (PPS) is a polysulfated polyglycosaminoglycan heparin analogue derived from beech wood hemicellulose. Pentosan polysulfate is available in two forms, a sodium salt and a calcium derivative. *In vitro*, effects have included increased TIMP 3 synthesis (Takizawa *et al* 2000), inhibition of aggrecan degradation (Munteanu *et al* 2002), increased synovial fluid hyaluronan (Francis *et al* 1993), stimulation of PG synthesis, and inhibition of MMP 3, but not MMP 2 and 9, synthesis (Rogachefsky *et al* 1993, Clegg *et al* 1998). Pentosan polysulfate binds to and is internalized by synoviocytes and chondrocytes (Ghosh *et al* 1996). Further, PPS inhibited aggrecanase mediated degradation of aggrecan in bovine explant cultures (Munteanu *et al* 2002). The anti-thrombotic effects of PPS are thought to improve subchondral bone blood flow (Ghosh 1999).

Although pharmacokinetic studies are limited, therapeutic plasma and synovial fluid concentrations have been detected following single intramuscular administration of calcium PPS (2mg/kg) in the horse (Fuller *et al* 2002). The efficacy of oral calcium PPS (10mg/kg) was evaluated in a double blind placebo controlled study in dogs with cranial cruciate ligament injury (Innes *et al* 2000). Functional outcome and radiographic progression were unaltered; however, reduced ECM breakdown was inferred from a significant decrease in 5D4 epitope of keratan sulfate in synovial fluid.

In a placebo controlled, blinded clinical study in horses, sodium PPS (3mg/kg) improved lameness and flexion scores, reduced effusion and synovial fluid total protein, reduced radiographically evident entheseophyte production and subchondral bone lysis, and reduced cartilage erosion and fibrillation (Frisbie *et al* 2003). However, serum markers of cartilage breakdown were increased (carboxy propeptides of type II procollagen and epitope 846 of chondroitin sulfate). PPS had no effect on clotting factors or platelets. However, in a study investigating the effects of sodium PPS on hematological and hemostatic values in horses, activated partial thromboplastin time was dose dependently increased (Dart *et al* 2001).

Glucosamine and Chondroitin sulfate

Structure

Glucosamine and chondroitin sulfate have been classified as both "nutraceuticals" and Symptomatic Slow Acting Drugs In Osteoarthritis (SYSADOA). Glucosamine is an

amino monosaccharide (2-amino-2-deoxy-alpha-D-glucose) consisting of a hexose sugar ring (glucose) with an amino group substituted for a hydroxyl group on carbon 2. Glucosamine, once modified as N-acetylglucosamine, is a precursor of the disaccharide units of GAGs such as HA and keratan sulfate. Isomerisation converts glucosamine to galactosamine, a structural component of chondroitin sulfate and dermatan sulfate. Most glucosamine in the body is in the form of glucosamine-6-phosphate (Platt 2001). Glucosamine is commercially available in three forms: glucosamine hydrochloride (GHCl), glucosamine sulfate (GS) and N-acetyl-D-glucosamine (NAG).

Chondroitin sulfate is a GAG consisting of alternating units of glucuronic acid and sulfated N-acetyl galactosamine. The two forms of chondroitin sulfate vary in the position of the sulfate residue attached to N-acetylgalactosamine (C-4-S and C-6-S).

Actions in Articular Tissues

Glucosamine

Initially, beneficial effects of glucosamine supplementation were attributed to the provision of raw materials required for "building blocks" of cartilage, namely GAGs. Theoretically, by supplying an exogenous form of glucosamine, the rate-limiting step of synthesis of glucosamine-6-phosphate is bypassed, thus increasing the rate of HA synthesis (Kim 1974). Exogenous glucosamine is preferentially utilized in the synthesis of GAGs when cells are cultured without glucose (Roden 1956). The preferential incorporation of glucosamine into galactosamine moieties of CS in articular cartilage explants supported the use of glucosamine as a source of cartilage matrix components

(Noyszewski *et al* 2001); however, this mode of action has been debated (Mroz *et al* 2004).

Glucosamine enters chondrocytes via facilitated glucose transporters (GLUT), acting as a competitive inhibitor of glucose transport (Windhaber *et al* 2003). Glucosamine has a greater affinity for certain glucose transporters, especially GLUT2, in other cell types (Uldry *et al* 2002); however, in chondrocytes, glucose uptake is non-insulin dependent as GLUT4, the major insulin regulating glucose transporter, has not been identified in cartilage (Shikhman *et al* 2004).

Potential chondroprotective effects include stimulation of GAG and PG production by GS (10-100μg/ml) in human OA chondrocytes *in vitro*, with a carry over effect evident for at least 4 days after the withdrawal of glucosamine from media (Bassleer *et al* 1998). Glucosamine is thought to stimulate the synthesis of aggrecan (Jimenez *et al* 1997, Dodge *et al* 2003). Glucosamine appears to have no influence on type II collagen production (Bassleer *et al* 1998, Dodge *et al* 2003). The lack of effect on collagen synthesis was also documented in a rabbit chymopapain induced OA model, despite a significant increase in GAG content of both affected and normal cartilage in the contra-lateral limb (Oegema *et al* 2002).

The effect of glucosamine on PG synthesis may be attributable to an increase in GAG precursors such as UDP-N-acetylglucosamine and galactosamine (Sandy *et al* 1998, Patwari *et al* 2000), or it may act as a direct substrate of glycosyltransferases, enzymes

involved in post-translational modification of GAGs. Glucosamine prevented IL-1 induced repression of galactose β-1,3-glucuronosyltransferase I (GlcAT-1), a key enzyme that catalyzes the addition of the first glucuronic acid residue to the trisaccharide backbone of GAGs (Gouze *et al* 2001). Hence, exogenous glucosamine could, by supplying precursors for the glycosyltransferases, bypass glutamine fructose-1-phosphate transaminase, the rate-limiting enzyme in the hexosamine pathway. Further contribution to GAG synthesis may be through promotion of incorporation of sulfur into cartilage, as administration of GS increased both serum and synovial fluid sulfate concentrations (Hoffer *et al* 2001).

Glucosamine also has an anti-inflammatory activity, suppressing inflammatory mediators and degradative enzymes such as NO, PGE_2, aggrecanases, and MMPs. The form of glucosamine appears to influence its activity, with GHCl and GS appearing to inhibit cartilage degradation more consistently than NAG *in vitro*. In LPS stimulated equine explants, GS (3.075mg/ml) inhibited NO production, and PG release at 30.75mg/ml, consistent with previous findings for equimolar concentrations of GHCl (Fenton *et al* 2000a,b). Although PG synthesis was also decreased, tissue PG content was significantly higher. A higher dose was required to inhibit PG release in rhIL-1 stimulated explants compared to LPS stimulated explants. N-acetyl-glucosamine had more variable effects, and generally did not inhibit NO production or PG release at any concentration. However, in another study, high concentrations of NAG were shown to be effective in suppressing IL-1 induced COX 2 and iNOS expression (Shikhman *et al* 2001). Both GS

and GHCl contain a free amine group on the second carbon atom, the presence of which may be important for activity of these compounds.

Glucosamine inhibits NO and PGE_2 production in equine cartilage stimulated with LPS, rhIL-1β and reIL-1β (Fenton *et al* 2000a,b; Orth *et al* 2002). In general, higher doses were required to inhibit NO production (1mg/ml) compared to PGE_2 production (0.5mg/ml) (Orth *et al* 2002). Glucosamine (4.5g/l) also decreased PGE_2 both in the presence and absence of IL-1 in rat chondrocytes (Gouze *et al* 2001). In human OA chondrocytes, GS (1.0g/L), but not NAG, inhibited COX 2 gene expression and protein synthesis and inhibited PGE_2 synthesis induced by IL-1 (Largo *et al* 2003). A potential benefit of glucosamine over NSAIDs was also demonstrated in this study, with glucosamine having no effect on COX-1 production. Glucosamine hydrochloride (100 μg/ml) inhibited PGE_2 production but not COX 2 mRNA expression in IL-1 stimulated normal and OA chondrocytes, and suppressed PGE_2 production in synoviocytes (500 μg/ml); however, this effect was not statistically significant (Nakamura *et al* 2004). NO production was suppressed in normal chondrocytes but not OA chondroyctes or synoviocytes.

Glucosamine dose dependently inhibits aggrecanase-mediated cleavage of aggrecan in bovine cartilage (Sandy *et al* 1998, Patwari *et al* 2000). Mannosamine, a structural isomer of glucosamine, also inhibited aggrecan cleavage and, along with glucosamine, was shown to inhibit IL-1 induced alterations in mechanical properties of cartilage (Patwari *et al* 2000). Long-term exposure to glucosamine and mannosamine inhibits aggrecanase-

mediated degradation of aggrecan in a dose dependent manner (up to 10mM) in retinoic acid stimulated bovine cartilage (Ilic *et al* 2003). The effect on aggrecanase activity has been documented in both IL-1 and retinoic acid stimulated cartilage, suggesting that glucosamine may have a site of action downstream of IL-1 signaling pathways; however, the precise mechanism by which glucosamine inhibits aggrecanase activity requires further elucidation.

Effects on MMP gene expression, protein synthesis and activity have been investigated in a number of studies. In LPS stimulated equine explants, gelatinase and collagenase activity was inhibited by GHCl at 0.25mg/ml (Fenton *et al* 2000b), with higher concentrations (2.5mg/ml) required to exhibit the same effect in IL-1 stimulated explants (Fenton *et al* 2002). However, stromelysin activity has been inhibited with a lower concentration (0.25mg/ml) in reIL-1β stimulated equine explants. Glucosamine hydrochoride had negligible effect on preformed MMP, but did inhibit both MMP protein and mRNA expression (Byron *et al* 2003). MMP 13 expression was inhibited to a greater degree by glucosamine than was MMP 1 and 3 activity (1.5mM-50mM and 3mM – 50mM respectively). Similar results were found in terms of mRNA expression; however, this was not a statistically significant difference. Glucosamine decreased MMP 9 but not MMP 2 activity, and inhibited MMP 13 production (Orth *et al* 2002). MMP 3 production and activity has been dose dependently inhibited by GS (1.0-150μM) in human OA chondrocytes (Dodge *et al* 2003); however, chondrocytes obtained from 40% of OA patients did not respond. Further, no effect on MMP 1 activity was demonstrated. Reduction in MMP 3 mRNA expression has also been documented in rat chondrocytes *in*

vitro (Gouze *et al* 2001). GHCl suppressed MMP 1, 3 and 13 activities in IL-1 stimulated normal human chondrocytes (100 μg/ml) and synoviocytes (100 μg/ml), but not OA chondrocytes (Nakamura *et al* 2004).

High doses of glucosamine may have a detrimental effect on chondrocyte viability *in vitro*. GAG concentration and cell viability were reduced in canine chondrocytes cultured in alignate in the presence of glucosamine (0.1mg/ml for 12 days) (Anderson *et al* 1999), conversely, no detrimental effect on chondrocyte metabolism was observed with long-term exposure to glucosamine at 1 mg/ml (Ilic *et al* 2003). In bovine cartilage explants, GHCl (2.5-25 mg/ml) induced a dose dependent decrease in PG synthesis and lactate production, with cell viability reduced by over 90% at the higher dose (de Mattei *et al* 2002). Similarly, cell viability was reduced by 10mg/ml GHCl in bovine cartilage explants in another study (Mello *et al* 2004). This suggests that the protective action against cytokine induced catabolic effects observed at higher doses in previous studies were due to unrecognized toxic effects (Fenton et al 2000a,b).

Other potential actions in chondrocytes include reduction of phospholipase A_2 and increased production of protein kinase C (Piperno *et al* 2000). Glucosamine (0.1-1mM) suppressed neutrophil function and immune activity in synovial tissue, including suppression of superoxide anion generation, inhibition of enzyme release from granules and inhibition of phagocytosis (Hua *et al* 2002). Suppression of T cell activation has also been documented (Ma *et al* 2002).

Chondroitin sulfate

Documented actions of CS include contribution to the pool of GAG, inhibition of degradative enzyme synthesis and stimulation of GAG and collagen synthesis. A stimulatory effect on HA has been documented in synovial membrane *in vitro* (Nishikawa *et al* 1985), and CS improved synovial fluid viscosity by increasing HA concentration in human patients with knee OA (Conte *el al* 1991, Ronca *et al* 1998).

Stimulation of PG production was documented in human chondrocytes in clusters, with no effect on either basal DNA activity or PGE_2 production (Bassleer *et al* 1992). Chondroitin sulfate was more active in counteracting IL-1 induced effects on PGE_2, PG (500-1000μg/ml) and type II collagen (100-1000μg/ml) during the period of cluster formation (day 0-16) than in the period after which clusters had formed (PG: 100-1000μg/ml, type II collagen and PGE_2 1000μg/ml) (Bassleer *et al* 1998). In a rabbit model of OA, cartilage PG content was significantly higher following administration of CS prior to induction of OA (Uebelhart *et al* 1998).

In equine explants, CS decreased NO production, PG degradation and MMP 13 production, albeit at different concentrations (0.25mg/ml, 0.5mg/ml, and 0.125mg/ml respectively) (Orth *et al* 2002). Chondroitin sulfate had no effect on PGE_2 production, and had a minimal not statistically significant effect on MMP 2 and 9. Collagenase, phospholipase A_2, and n-acetylglucosaminidase activity were decreased in synovial fluid obtained from human patients with knee OA treated with CS (Ronca *et al* 1998). A protective effect in terms of PG concentration was recently established using human

chondrocyte cultures under conditions of IL-1 stimulation and cyclic pressurization (Nerucci *et al* 2000), suggesting that CS may be more effective when chondrocytes are exposed to mechanical forces. Chondroitin sulfate (100 μg/ml) may also protect chondrocytes from NO mediated apoptosis following stimulation with IL-1 (Conrozier 1998).

Other potential mechanisms of action have been postulated. Chondroitin sulfate partially inhibits complement (Paroli *et al* 1991) and elastase activity in human leukocytes (Baici *et al* 1994). Leukocyte elastase activityiss inhibited more efficiently by low molecular weight CS compared to intact CS (Cho *et al* 2004). In a collagen-induced arthritis model, C-4-S significantly reduced the production of free radicals and restored endogenous antioxidant levels (Campo *et al* 2003a, b). Further, CS inhibits intra-articular bradykinin induced PG depletion in rats in a dose dependent manner (Omata *et al* 1999).

Glucosamine and chondroitin sulfate in combination

The combination of glucosamine and CS appears to be more effective than either product alone. In equine explants, GHCl and CS in combination decreased LPS induced NO production (0.5mg/ml and 0.25mg/ml), and inhibited MMP 13 production (0.5mg/ml and 0.125mg/ml) (Orth *el al* 2002). The same dose of glucosamine had minimal or no effect alone. The combination decreased PG degradation (1mg/ml and 0.25mg/ml), with a higher dose of CS alone required to exhibit a similar effect. In bovine articular cartilage *in vitro*, Cosequin® (GHCl, LMWCS, manganese ascorbate) increased PG synthesis and

enhanced a mild stimulatory effect of a NSAID on PG synthesis, as well as inhibiting the NSAID induced acceleration in ECM degradation (Lippiello *et al* 2002).

In a canine cranial cruciate ligament model of OA, the same product reduced cartilage degradation and increased CS epitopes (3D3 and 7D4) and GAGs in synovial fluid (Johnson *et al* 2001). In a rabbit instability model of OA, severity of cartilage lesions in terms of both area and severity was significantly reduced (Lippiello *et al* 2000). The combination had a synergistic effect, with a greater effect than either compound alone.

In vivo joint stress was simulated *in vitro* by matrix depletion with enzymes, heat stress, and mechanical compression (Lippiello 2003). Exposure of cartilage explants to pronase induced upregulation of PG synthesis, whereas hyaluronidase, chondroitin ABC lyase and stromelysin had an inhibitory effect. Cosamin® (GHCl, LMWCS and manganese ascorbate) supplementation had no influence on the inhibitory effect of hyaluronidase and chondroitin ABC lyase. PG synthesis was stimulated in pronase treated aged joints with high doses (400μg/ml), while a lower dose (100μg/ml) reversed inhibition of PG synthesis induced by stromelysin. Heat stress similarly decreased PG synthesis, an effect prevented by administratin of GHCl and CS in combination. Cartilage from aged animals was more responsive to stress in terms of PG synthesis and also to supplementation with Cosamin®, with a 1000% increase in PG synthesis.

Effect on IL-1 signaling pathways

Recently, the effects of glucosamine on IL-1 signaling pathways have been investigated. Using human OA cartilage, the effects of GS, galactosamine hydrochloride and NAG on AP-1 and NFκB were investigated (Largo *et al* 2003). Glucosamine sulfate alone did not modify NFκB binding; however, significantly inhibited NFκB binding in a dose dependent manner in IL-1 stimulated cartilage, with maximal inhibition at 1000mg/L. Further, GS prevented IL-1 induced translocation of p50 and p65 subunits of NFκB to the nucleus, and prevented IL-1 induced degradation of IκB. No effect on AP-1 binding was observed, consistent with findings using rat chondrocytes where glucosamine (4.5gm/L) inhibited NFκB but not AP-1 pathways (Gouze *et al* 2002). Galactosamine hydrochloride and NAG had no effect in human OA cartilage (Largo *et al* 2003), in support of other studies where NAG had no effect on NFκB translocation or JNK, ERK or p38 activation (Shikhman *et al* 2001). As NFκB induces transcription of COX 2 and iNOS, the anti-inflammatory effects of glucosamine on PGE_2 and NO production observed in these studies (Gouze *et al* 2002) may be due to inhibition of NFκB binding to its response elements.

Other potential beneficial influences of glucosamine on IL-1 signaling have been documented at the level of IL-1 receptors. Glucosamine increased expression of IL-1RII (Gouze *et al* 2002). IL-1RII is a truncated protein and functions as a decoy receptor, by effectively trapping IL-1, the inflammatory effect of which requires signaling through IL-1R1. There was no effect on IL-1Ra mRNA expression.

In other cell types such as mesangial cells and adipocytes, glucosamine modulation of gene expression involves the transcription factor Specificity Protein (Sp1) (Goldberg *et al* 2000). This transcription factor has binding sites in promoters of genes coding for MMPs and aggrecan (Valhmu *et al* 1995); however, the regulation of Sp transcription factors in cartilage has not been determined. Whether CS is capable of exerting a similar effect on IL-1 signaling requires further investigation.

Pharmacokinetics

Glucosamine

Glucosamine is a small water-soluble molecule (molecular weight 179), with a pKa that favors its intestinal absorption and intracellular transportation (Kelly 1998). Absorption is carrier-mediated, whereas absorption of NAG occurs via diffusion (Tesoriere *et al* 1972). Quantitative aspects of glucosamine absorption have been debated. Early pharmacokinetic and bioavailability studies utilized colorimetric, chemical or radiolabeling techniques; however, limitations were apparent with each analytical method. Colorimetric assays failed to differentiate between glucosamine and other hexosamines, and required the administration of doses far exceeding levels considered therapeutic and/or achievable in animals (Setnikar *et al* 2001). Following intravenous (IV) and oral (PO) administration of crystalline GS in man, plasma and urinary concentrations were assayed using plasma deproteinised with sulfosalicylic acid or ion exchange chromatography (Setnikar *et al* 1986). Oral GS at four times the recommended daily dose, resulted in plasma levels were still below the limit of detection (3μg/ml) with

ion exchange chromatography. Thus the ADME profile (absorption, distribution, metabolism and excretion) of glucosamine required further elucidation.

Limitations with radiolabeling techniques were also evident. Accurate determination of bioavailability of glucosamine was precluded as both the labeled compound and its metabolites were quantified. However, such techniques did enable preliminary investigation of pharmacokinetics in a number of species. Incorporation into plasma proteins, biotransfomation in the liver and urinary excretion was demonstrated following IV dosing in a rat (12.6mg/kg) (Setnikar *et al* 1984). Tissue distribution was rapid, with early incorporation into articular cartilage (confirmed by autoradiographs of sagittal sections of necropsied animals). Most organs, including articular cartilage, contained some radioactivity even 144 hours after administration. The equivalent dose administered orally resulted in peak plasma concentration by 4 hours, 95% absorption and an estimated bioavailability of 40% based on urinary excretion within the first six hours. Again, early incorporation into articular cartilage was observed (within 30 minutes), and concentrations from 8 hours were similar to that in plasma.

Another study in the rat utilizing oral doses at 4.6, 46, 125 times the recommended daily dose, demonstrated that absorption and elimination kinetics were independent of the dose, however at all sampling times radioactivity content was dose related. Significantly higher levels of radioactivity existed in cartilage compared to plasma at 72 and 120 hours, suggesting substantial incorporation of glucosamine into articular cartilage. Multiple dosing (SID PO 12.6mg/kg for 6 days) lead to accumulation of glucosamine in

cartilage, with 3-5 times greater radioactivity at 144 hours after the last of the multiple doses compared to after a single dose. Furthermore, at the same time period, cartilage radioactivity was nearly 3 times greater than in plasma after both single and multiple dose administration (Setnikar *et al* 2001).

Radiolabeling studies in man have demonstrated similar pharmacokinetics (Setnikar *et al* 1986 and 1993). Nearly 90% absorption was noted following oral administration, and, whilst no free glucosamine was detectable in plasma (below LOQ), the incorporation of radioactivity into plasma proteins was found to follow similar pharmacokinetic patterns as parenteral administration, albeit at a concentration approximately 5 times lower.

A similar ADME profile occurs in dogs. Two hours following IV administration (12.6mg/kg), radioactivity in articular cartilage was 13 times deproteinised plasma levels and twice plasma protein concentrations (Setnikar *et al* 1986). Tropism for articular cartilage was again apparent with radioactivity still detectable 144 hours after PO administration. Conversion of GS into radiolabelled galactosamine moieties of CS and keratan sulfate in cartilage was also demonstrated (Dodge *et al* 2001).

High Performance Liquid Chromatography (HPLC) allows for accurate determination of glucosamine in orally available products and pharmacokinetic studies. As glucosamine lacks a chromophore absorbing in the ultraviolet light range, modifications were required. Pre-column derivatization of GHCl with phenylisothiocyanate overcame this problem and was shown to be specific (thereby eliminating interference by degradation products),

accurate and precise. Plasma concentrations were measured between 1.25 – 20 μg/ml in dogs (Liang *et al* 1999). Use of a plasma concentration versus time curve in a dog (2000 mg GHCl PO) established a maximum plasma concentration at less than 2 hours, with none detectable at 8 hours.

Due to interference from similarly derivatized endogenous amines, this technique was not applicable to studies in the rat, prompting development of a technique using HPLC with pre-column derivatization and ion exchange purification. The LOQ was found to be 1.25 μg/ml, sufficient for pharmacokinetic studies with 350mg/kg PO; however, lower doses could not be determined (Aghazadeh-Habashi *et al* 2002b). Rapid elimination and distribution of glucosamine was also apparent (Aghazadeh-Habashi *et al* 2002a). Intraperitoneal dosing exhibited complete bioavailability, with poor oral bioavailability of glucosamine thought due to loss in the gastrointestinal tract rather than hepatic first pass effect as previously thought (Setnikar *et al* 1993). Other analytical methods, such as refractive index detection, have also been utilized and found to be accurate, precise and useful for the quantification of GS, although with less accuracy than HPLC (El-Saharty *et al* 2002).

More sensitive and accurate techniques that eliminate interference by degradation products have enabled the determination of plasma concentrations achievable after oral administration in the dog and horse (Adebowale *et al* 2002, Du *et al* 2004). Results of single dose pharmacokinetics of glucosamine using these methods support its absorption, with lower oral bioavailability in the horse than in the dog. In the dog, glucosamine was

rapidly absorbed with a Cmax between 7.1-12.1 μg/ml. In the horse, after IV administration (Cmax 349 μg/ml), concentrations decreased rapidly in a biphasic manner, and a high volume of distribution was observed, attributed to the extensive uptake of glucosamine into tissues. In the horse, oral doses at currently recommended rates resulted in plasma levels that were below the limits of quantification, necessitating the use of doses approximately 5-10 times greater. Administration of 125mg/kg PO resulted in a Cmax of 10.6 μg/ml.

Recently, a single dose of 20mg/kg GHCl was admininstered to horses both IV and via nasogastric intubation (Laverty *et al* 2005). As expected, mean maximum serum concentrations were greater following IV compared to PO administration (288 +/- 53μM and 5.8 +/- 1.7μM respectively). Synovial fluid levels were also measured 1-hour later at 9-15 μM and 0.3-0.7 μM for IV and PO administration; however, glucosamine was still detectable in synovial fluid from most horses 12 hours after dosing.

Chondroitin sulfate

Similar analytical methods have also been used for the quantification of CS following oral administration. Early studies of intact CS utilized capillary electrophoresis (Pervin *et al* 1994, Volpi 1996) or fluorometric labeling followed by HPLC on hydroxyapatite (Narita *et al* 1995) or size-exclusion columns. The latter technique has been used to quantitatively measure CS both in raw materials and dosage forms (Choi *et al* 2003), and also to determine plasma concentrations (Conte *et al* 1995). Limitations, such as lack of sensitivity to distinguish between CS disaccharides, were apparent in early studies. The

methodology used appeared to influence levels of CS that were measured, with HPLC more accurate than spectrophotometric methods for quantification of CS in dosage forms (Choi *et al* 2003). This prompted the development of other analytical methods incorporating enzymatic digestion to detect the disaccharides, with applications including their detection in plasma (Huang *et al* 1995, Kinoshita *et al* 1999) and urine (Sakai *et al* 2002).

Early studies did not support the oral absorption of CS, as evidenced by the lack of change in serum GAG levels when measured with dimethylene blue (Baici *et al* 1992). Such studies have since been criticized due to the low sensitivity inherent with colorimetric methods, and as dimethylene blue determines all uncharged GAGs not just CS (Lualdi *et al* 1993). Other studies attributed lack of absorption of CS following oral administration to an inability to cross the gastrointestinal tract due to its high molecular weight (Andermann *et al* 1982). The absorption of CS was evaluated using $CaCO_2$ cell monolayers, an *in vitro* model of intestinal epithelium (Cho *et al* 2004). LMWCS traversed the membrane better than a higher molecular weight CS. Using a spectrophotometric assay, differential absorption of CS along the intestinal tract of rats was depicted (Barthe *et al* 2004). CS was transported across the small intestine in its intact form, probably by endocytosis. A greater amount was transported in the large intestine in the form of its constituent disaccharides, with degradation in the colon and cecum.

Similar to glucosamine, radiolabeling techniques have been used to investigate the metabolic fate of CS. In rats and humans, oral CS was rapidly absorbed and tropism for articular cartilage was demonstrated with scintigraphy (Ronca *et al* 1998). In the rat and dog, oral absorption of a single dose of 50% C-4-S and 50% C-6-S was greater than 70%, with plasma radioactivity levels increasing rapidly (Palmierei *et al* 1990). In the rat, distribution of radioactivity in tissues was the same regardless of the route of administration (IM and PO). Chondroitin sulfate rapidly accumulated in cartilage, with cartilage radioactivity levels at 72 hours exceeding levels in other tissues including plasma. Cartilage levels were greater with oral rather than IM administration in the rat. In the dog, synovial fluid concentrations were also measured (depicted as total radioactivity/ml independent of the nature of radioactive compounds which may have been present). Five hours after oral dosing, radioactivity was 66.5% higher in synovial fluid than plasma (10.1 μg/ml and 6.0 μg/ml respectively). In plasma and synovial fluid, molecular weights higher than that administered were observed, presumably due to protein binding. Chromatography of rat articular cartilage at 24 hours after oral administration yielded both high and low molecular weight compounds, indicative of the metabolism of chondroitin sulfate.

Both radiolabeling techniques and size exclusion chromatography were combined to investigate pharmacokinetic properties in the rat and dog (Conte *et al* 1991 and 1995), and supported more than 70% absorption found in other studies (Palmieri *et al* 1990). Gel filtration of plasma and synovial fluid of dogs at 3 and 5 hours post administration depicted the presence of both native labeled compound and N-acetyl-galactosamine, with

radioactivity 66.5% higher than in plasma. Radioactivity accumulated in both synovial fluid and articular cartilage in rats, with mostly high molecular mass CS present in cartilage at 24 hours. Both synovial fluid and cartilage radioactivity levels exceeded plasma levels at 24 hours, with accumulation in cartilage depicted by greater radioactivity at 48 compared to 24 hours. Radioactivity in cartilage exceeded radioactivity in other all other tissues by 48 hours.

Pharmacokinetics have been investigated in humans with a single dose of 0.8g CS once daily or in divided doses twice daily (Conte *et al* 1995). Both dosage regimes resulted in a significant increase in plasma concentration, with no significant difference in time to peak concentration noted. However peak plasma concentration (Cmax) was higher with SID dosing (2.6 μg/ml SID and 1.2 μg/ml BID). Plasma levels were almost constant (around 1.8 ug/ml), with a plateau reached in 2-3 days with SID dosing throughout a 30-day trial conducted in patients with first and second degree OA (based on American Rheumatism Association designation).

Oral bioavailability and pharmacokinetic parameters were investigated in man using a single (4g) oral dose of CS of bovine origin (Condrosurf®) and shark origin (Volpi 2002 and 2003). With the bovine CS, plasma levels CS increased more than 200% from endogenous levels. Basal endogenous disaccharide composition of plasma was approximately 60% non sulfated disaccharide and 40% 4-sulfated disaccharide, with administration of CS decreasing relative amount of non sulfated disaccharide and increasing both 4- and 6-sulfated disaccharide. Maximum plasma concentration was

12.73 μg/ml, higher than that obtained with CS from shark cartilage (Cmax 4.9 μg/ml). Absorption was also slower.

A recent study utilizing pre-column derivatization, HPLC and fluorometric detection showed good resolution of CS disaccharides, with a level of sensitivity of 20-30ηg (Volpi 2000). This technique was recently adapted for the detection of CS disaccharides in dog and horse plasma, and was shown to be valid and specific with high recovery (limit of detection of 1μg/ml) (Du *et al* 2002). An integral component of this study was the investigation of the pharmacokinetics of CS following intravenous administration in dogs (400mg) and horses (3 g). Plasma disaccharide concentrations of 350 μg/ml (dog) and 110 μg/ml (horse) were rapidly achieved, with concentrations below 20 μg/ml by 20 hours post administration in the horse. Oral absorption (3g) has been demonstrated in the horse, with a C_{max} of 36.5 μg/ml (Eddington *et al* 2001). Absorption was rapid (t_{max} 1.32h), with an apparent bioavailability of 22%. Different forms of CS were compared, with the 16.9kDa forms, the active ingredient in Cosequin®, having statistically higher plasma concentration and area under the plasma concentration curve (AUC) compared to an 8kDa product.

Glucosamine and Chondroitin sulfate in combination

The bioavailability and pharmacokinetics of GHCl and LMWCS in combination have been investigated in both the horse and dog (Du *et al* 2004, Adebowale *et al* 2002). Plasma concentrations of glucosamine (Liang *et al* 1999) and chondroitin sulfate (Du *et al* 2002) were quantified using techniques previously described. Single dose

pharmacokinetics was investigated in the horse, with a C_{max} of 349 μg/ml and 10.6 μg/ml following IV and PO administration for glucosamine and 210 μg/ml and 36.5 μg/ml for CS.

In the dog, single oral doses of 1500mg GHCl and 1200mg CS, or 2000mg and 1600mg were administered, and multiple dose pharmacokinetics investigated with BID doses of 1500mg GHCl and 1200mg CS from days 1-7 then 3000mg GHCl and 2400mg CS on days 8-14. The mean bioavailability of glucosamine after single and multiple dosing (12.1-12.7% and 9.7-10.6%) was lower than that previously estimated in radiolabel studies. Radiolabeling may overestimate systemic bioavailability due to failure to detect presystemic metabolism in gastrointestinal tract or liver during absorption as drug and metabolites are not differentiated. There was no significant difference found between single and multiple dose pharmacokinetics. Higher levels of constituent disaccharides ΔDi-4S and ΔDi-6S were found compared to ΔDi-OS, correlating to the low molecular weight chondroitin used in the study (Cosequin® - contains approximately 60% C-4-S and 40% C-6-S). The absorption of glucosamine and CS were rapid with C_{max} of 8.95-12.4 μg/ml and 19.0-21.5 μg/ml respectively (t_{max} around 1.5h). Unlike glucosamine, CS disaccharides accumulated in plasma after multiple dosing depicting a significant carry over effect. C_{max} was 96.3 and 208 μg/ml with PO 1200mg on day 7 and 2400mg on day 14 respectively, with 200% and 278% bioavailability of total disaccharides on a molar basis after multiple dosing with 1200mg and 2400mg respectively.

Clinical Trials

Human

A number of clinical trials have been performed in man with mostly favorable, albeit equivocal, results. Flaws in terms of study design (low number of participants, lack of placebos, inclusion criteria) and assessment of outcome (subjective) were evident in early studies. However moderate symptomatic effects and a good safety profile were demonstrated in a number of short-term trials (Busci *et al* 1998, Leffler *et al* 1999, Muller-Fassbender *et al* 1994, Noack *et al* 1994).

Recently, a meta-analysis and quality assessment of 15 randomized double blind placebo controlled studies in patients with hip or knee OA was reported (McAlindon *et al* 2000). Of these trials, all but one showed positive results in terms of decreasing pain and improving mobility, with overall moderate effects for glucosamine and large effects for chondroitin. However, deficiencies were noted in terms of randomization, blinding and completion rates. Furthermore, publication bias was suggested with most of the trials supported by manufacturers' of these products.

In two randomized double blind placebo controlled trials in patients with knee OA conducted over a six-month period, no additional beneficial analgesic effects of glucosamine were seen compared to placebo (Hughes *et al* 2002, Rindone *et al* 2000); however, a statistically significant difference in degree of knee flexion was noted in the first study. Symptoms were scored based on both subjective assessment and recommended grading scales (visual analog and WOMAC score (Western Ontario and

McMaster University OA index)). Of note, in both studies patients generally had more severe OA than those in other trials.

Two recent long-term randomized double blind placebo controlled trials have identified a beneficial effect of glucosamine sulfate (1500mg/day) in knee OA (Reginster *et al* 2001, Pavelka *et al* 2002). Patients had mild to moderate OA based on American College of Rheumatology classification criteria (Altman *et al* 1986). In the first study, glucosamine resulted in a 20-25% improvement with respect to pain and physical function, with worsening WOMAC scores evident in placebo treated patients. Placebo patients also had progressive joint space narrowing (measured as mean joint space width of the medial compartment of the tibiofemoral joint and minimum joint space width), while glucosamine patients had no significant joint space loss. No significant adverse effects were seen, and no alteration in glycemic homeostasis or other routine laboratory tests were identified. Based on analysis of mean joint space width in the same population, patients with mild OA appeared to be the most responsive to supplementation with glucosamine (Bruyere *et al* 2003). Similar results in terms of symptomatic improvement, slowing of radiographic progression and lack of perceivable joint space narrowing were found in the second long-term study. Since these trials have been published, results have been criticized. These trials utilized a standing antero-posterior fully extended knee view, the recommended "gold standard" (Altman *et al* 1987). It was postulated that by providing symptomatic relief, knee positioning might have been altered, thereby introducing potential systematic error in using measurement of joint space width as an index of OA progression. However, further analysis revealed that pain was not a

confounder in joint space narrowing assessment on this radiographic view (Pavelka *et al* 2003). Further, patients with high cartilage turnover, as measured by urinary collagen type II C-telopeptide fragments, appeared to be most responsive to supplementation with glucosamine (Christgau *et al* 2004)

Fewer trials have been conducted to determine the efficacy of chondroitin sulfate. In a one-year randomized double blind placebo controlled study of knee OA (Uebelhart *et al* 1998), CS significantly reduced pain and increased overall mobility. In addition, joint space narrowing was reduced and biochemical markers stabilized. Results of a similar trial conducted over 6 months were also supportive (Busci *et al* 1998), with CS well tolerated in both trials. In a 3-month trial, CS resulted in significant improvement in clinical symptoms, including joint mobility, with no difference in efficacy appreciated between different dosage regimes (SID versus divided TID) (Bourgeois *et al* 1998). In a 3-year randomized double blind placebo-controlled trial, CS was found to be protective against radiographic progression of finger joint OA (Verbruggen *et al* 1998). Recently, intermittent administration of CS (3 months twice over 1 year) was also found to reduce pain and joint space narrowing in patients with knee OA, suggestive of a prolonged chondroprotective effect (Uebelhart et al 2004).

In a placebo-controlled study of patients with knee OA, synovial fluid parameters improved following CS administration of 3 grams orally once daily. After 5 days, there was a significant decrease in the lysosomal enzyme N-acetylglucosaminidase, and no significant difference in leukocyte count and protein levels. HA levels increased

concurrent with an alteration in molecular mass distribution, resulting in an increase in high molecular weight fractions. The molecular mass of sulfated GAGs also changed. High molecular weight molecules, indicators of cartilage breakdown, decreased and low molecular weight molecules increased, suggestive of the incorporation of exogenous CS (Conte *et al* 1991). Such changes in synovial fluid parameters were supported by another study (Ronca *et al* 1998).

Trials utilizing both glucosamine and chondroitin sulfate in combination have been limited, and have used commercially available products that also contain manganese ascorbate. In a randomized placebo controlled study (Cosamin DS®- GHCl 1000mg, CS 800mg and manganese ascorbate), patients with radiographically mild to moderate OA had significant improvement at 4 and 6 months, whereas there was no significant improvement in patients with radiographically severe OA based on the Lesquene Index of severity (Das *et al* 2000). In a 16 week randomized double blind placebo controlled study of patients with knee or lower back DJD, therapy relieved symptoms of knee OA (based on patient assessment, visual analog scale and physical examination score), but not spinal DJD (Leffler *et al* 1999). In a meta-analysis of oral glucosamine and CS in knee OA, structural efficiacy of glucosamine and sympotmoatic efficacy of both products were demonstrated. This study included analysis of a number of the studies described previously (Richy *et al* 2003).

Recently the efficacy of glucosamine has been compared to other commonly used OA drugs. Glucosamine was found to have greater benefits in terms of reduction of pain

compared to a NSAID (ibuprofen) in a short-term trial of patients with temporomandibular joint OA (Thie *et al* 2001). In another study comparing CS and a NSAID, although NSAID treated patients had prompt reduction of clinical symptoms, signs reappeared following cessation of treatment. Of interest, while the therapeutic response to CS appeared later, benefits lasted up to 3 months after cessation of treatment (Morreale *et al* 1996).

The safety profile of glucosamine and chondroitin has been supported by a number of clinical trials, including those reported above (Towheed *et al* 2001). Initially glucosamine was thought to be associated with the development of diabetes; however, continuous intravenous infusion was required to induce insulin resistance (Patti *et al* 1999, Monauni *et al* 2000). In a recent placebo-controlled study with short-term intravenous infusion of glucosamine, no effect on insulin-induced glucosamine uptake was evident (Pouwels *et al* 2001). Further, results of a recent placebo controlled double blind randomized clinical trial involving patients with Type 2 diabetes, showed that oral glucosamine and CS supplementation had no clinically significant effect on glucose metabolism (Scroggie *et al* 2003). Reported side effects are rare with angioedema reported in one patient due to an immediate hypersensitivity reaction to GS (Matheu *et al* 1999).

Small Animal

In recent years, a number of trials have been performed in small animals, predominantly in the dog. The majority of these trials have utilized GHCl and LMWCS in combination (Cosequin®). In a rabbit instability model of OA, the combination had a

chondroprotective effect, with absence of severe lesions (Mankin grade 7 or greater) along with significant reduction in total linear involvement and total grade histologically. (Lipiello *et al* 2000) This effect was greater than that seen for either agent alone. In a double blind placebo controlled study, dogs were administered Cosequin® or placebo for 21 days prior to the induction of short-term synovitis in the carpus with intra-articular chymopapain (Canapp *et al* 1999). Preadministration significantly reduced both soft tissue phase scintigraphic uptake at 48 days and bone uptake at 41 and 48 days, concurrent with a reduction in the degree of lameness.

Due to the structural similarity of GAGs and heparin, the concurrent use of these products with other platelet inhibitors, such as phenylbutazone or aspirin, is often cautioned (Davidson 2000, Malone 2002). A good safety profile has been demonstrated in the cat and dog (McNamara *et al* 1996 and 2000) following oral administration of Cosequin® at doses exceeding the recommended daily dose for 30 days. Biochemical, hematological and hemostatic indices remained within normal limits, with only minor but not clinically significant changes in hemoglobin content and total white cell count. There was no effect on clotting times. In a study in rats, the therapeutic margin with regard to prolonged administration was more than 10-30 times more favorable for GS than for the NSAID indomethacin (Setnikar *et al* 1991).

In a double blind placebo controlled study of oral Cosequin® in a cranial cruciate rupture model of OA in dogs, treated dogs had decreased mean modified Mankin scores (Hulse *et al* 1998). Using the same model of OA, long-term (5 month) administration of the same

product resulted in modulation of articular cartilage metabolism reflected by an increase in synovial fluid 3B3 and 7D4 epitope concentrations (Johnson *et al* 2001) - chain length and sulfation patterns of chondroitin sulfate alter early in OA, exposing unique epitopes that can be detected by immunoassay with monoclonal antibodies (Hardingham 1998). Systemic effects were also noted with similar changes in the contra-lateral non-operated joint. There was however a delay of four weeks before GAGs with 3B3 epitope were released into the synovial fluid. A 42% increase in serum GAGs was noted in dogs in a 30-day trial using the same product (Lippiello *et al* 1999). Further, when this serum was used to incubate cartilage segments *in vitro*, GAG biosynthetic rate significantly increased by 50%, with a concomitant reduction in proteolytic degradation of 59%.

Recently, a multi-centered, double blind, randomized clinical trial was initiated to compare a glucosamine/chondroitin sulfate preparation (containing C-4-S, GHCl, NAG, ascorbic acid and zinc sulfate) to carprofen, a NSAID (McCarthy *et al* 2003). Dogs included in the study had radiographic evidence of hip or elbow OA and were treated for 70 days, with reassessment one month following cessation of treatment. Preliminary results were encouraging with improvement in clinical parameters such as lameness.

Equine

To date, clinical trials in the horse have been limited. A randomized double blind placebo controlled clinical trial was conducted in horses 5-15 years of age with progressive forelimb lameness of 3-12 months duration due to navicular disease. Horses were administered Cosequin® (9gms GHCl, 3gms LMWCS, 600mg manganese) or placebo

twice daily for 56 days. A statistically significant improvement in overall clinical score, overall clinical condition and total lameness was observed, with no side effects reported (Hanson *et al* 2001). Another study into the safety profile of this oral dosage form was conducted in which horses were administered 5 times the recommended daily dose for 34 days (Kirker-Head *et al* 2001). No clinically significant changes in hematological, biochemical or synovial fluid values were observed, and no adverse effects noted. Of interest, hematocrit, hemoglobin levels, and total white cell count were significantly increased.

Cosequin® was also used in a 6 week study of possible beneficial effects in the treatment of OA in 6-20 year old horses (Hanson *et al* 1997). Physical examination, intra-articular analgesia, radiographs and/or fluoroscopy were used to confirm a diagnosis of OA of the distal interphalangeal, metacarpophalangeal, tarsometatarsal or carpal joints. Within two weeks, a significant improvement was noted in lameness grade, flexion test grade and stride length. By four weeks, there was no further improvement in lameness grade and no significant changes in other parameters. Age was not found to be a significant factor. A number of problems with trial design should be recognized, including lack of placebos and lack of blinding of assessors to treatment method. Furthermore, as most horses were able to return to exercise and competition after the initial two wks of treatment, this may explain the leveling in improvement seen after 2 weeks. Again, no side effects were noticed.

Other studies using Cosequin® have had conflicting results. Lameness measured by force plate analysis resolved (Hanson 1996); however, no beneficial effects were seen in a Freunds adjuvant model of synovitis (White *et al* 1994). The efficacy of IM NAG and CS was found to be significantly less than that of a PSGAG using the same model (White *et al* 2003). Recently a double blind placebo controlled study was performed using precursors of glucosamine (Cortaflex®: containing glutamine, glutamic acid, glycine and glucuronic acid-loading dose 60mls/day 5 days then 30mls per day) (Clayton *et al* 2002). Horses included in the study had OA of the distal intertarsal and/or tarsometatarsal joints. Using force plate analysis, an improvement in gait symmetry measured by vertical ground reaction force was evident.

In two studies of the effect of supplementation with glucosamine on serum markers, no significant influence on keratan sulfate, osteocalcin or pyridinoline crosslinks were observed (Fenton *et al* 1999, Caron *et al* 2002). Horses in these studies were clinically normal with no evidence of OA.

The efficacy of exogenous intramuscular and oral chondroitin sulfate in an experimentally induced model of equine arthritis has been investigated (Dorna *et al* 1998). Both routes of administration were associated with improved joint function and reduced lameness scores. Time of onset for clinical improvement was slower with oral administration however the maximum level of improvement achieved was comparable for both routes of administration.

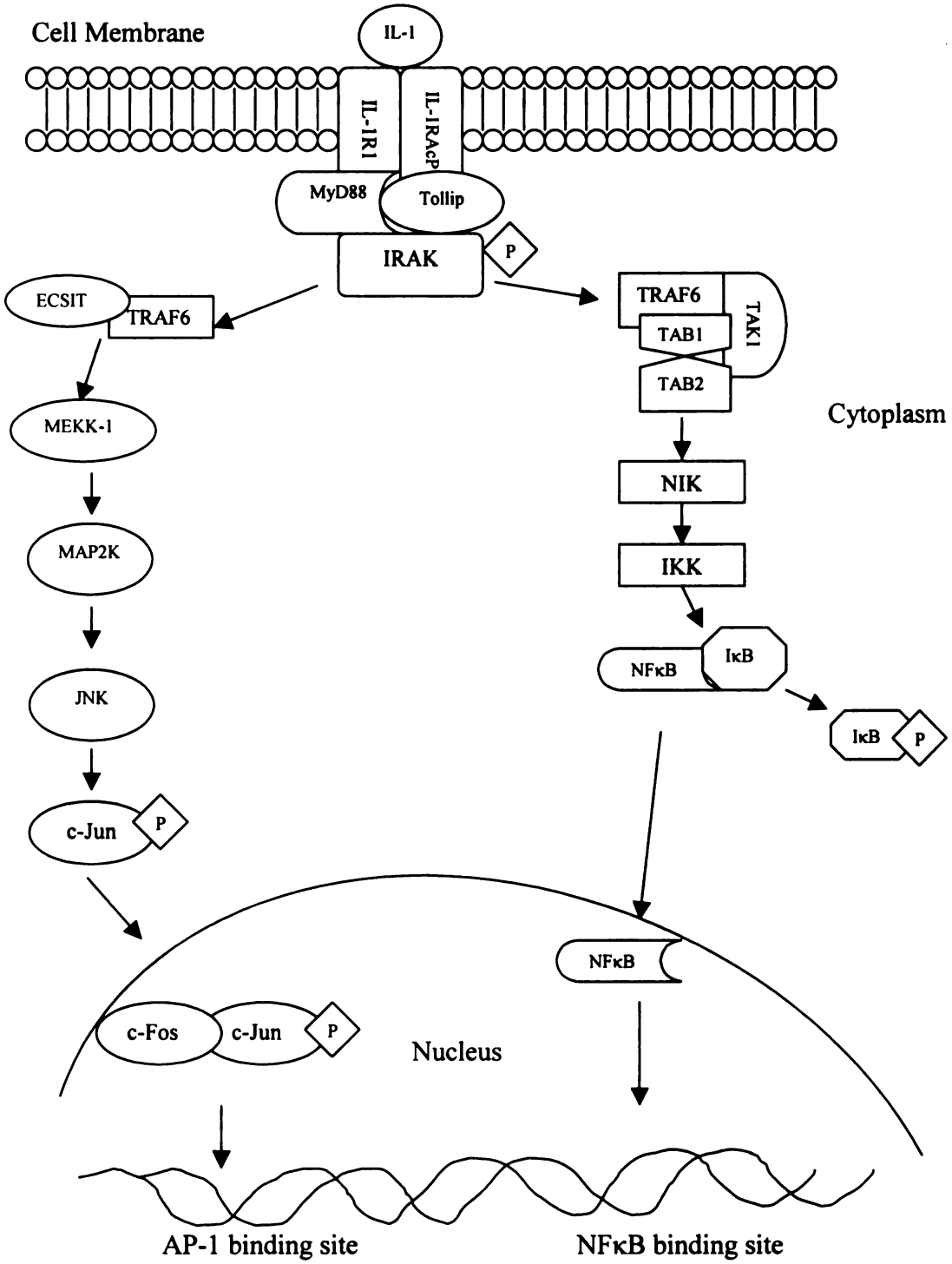

Figure 1 (chapter 1): Interleukin-1 Signaling Pathways.

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CHAPTER 2: DETERMINATION OF A SUBSATURATING DOSE OF RECOMBINANT EQUINE INTERLEUKIN-1β FOR GENES IMPLICATED IN CARTILAGE DEGRADATION IN OSTEOARTHRITIS

Summary

Objective: To determine a subsaturating dose of recombinant equine interleukin-1β (reIL-1β) corresponding to approximately half maximal induction of expression of genes involved in cartilage degradation.

Design: Equine chondrocytes in pellet cultures were exposed to incremental doses of reIL-1β at concentrations ranging from 0 to 20,000 ρg/ml. RNA was isolated after a 6-hour incubation and effects on gene expression of matrix metalloproteinase 13 (MMP 13), inducble nitric oxide synthase (iNOS), cyclooxygenase 2 (COX 2), aggrecanase-1 (Agg 1), tissue inhibitor of metalloproteinases 1 (TIMP 1), type II collagen, nuclear factor kappaB (NFκB), and interleukin receprot antagonist (ILRa) assessed with quantitative real-time polymerase chain reaction (Q-RT-PCR).

Results: reIL-1β resulted in dose dependent saturable up regulation of expression of iNOS, COX 2, Agg 1, MMP 13 and ILRa. The effect on TIMP 1 mRNA was minimal, except at high doses of reIL-1β. At low doses (10-500 ρg/ml), type II collagen gene expression increased; however, higher doses reduced gene expression. NFκB gene expression was upregulated at all doses except the highest dose (20,000 ρg /ml).

Conclusion: A subsaturating dose of 500 ρg/ml was determined to result in approximately half-maximal effect on the majority of the genes of interest.

Introduction

The hallmark of osteoarthritis (OA) is the progressive and permanent degeneration of articular cartilage. Establishment of the role of cytokines in OA (Martel-Pelletier *et al* 1999) prompted the use of cytokines, including interleukins, to create *in vitro* models of OA. Interleukin-1 (IL-1) is a primary mediator of the degradative processes characteristic of OA, with elevated levels of IL-1-like biological activity in the synovial fluid of horses with clinical OA supportive of its role in the disease (Morris *et al* 1990, Alwan *et al* 1991). Pharmacological inhibition of the effects of IL-1 on equine cartilage, as a means to slow or arrest OA progression, illustrates its importance in OA (Caron *et al* 1996a, Frisbie *et al* 2000, Frean *et al* 2000).

The response of cartilage to IL-1 stimulation is multifactorial. Extracellular matrix synthesis is inhibited and the production of inflammatory mediators and matrix degrading enzymes predominate, leading to proteoglycan loss, cleavage of type II collagen and degradation of aggrecan, events which are synonymous with OA. *In vitro*, IL-1 stimulation induces synthesis of phospholipase A_2, COX 2, PGE_2, and iNOS; and inhibits collagen synthesis (type II, IX, and XI) (Cook *et al* 2000, Murrell *et al* 1995, Tung *et al* 2002a). Further, IL-1 induces MMP synthesis (Caron *et al* 1996b, Reboul *et al* 1996, Richardson *et al* 2000), GAG release into media and aggrecanase activity (Cawston *et al* 1999). Effects on PG homeostasis include both dose dependent induction of PG degradation (Frean *et al* 2000) and inhibition of PG synthesis (Morris *et al* 1994, Frisbie *et al* 1997, MacDonald *et al* 1992, Platt *et al* 1994).

Stimulation of cartilage with recombinant human interleukin-1 (rhIL-1) *in vitro* suppresses synthesis of type II collagen (Goldring *et al* 1988) and aggrecan (Richardson *et al* 2000), and induces synthesis of MMPs, PGE_2 and NO (Morris *et al* 1994, Caron *et al* 1996b), and induces ECM degradation (MacDonald *et al* 1992, Frisbie *et al* 2000). An age effect was identified, with an overall reduced response to IL-1 in cartilage obtained from older humans and horses (Ismaiel *et al* 1992, May *et al* 1992b, Morris *et al* 1994, MacDonald *et al* 1992). Further, the response to IL-1 varies not only between species, but also with the source of IL-1, with dissimilar and disproportionate stimulation of PGE_2 and MMP synthesis by chondrocytes and synoviocytes following stimulation with rhIL-1 and a partially purified form of equine IL-1 (May *et al* 1990). The form in which chondrocytes are propagated in culture also has an influence on the concentration of cytokine required to produce a given stimulus. Typically, a higher dose is employed for the stimulation of chondrocytes in explants than for monolayer cultures.

Recombinant equine interleukin-1 (reIL-1) is a species-specific interleukin developed following concerns that, due to limited sequence identity between equine and human IL-1β, the response of equine cartilage to rhIL-1 may not be entirely representative of the *in vivo* situation (Tung *et al* 2002a). Human synoviocytes and chondrocytes required only 5% of receptors to be occupied for half maximal stimulation of MMPs by rhIL-1β (Martel-Pelletier *et al* 1992). In contrast, 2-3 times

greater concentration was needed to saturate all receptor sites to initiate low-level MMP secretion in other species (Chin *et al* 1990).

In equine culture systems, purified reIL-1β resulted in dose saturable up regulation of MMP 1, 3, 13, TIMP1, and COX 2 gene expression (Tung *et al* 2002a); and dose dependently increased PGE_2 synthesis and PG release into media (Takafuji *et al* 2002). While catabolic and anabolic effects were in agreement with previous studies utilizing rhIL-1 (MacDonald *et al* 1992, Platt *et al* 1994, Morris *et al* 1994), concentrations of reIL-1 were 40-100 times lower.

The choice of an appropriate *in vitro* model of equine OA that appropriately reflects the *in vivo* disease process has been debatable. Both IL-1 and lipopolysaccharide (LPS) have been used *in vitro* to stimulate OA, and result in similar metabolic responses and cartilage degradation (Fenton *et al* 2000). However, horses are particularly sensitive to the effects of LPS relative to other species (MacDonald *et al* 1994). The use of a species-specific interleukin, at a subsaturating dose, appears to be a useful means of study of a number of pathophysiological events in equine OA. The objective of the study reported here was to determine the ideal dose of reIL-1β to result in half-maximal induction of a variety of genes implicated in cartilage degradation in OA using chondrocytes in pellet culture.

Materials and Methods

Tissue Sources-Pellet Cultures

Grossly normal metacarpophalangeal articular cartilage was obtained from horses between 2 and 8 years of age that died or were euthanized for reasons other than joint disease. Chondrocyte isolation and propagation in pellet culture was conducted as previously described with minor modifications (Caron *et al* 1996b). Breifly, cartilage was dissected from the subchondral bone and incubated in physiologic saline (0.9% NaCl) solution containing penicillin (500 U/ml) and streptomycin[a] (500 mg/ml) (25 °C, 1 hour). Chondrocytes were isolated by sequential digestion with pronase[b] (1mg/ml, 1 hour) and collagenase[a] (0.3mg/ml, 18 hours) as previously described. After digestion, cells were separated by sequential centrifugation (300 x g, 10 minutes, repeated 3 times), washed, and re-suspended in 20ml Dulbecco's modified Eagle's medium (DMEM):nutrient mixture F-12 (Ham) (1:1)[b]. The media was supplemented with insulin-transferrin-sodium selenite supplement[c] (5 μg/ml insulin, 5 μg/ml transferrin, 5 ηg/ml sodium selenite), 50 ug/ml ascorbic acid[b], amino acids[b], 2 μg/ml lactalbumin hydrolysate[b], 5 μg/ml linoleic acid[b], 40 ηg/ml thyroxine[b], and 100 U/ml penicillin/streptomycin[a]. The concentration of amino acids was 50% of those previously reported (Rosselot *et al* 1992).

Cell concentration was determined with a hemocytometer and aliquots of 3 X 10^6 cells were transferred to 15 ml polypropylene centrifuge tubes in 1 ml supplemented serum free media described above. Following centrifugation (300 X g, 5 minutes), pellets were incubated under standard cell culture conditions (37 °C, 95% RH, 5%

CO_2). Medium was changed every 3 days. Pellet cultures were maintained in serum free media without supplementation with insulin-transferrin-sodium selenite supplement, linoleic acid, thyroxine and ascorbic acid for 2 days prior to the start of an experiment for equilibration. At the conclusion of experiments pellets were collected for isolation of total RNA.

RNA Isolation

Total RNA was extracted, using a commercial extraction preparation[d] and RNA isolation kit[e] and following manufacturer's instructions with minor modifications (Reno *et al* 1997). Briefly, 1 ml of this agent was added to each pellet after removal of media, incubated (25°C, 5 min), placed on plate shaker (25°C, 5 min) and transferred to microcentrifuge tubes. Following centrifugation (10,000 X g, 10 min), 200 µl chloroform was added to extract total RNA followed by agitation and a second incubation (25°C, 2 min). The aqueous phase containing RNA was collected after centrifugation (4°C, 12,000 X g, 15 min) and RNA precipitated with an equal volume of 75% ethanol. RNA was then purified further with RNeasy mini spin columns[e]. Pellets were re-suspended in RN-ase free water (0.1% diethylpyrocarbonate, supplied) and centrifuged (10,000 x g, 1 min) to elute RNA. RNA was analyzed by electrophoresis through 1% agarose gels containing 10µg/ml ethidium bromide in 1 x MOPS (3-(N-morpholino) propanesulfonic acid to validate spectrophotometric determination and RNA integrity. RNA was quantified by UV spectrophotometry[f], and adjusted with RN-ase free water to 1 µg/µl solutions.

Quantitative Real Time PCR

One microgram of each RNA sample was treated with Dnase[g] to degrade contaminating single and double stranded DNA. Treated RNA was converted to single stranded cDNA using Superscript II reverse transcriptase[g] as recommended by the manufacturer. Final cDNA pellets were resuspended in 20 µl of RNase-free water. One microliter of each cDNA template primed each PCR reaction[h] using Taq DNA polymerase[g]. PCR derived cDNA was analyzed by electrophoresis through 1.5% agarose gels in Tris-HCL-acetate-EDTA (TAE) gels containing 10µg/ml ethidium bromide. cDNA was quantified by UV spectrophotometry, and adjusted with Rnase-free water to 50ηg/µl.

Reference amplicons were developed using equine cartilage RNA-derived cDNA using specific primers designed by Primer Express software Version 2.0[i] and were synthesized by a commercial facility[j]. Nucleotide sequences used for primer design were obtained from public databases (Genbank[k]) as full length or partial equine sequences were available for glyceraldehyde phosphate dehydrogenase (GAPDH), inducible nitric oxide synthase (iNOS), matrix metalloproteinase 13 (MMP 13), tissue inhibitor of metalloproteinases-1 (TIMP 1), aggrecanase-1 (Agg 1), cyclo-oxygenase 2 (COX 2), type II collagen, and interleukin-1 receptor antagonist (ILRa). When no equine sequence was available (nuclear factor kappaB (NFκB), equine expressed sequence tag (EST) sequences corresponding to the target gene were used based on similarity to the corresponding mouse sequence. BLASTN[k] searches for all of the primer and amplicon sequences were conducted to ensure gene specificity.

Optimal concentrations of each set of primers were determined with a primer matrix (lowest standard deviation with no change in cycle to threshold (Ct)). Primer sequences used for each gene are summarized in Table I.

Fifty nanograms of sample cDNA primed each real-time PCR reaction. All analyses were conducted in a PE 7700 DNA sequence detection system[l]. The cDNA templates were combined with optimal concentrations of primers and SYBR Green PCR dye mix[i] in a total volume of 50 µl and the amplification conducted as recommended by the manufacturer. The PCR conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles of extension at 95°C for 15 sec and 1 min at 60°C. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curves. The software automatically recorded the Ct. Analysis of each sample was performed in duplicate, and a standard deviation of <0.5 set for control of pipetting error. Dissociation curves were checked for each sample to verify that the correct product was amplified with specific melting temperatures compared to those calculated by Primer Express. For each set of amplification reactions, GAPDH was used as an endogenous control for normalization of RNA loading, cDNA synthesis efficiency, and amplification efficiency. Reference amplicons for each gene and primer sets were functional based on test amplifications using equine cartilage RNA-derived cDNA. The supplemented serum free control was used as a calibrator (ie the fold change for control is 1.0). Replicated data was normalized with GAPDH and the fold change in gene expression relative to serum free control treatment was calculated using the Delta Delta Ct method (Livak *et al* 2001).

Stimulation with reIL-1β

ReIL-1β was purified as described previously (Tung *et al* 2002). After an 8-12 day period to establish a stable metabolism, reIL-1β was added in increments to achieve final concentrations ranging from 0 to 20,000 ρg/ml (0, 10, 50, 100, 500, 1000, 5000, 10000, 20000 ρg/ml). Pellets were harvested after a 6-hour incubation and RNA isolated for Q-RT-PCR. The subsaturating dose of reIL-1 to be used was determined using tissues of 5 horses.

Results

Inflammatory mediators

Exposure of pellet cultures to graduated concentrations of reIL-1β resulted in dose dependent saturable up regulation of expression of both iNOS and COX 2 (Figure 1). The dose of cytokine corresponding to approximately half maximal induction of expression of COX 2 and iNOS was approximately 500 ρg/ml. This dose resulted in 2 to 5-fold increase in expression of both genes.

Extracellular Matrix Proteins

The effect on type II collagen gene expression was dose dependent (Figure 2). At low doses (10-50 ρg/ml), gene expression was increased, however, higher doses inhibited gene expression. There was large variability in expression levels between horses at intermediate doses. Less variability was apparent with higher doses (5,000 – 20,000 ρg/ml), with down regulation of gene expression at these doses for all horses. A dose

of 500 ρg/ml was selected as a subsaturating dose, with an intermediary effect on type II collagen gene expression in this model.

Transcription Factors

The effect of increasing reIL-1β dose on NFκB gene expression was variable (Figure 2). The response appeared to be dose dependent, with increased gene expression at all doses apart from the highest dose (20,000 ρg/ml), which consistently reduced NFκB expression in all horses. However, maximal elevation in expression appeared biphasic, occurring at 50 and 10,000 ρg/ml. One horse (3 year old Quarterhorse) was particularly sensitive to reIL-1β stimulation in terms of NFκB expression. As for other genes investigated, 500 ρg/ml appeared a suitable sub-saturating dose for NFκB.

Degradative Enzymes and their inhibitors

The response to reIL-1β stimulation for aggrecanase-1, MMP 13, TIMP 1 and ILRa was gene dependent (Figure 3). All horses had reduced MMP 13 expression at low doses (10 to 100 ρg/ml). A dose of 500 ρg/ml resulted in approximately half-maximal stimulation of MMP 13; however, gene expression did not increase markedly with increasing doses up to 10,000 ρg/ml. Gene expression appeared to be dose saturable, with reduced expression at the highest dose.

Stimulation with reIL-1β resulted in dose dependent saturable up regulation of Agg 1 gene expression. For all but one horse, a dose of 1000 ρg/ml resulted in maximal

induction of gene expression. Gene expression subsequently decreased at higher doses. Thus, although a dose of 500 ρg/ml resulted in maximal gene expression in one of the 5 horses used, 500 ρg/ml proved a suitable dose.

ReIL-1β stimulation had little effect on TIMP 1 gene expression, except at the highest dose. Although response at this dose was variable between horses, 3 of 5 horses had maximal induction of TIMP 1 expression at this dose. The dose of reIL-1β corresponding to maximal IL-1Ra expression varied between horses, with maximal expression between 10 to 500 ρg/ml. Doses higher than 500 ρg/ml subsequently reduced IL-1Ra expression relative to the maximal dose.

Discussion

Exposure of chondrocytes to reIL-1β resulted in upregulation of gene expression of inflammatory mediators, COX 2 and iNOS, and degradative enzymes, MMP 13 and Agg 1. This IL-1 induction of gene expression was in agreement with previous studies utilizing both rhIL-1 (Caron *et al* 1996, Morris *et al* 1994, Richardson *et al* 2000) and reIL-1 (Tung *et al* 2002a), with expression of these genes being both dose dependent and saturable.

Minimal effect on TIMP 1 expression was apparent, except at the highest dose of reIL-1β. While this is in agreement with some studies in which IL-1 had no effect on TIMP 1 mRNA levels (Martel-Pelletier *et al* 1991), other studies have documented

an increase in TIMP expression with exposure to IL (Shingu *et al* 1993), including reIL-1β (Tung *et al* 2002a).

IL-1 stimulation dose-dependently increased IL-1Ra gene expression; however this effect appeared to be saturable, with reduced expression at higher doses. IL-1 induced IL-1Ra synthesis in human articular chondrocytes, and OA cartilage spontaneously produced higher levels of IL-1Ra than normal cartilage (Maneiro *et al* 2001). A relative deficit in IL-1Ra production has been demonstrated in OA synovium (Pelletier *et al* 1996). IL-1Ra functions as a competitive inhibitor of IL-1R, and is capable of inhibiting a number of pathological events in OA including synoviocyte PGE_2 synthesis, chondrocyte collagenase production, and degradation of ECM (Pelletier *et al* 1999). Elevated IL-1Ra expression at low doses in this study may be an attempt by cartilage to protect against and inhibit the effect of IL-1. Higher doses decreased expression, to levels equivalent to or slightly greater than unstimulated controls. Other factors may influence ILRa expression, as nitric oxide has been shown to reduce ILRa expression by chondrocytes stimulated with rhIL-1β (Pelletier *et al* 1996).

The effect of IL-1 stimulation on NFκB expression in equine cartilage has not been previously documented. NFκB is a transcription factor in the IL-1 signaling pathway. NFκB binding sites are present on the promoter regions of a number of inflammatory genes, including IL-1, MMPs, iNOS, and COX 2 (Mengshol *et al* 2000, Tak *et al* 2001). IL-1 induced activation of NFκB in rat articular chondrocytes

(Gouze *et al* 2002), and induced a time dependent increase in NFκB activity in human OA cartilage *in vitro* (Largo *et al* 2003). In this model, reIL-1β induced NFκB gene expression at all doses except the highest dose. This effect was variable depending on the dose, hence dose related kinetics were not obvious and require further elucidation. Further, variability in response between different horses was high. However, elevation in NFκB expression concurrent with elevation in inflammatory mediators and degradative enzymes such as iNOS, COX 2 and MMP13, may be one mechanism by which IL-1 exerts its effects in articular cartilage.

The response of type II collagen gene expression was dose dependent. Increased expression at low doses may be representative of an attempt of cartilage at repair, as occurs with the initial stages of OA (Aigner *et al* 2002). Higher doses reduced type II collagen expression, in agreement with previous studies (Cook *et al* 2001, Goldring *et al* 1988), consistent with extracellular matrix degradation as has been previously reported (Tyler *et al* 1988, Richardson *et al* 1997 and 2000).

The response of individual horses to IL-1 stimulation varied, in agreement with previous studies in which cartilage derived from some humans was susceptible, and others refractory to IL-1 stimulation (Ismaiel *et al* 1992). Further, an age related response to IL-1 stimulation has been documented previously (MacDonald *et al* 1992). Age may have had an influence in our study because of the diversity in the ages of horses from which cartilage was obtained; however, 3 of the 5 horses used

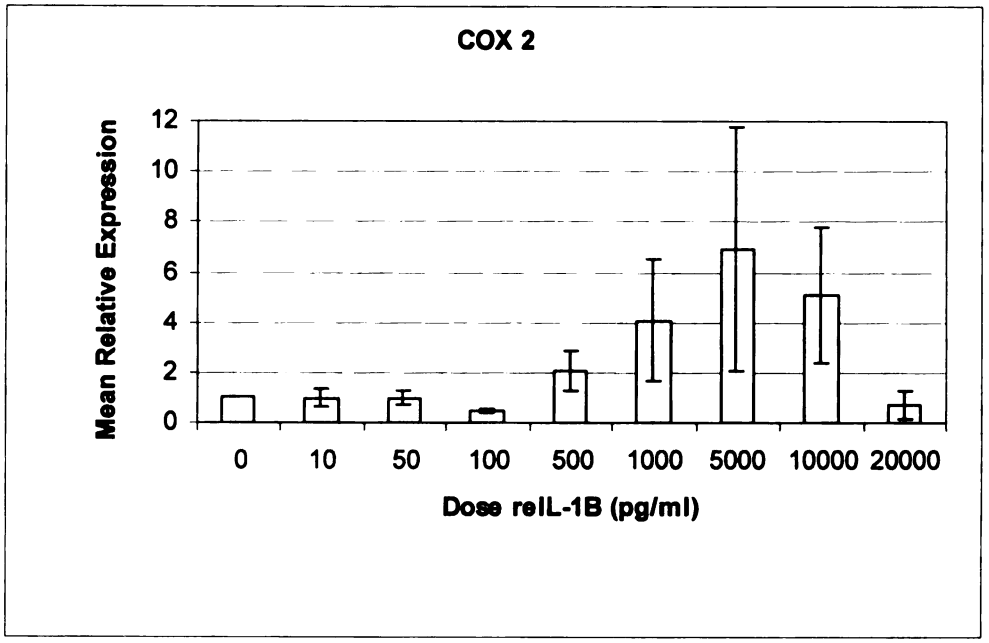
were the same age. The subsaturating dose of reIL-1β determined in this study (500 ρg/ml) is lower than that used in other studies utilizing rhIL-1β (Caron *et al* 1996b, Fenton *et al* 2000) and reIL-1β (Tung *et al* 2002a,b). In these studies, doses between 1 and 100 ηg/ml were tested, with dose saturable kinetics demonstrated for MMP 1, 3, 13, TIMP 1, COX 2 and iNOS. However, in a further study with monolayer cultures, a subsaturating dose of reIL-1β of 50 ρg/ml resulted in half-maximal stimulation of MMP 1 (Tung *et al* 2002c). In that study, the 50 ρg/ml dose gave approximately 2-fold increase in gene expression of MMP 3, 13 and TIMP 1. In the study presented here, the equivalent dose failed to upregulate MMP13, Agg 1 and COX 2 but did upregulate iNOS, ILRa and NFκB.

Equivalent or higher doses of IL-1 that consistently saturated gene expression in this study have been previously used to determine both response to IL-1 stimulation and the ability of compounds to attenuate the response to IL-1 (Fenton *et al* 2002). Such studies have been criticized as a detrimental effect on cell viability has been documented *in vitro* using 20 ηg/ml of IL-1 (Dvorak *et al* 2002, Cook *et al* 2000). The aim of this study was to determine a dose that may be more representative of the *in vivo* disease process; however, choice of a subsaturating dose may depend on a number of factors including the species tested, culture conditions, and methodology used to determine gene expression. For instance, expression was quantified with Q-RT-PCR, a method that is more sensitive for determination of mRNA expression in articular cartilage than Northern hybridization (Fehr *et al* 2000).

The development of a subsaturating dose of reIL-1β for genes implicated in cartilage degradation in OA provides a model that may be more representative of the *in vivo* disease process. While individual horse and gene variation are apparent, this dose of 500ρg/ml is thought to be intermediary in its effects on gene expression and will be used in subsequent experiments.

Table I (chapter 2): Forward and reverse primer sequences (5'→3') used for quantitative real-time polymerase chain reaction.

Gene	Genbank Accession	Forward primer	Reverse Primer
Agg 1	AF368321	TTTCCCTGGCAAGGACTATGA	GCGGACAATGGCGTGAGT
COX 2	AB041771	GGAGCTGTATCCTGCCCTTCT	CCATGGTCTCCCCGAAGAT
GAPDH	AF157626	CCCACCCCTAACGTGTCAGT	TCTCATCGTATTTGGCAGCTTTC
iNOS	AY027883	GGCCTTGGCTCCAGCAT	GGTGAGACAACTTCTGGTCAATGT
IL-1Ra	AF072535	TGCTCTATTCCTGGGACTCCAT	TGGTTCCTAATCTCATCACCAGACT
MMP 13	AF034087	TGAAATCATACTACTATCCCCTTAATCCT	TCTCGGAGCCTGTCAACCA
NFκB	BI960845 (84% homology to mouse sequence AF155373)	CATCGAGCAGATAGCTCACGTT	AGGTGGTTGGTGAGATTGACAA
TIMP 1	U95039	AGCCTCTGCGGATACTTCCA	TTGTCCGGCGATGAGAAAC
Type II collagen	U62528	AAGAGCGGAGACTACTGGATTGAC	TCCATGTTGCAGAAAACCTTCA



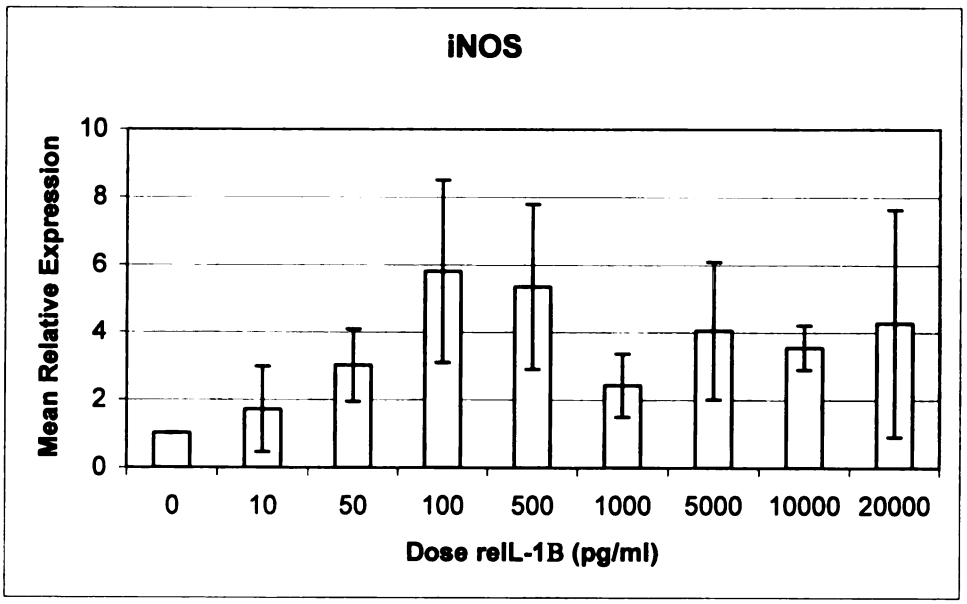
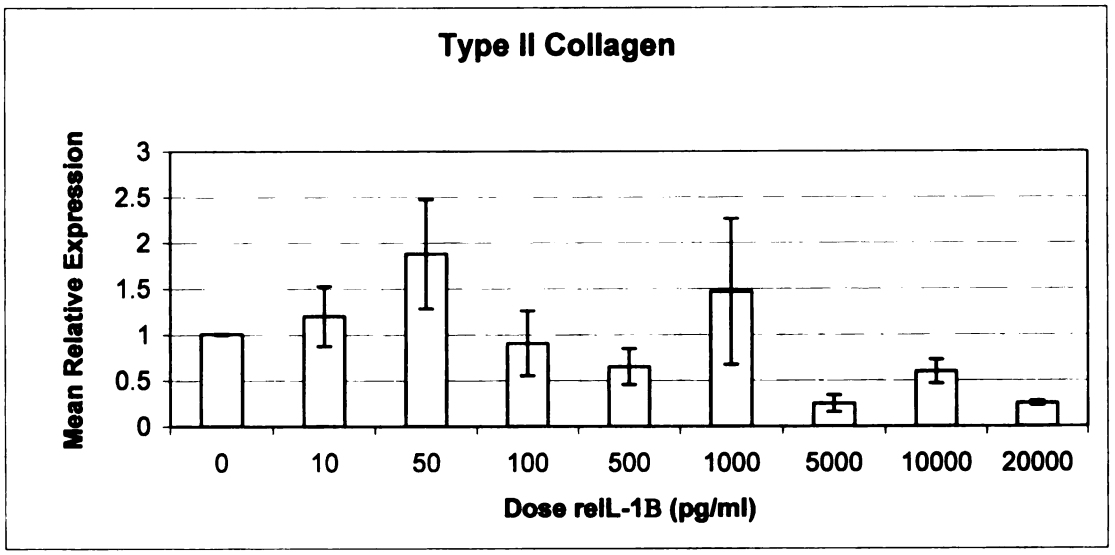


Figure 1 (chapter 2): Relative expression of inflammatory mediators, COX 2 and iNOS, in equine chondrocyte cultures in response to graduated doses of recombinant equine interleukin-1β (reIL-1β).



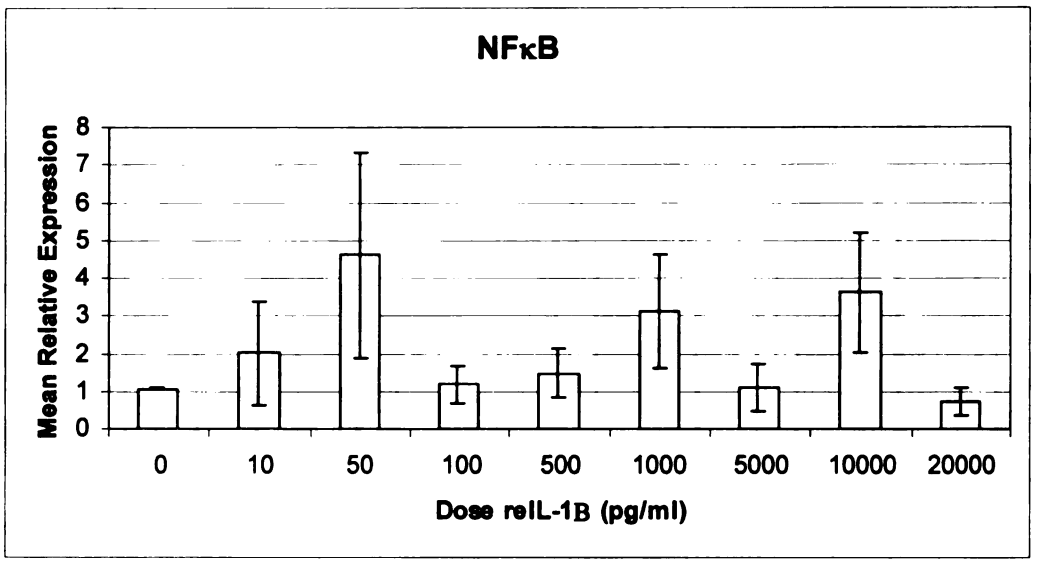
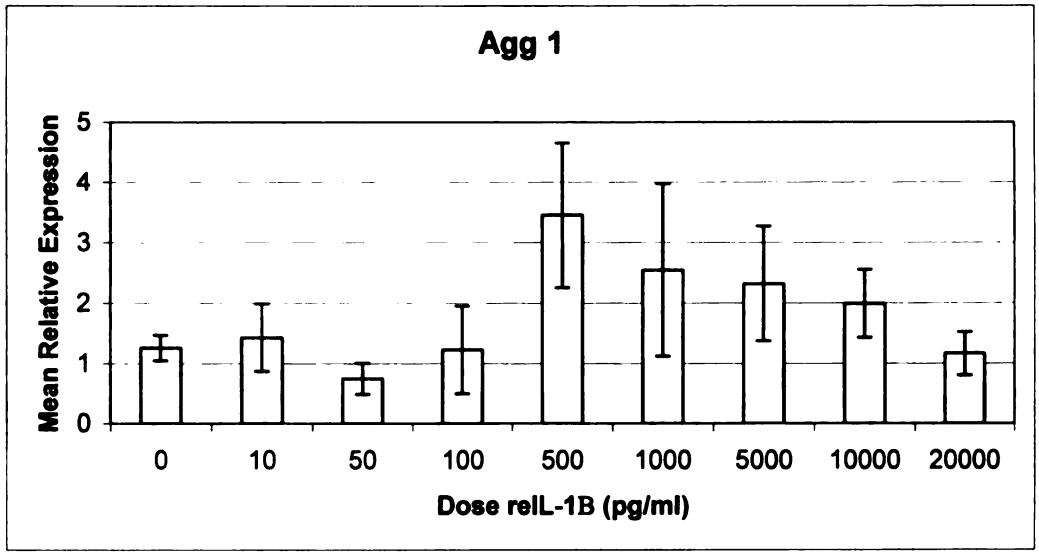


Figure 2 (chapter 2): Relative expression of extracellular matrix protein, type II collagen, and transcription factor, NFκB, in equine chondrocyte cultures in response to graduated doses of recombinant equine interleukin-1β (reIL-1β).



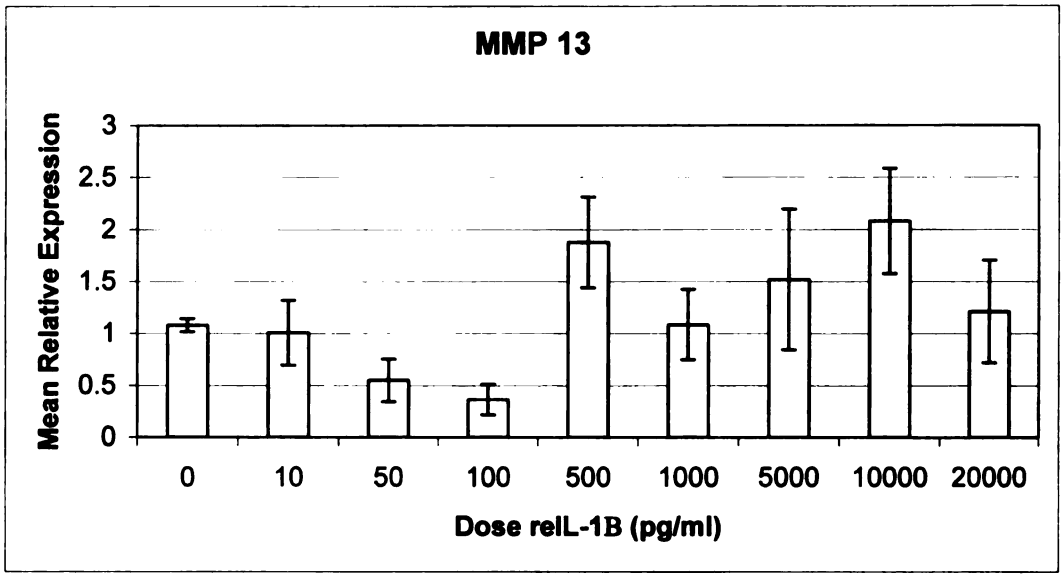
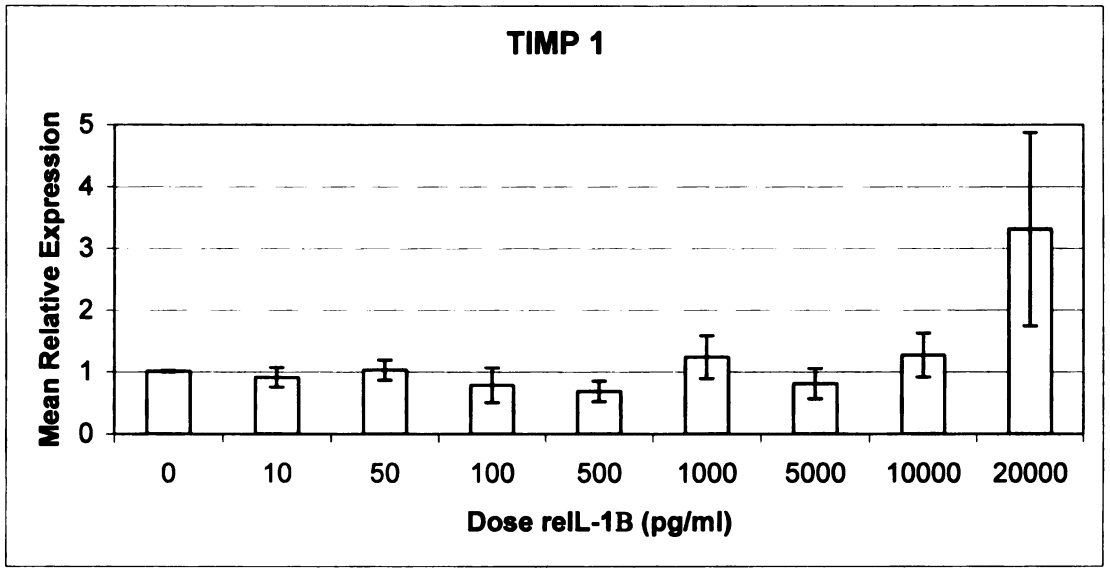


Figure 3A (chapter 2): Relative expression of degradative enzymes MMP 13 and Agg 1 in equine chondrocyte cultures in response to graduated doses of recombinant equine interleukin-1β (reIL-1β).



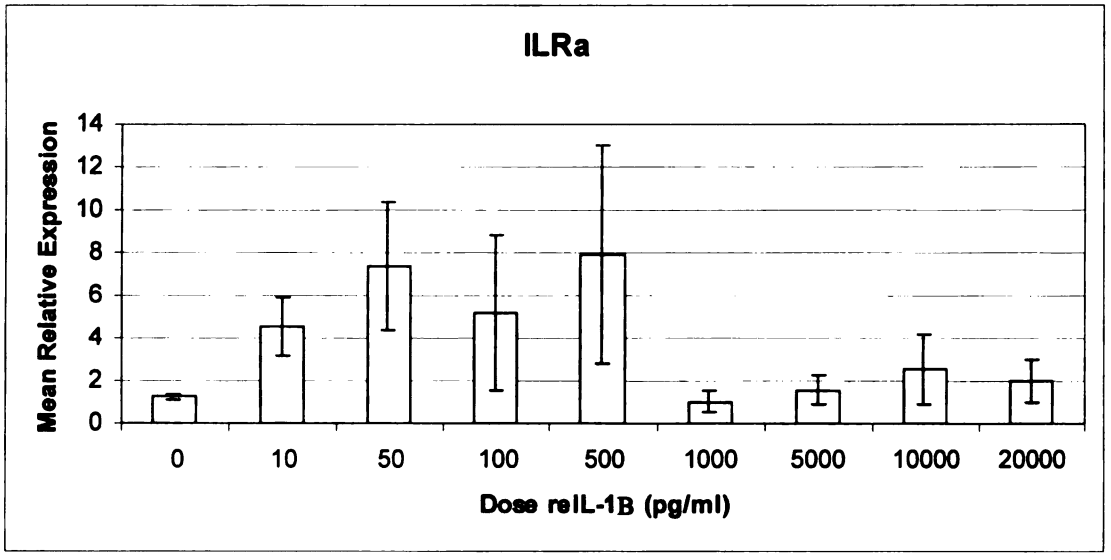


Figure 3B (chapter 2): Relative expression of inhibitors (TIMP 1, ILRa) in equine chondrocyte cultures in response to graduated doses of recombinant equine interleukin-1β (reIL-1β).

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CHAPTER 3: GLUCOSAMINE AND CHONDROITIN SULFATE REGULATION OF MEDIATORS OF OSTEOARTHRITIS IN RECOMBINANT EQUINE INTERLEUKIN-1β STIMULATED EQUINE CHONDROCYTES IN PELLET CULTURE.

Summary

Objective: To determine if glucosamine (GLN) or chondroitin sulfate (CS), at concentrations approximating those achieved by oral administration, influence gene expression of selected mediators of osteoarthritis in cytokine-stimulated equine articular chondrocytes.

Methods: Using equine chondrocytes in pellet culture stimulated with a sub-saturating dose of recombinant equine interleukin - 1β, the effects of preincubation with GLN (2.5 - 10.0 μg/ml) and CS (5.0 - 50.0 μg/ml) on gene expression of matrix metalloproteinases (MMPs) 1,2,3,9,13, aggrecanase (Agg) 1,2, inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX 2), nuclear factor κB (NFκB) and cjun-N-terminal kinase (JNK) were assessed with quantitative real-time polymerase chain reaction (Q-RT-PCR).

Results: Glucosamine significantly reduced reIL-1β induced mRNA expression of MMP 13, Agg 1, and JNK at 10.0 μg/ml. A trend for reduction in cytokine-induced expression was also observed for iNOS (P = 0.06) and COX 2 (P = 0.08). Chondroitin sulfate had no effect on gene expression at the concentrations tested.

Conclusion: Concentrations of GS achieved following oral administration in horses exerts pre-translational regulation of some putative mediators of osteoarthritis, an effect that may contribute to the cartilage-sparing properties of this aminomonosaccharide.

Based on this study, the influence of CS on pre-translational regulation of these selected genes is limited.

Introduction

Osteoarthritis (OA) remains an important and expensive cause of lameness in affected horses. Although a variety of factors can initiate the disease process, ultimately all articular tissues are affected. The hallmark of OA is the degeneration of the articular cartilage matrix, attributed to an excess production of proinflammatory cytokines (Martel-Pelletier *et al* 1999). Interleukin-1 β (IL-1β) is widely accepted as one of the cytokines that plays a pivotal role in the pathophysiology of OA (Pelletier *et al* 1993, Tung *et al* 2002). This cytokine induces a number of catabolic events in both synoviocytes and chondrocytes, including induction of genes of matrix degrading proteinases such as the metalloproteinases (MMPs) and aggrecanases, as well as a number of other inflammatory mediators including inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX 2) (Tung *et al* 2002, Cook *et al* 2000, Morris *et al* 1994, MacDonald *et al* 1992, Richardson *et al* 2000). Accumulating evidence indicates that many of the effects of IL-1β on mediators of osteoarthritis pathophysiology are effected through the activation of transcription factors such as nuclear factor kappa B (NFκB) and activator protein –1 (AP-1) (Mengshol *et al* 2000, Vincenti *et al* 2002).

Oral administration of nutraceuticals containing glucosamine or chondroitin sulfate or both has enjoyed considerable vogue as a symptomatic treatment of OA in man and

domestic animals. On-going research in this area indicates that these nutraceuticals may possess chondroprotective or cartilage-sparing properties. For example, a chondroprotective effect has been supported by two studies indicating that glucosamine prevented knee joint space narrowing in OA patients over a three-year period (Pavelka *et al* 2002, Reginster *et al* 2001). Similarly, in a randomized, double-blind, placebo-controlled trial, chondroitin sulfate was found to be protective against radiographic progression of finger joint OA (Verbruggen *et al* 1998).

Certain effects of these substances on cellular metabolism have received recent attention. Glucosamine acts as a substrate for and enhances the production of proteoglycans and glycosaminoglycans (Bassleer *et al* 1998b). Further, glucosamine prevents the repression of galactose β-1,3-glucuronosyltransferase I, a key biosynthetic enzyme in glycosaminoglycan synthesis (Gouze *et al* 2001). Glucosamine inhibits proteoglycan loss, prostaglandin production, iNOS, and MMP activity in equine cartilage explants stimulated with LPS or human and equine recombinant interleukin-1 (Fenton *et al* 2000a, b, Orth *et al* 2002, Byron *et al* 2003). Although purported to have favorable effects on chondrocyte metabolism, chondroitin sulfate has been less well characterized than glucosamine with respect to its anti-catabolic functions. In an *in vitro* culture system using human chondrocytes in clusters, chondroitin sulfate was protective against IL-1 induced deleterious effects on sulfated proteoglycan, and type II collagen synthesis (Bassleer *et al* 1998a). Chondroitin sulfate stimulates hyaluronic acid production by synoviocytes (Nishikawa *et al* 1985).

Recent reports suggest that at least some of the anti-catabolic effects of glucosamine are exerted at the pre-translational level. Modulation of cytokine and endotoxin-stimulated induction of MMPs and aggrecanases in chondrocytes has been demonstrated *in vitro* (Byron *et al* 2003, Sandy *et al* 1998, Dodge *et al* 2003). Specifically, glucosamine was shown to reduce expression of MMP 1, MMP 3, and MMP 13 in monolayer cultures of equine chondrocytes (Byron *et al* 2003) and MMP 3 in both rat chondrocytes (Gouze *et al* 2001) and human osteoarthritic chondrocytes (Dodge *et al* 2003). Recent data suggest that inhibition of IL-1 induced synthesis of inflammatory mediators and matrix degrading proteinases by glucosamine may occur via reducing the activity of certain cell signaling pathways. For example, the transcription factors NFκB and AP-1 are increased in OA cartilage and IL-1 induced NFκB and AP-1 activity is associated with enhanced transcription of the aforementioned mediators of cartilage degradation (Vincenti *et al* 2001, Mengshol *et al* 2001). Glucosamine inhibits NFκB binding in a dose dependent manner as well as preventing IL-1 induced translocation of p50 and p65 subunits of NFκB to the nucleus in osteoarthritic human cartilage (Largo *et al* 2003).

To date, most in vitro research in this area has been performed with concentrations of GLN and CS that exceed those obtained by oral administration (Fenton *et al* 2000a, b, Orth *et al* 2002, Byron *et al* 2003, Sandy *et al* 1998, Largo *et al* 2003, Goouze *et al* 2002, Fenton *et al* 2002). Depending on the species and molecular weight of CS, concentrations of CS in serum after oral administration range from 19 to 208 μg/ml, with a cumulative effect after multiple dosing (Adebowale *et al* 2002, Du *et al* 2002, 2004). Glucosamine concentrations after oral dosing are in the 1.25 to 20 μg/ml range, with concentrations up

to 350 μg/ml possible after intravenous administration (Liang *et al* 1999, Adebowale *et al* 2002, Du *et al* 2002, 2004). Thus, the objective of the study reported here was to determine the effect concentrations of GLN (2.5 – 10.0 μg/ml) and CS (5.0-50.0 μg/ml) that more closely approximate those achieved following oral administration have on gene expression of a number of mediators of cartilage catabolism in OA using equine chondrocytes in pellet culture stimulated with recombinant equine IL-1β (reIL-1β).

Materials and Methods

Tissue Sources- Pellet Culture

Grossly normal metacarpophalangeal articular cartilage was obtained from horses between 2 and 8 years of age that died or were euthanized for reasons other than joint disease. Chondrocyte isolation and propagation in pellet culture was conducted as previously described with minor modifications (Byron *et al* 2003, Caron *et al* 1996). Briefly, cartilage was dissected from the subchondral bone and following incubation in penicillin (500 U/ml) and streptomycin[a] (500 mg/ml) (25 °C, 1 hour), chondrocytes were isolated by sequential digestion with pronase[b] (1mg/ml, 1 hour) and collagenase[a] (0.3mg/ml, 18 hours). After digestion, the cells were separated by sequential centrifugation (300 x g, 10 minutes, repeated 3 times), washed, and re-suspended in 20ml Dulbecco's modified Eagle's medium (DMEM):nutrient mixture F-12 (Ham) (1:1)[b]. The media was supplemented with insulin-transferrin-sodium selenite supplement[c] (5 μg/ml insulin, 5 μg/ml transferrin, 5 ηg/ml sodium selenite), 50ug/ml ascorbic acid[b], amino acids[b], 2 μg/ml lactalbumin hydroslyate[b], 5 μg/ml linoleic acid[b], 40 ηg/ml thyroxine[b], and

100 U/ml penicillin/streptomycin[a]. The concentration of amino acids was 50% of those previously reported (Rosselot *et al* 1992).

Cell concentration was determined with a hemocytometer and aliquots of 6 X 10^6 cells were transferred to 15 ml polypropylene centrifuge tubes in 1 ml supplemented serum free media described above. Following centrifugation (300 X g, 5 minutes), pellets were incubated under standard cell culture conditions (37°C, 95% RH, 5% CO_2). Medium was exchanged every 3 days. Pellet cultures were maintained in serum free media without supplementation with insulin-transferrin-sodium selenite supplement, linoleic acid, thyroxine, and ascorbic acid for 2 days prior to the start of an experiment for equilibration. At the conclusion of experiments pellets were collected for isolation of total RNA.

RNA Isolation

Total RNA was extracted, using a commercial extraction preparation[d] and RNA isolation kit[c] and following manufacturer's instructions with minor modifications (Reno *et al* 1997) Briefly, 1 ml of this agent was added to each pellet after removal of media, incubated (25°C, 5 minutes), placed on a plate shaker (25°C, 5 minutes) and transferred to microcentrifuge tubes. Following centrifugation (10,000 X g, 10 minutes), 200 µl chloroform was added to extract total RNA followed by agitation and a second incubation (25°C, 2 minutes). The aqueous phase containing RNA was collected after centrifugation (4°C, 12,000 X g, 15 minutes) and RNA precipitated with an equal volume of 75% ethanol. RNA was then purified further with RNeasy mini spin columns[c]. Pellets

were re-suspended in RN-ase free water (0.1% diethylpyrocarbonate, supplied) and centrifuged (10,000 x g, 1 minute) to elute RNA. RNA was analyzed by electrophoresis through 1% agarose gels containing 10μg/ml ethidium bromide in 1 x MOPS (3-(N-morpholino) propanesulfonic acid) to validate spectrophotometric determination and RNA integrity. RNA was quantified by UV spectrophotometry[f], and adjusted with RN-ase free water to 1 μg/μl solutions.

Quantitative Real Time PCR

Two micrograms of each RNA sample was treated with DNase I[g] to degrade contaminating single and double stranded DNA. Treated RNA was converted to single stranded cDNA using Superscript II reverse transcriptase[g] as recommended by the manufacturer. cDNA was quantified by UV spectrophotometry, and adjusted with Rnase-free water to 25ηg/μl.

Reference amplicons were developed using specific primers designed by Primer Express software[h] and synthesized by commercial facilities[i] (Tables I and II) Nucleotide sequences used for primer design were obtained from public databases (Genbank[j]) as full or partial length equine sequences were available for 18s ribosomal subunit (18s), β-actin, β_2-microglobulin, glyceraldehyde phosphate dehydrogenase (GAPDH), inducible nitric oxide synthase (iNOS), matrix metalloproteinases 1,2,3 and 13 (MMP 1, 2, 3, 13), aggrecanases-1 and –2 (Agg 1, 2), cyclooxygenase 2 (COX 2), ribosomal protein L19 (RPL19), and ubiquitin. When no equine sequence was available [cJun N-terminal kinase (JNK), nuclear factor kappaB (NFκB), MMP 9], equine expressed sequence tag (EST)

sequences corresponding to the target gene were used, based on similarity to the corresponding bovine, human or mouse sequence. BLASTN[j] searches for all of the primer and amplicon sequences were conducted to ensure gene specificity. Optimal concentrations of each set of primers were determined with a primer matrix [lowest standard deviation with no change in cycle to threshold (C_T)].

Fifty nanograms of sample cDNA primed each real-time PCR reaction. All analyses were conducted in an ABI PRISM 7700 sequence detection system[h]. The cDNA templates were combined with optimal concentrations of primers and SYBR Green PCR dye mix[h] in a total volume of 50 µl and the amplification conducted as recommended by the manufacturer. The PCR conditions were 2 min at 50°C and 10min at 95°C followed by 40 cycles of extension at 95°C for 15 sec and 1 min at 60°C. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curves. The software automatically recorded the C_T. Analysis of each sample was performed in duplicate, and a standard deviation of <0.5 between replicates set as a criteria for inclusion of data. Reference amplicons for each gene and primer sets were functional based on test amplifications using equine cartilage RNA-derived cDNA. Replicated data was normalized against the geometric average of 3 endogenous controls (18s, GAPDH, β_2-microglobulin) (Vandesompele *et al* 2002), and the fold change in gene expression relative to serum free control was calculated using the $2(-\Delta\Delta C_T)$ method (Livak *et al* 2001).

Determination of a Sub-saturating Dose of reIL-1β

ReIL-1β was purified as described previously (Tung *et al* 2002). Chondrocyte pellet cultures were established as described above. After an 8-12 day period to establish a stable metabolism, reIL-1β was added in incremental doses to achieve concentrations ranging from 0 to 20,000 ρg/ml. Pellets were harvested after a 6-hour incubation and RNA isolated for Q-RT-PCR, with data normalized to GAPDH. Effects on expression were quantified for MMP 13, iNOS, COX 2, and Agg 1. This was repeated using tissue from 5 different horses.

Selection of housekeeping genes for use as endogenous controls in subsequent studies

Six housekeeping genes (Table I) were selected based on the availability of full or partial length equine sequences for commonly used endogenous controls. Validation of the effect of experimental treatment on the expression of each housekeeping gene was determined for each horse using the $2(-\Delta C'_T)$ method, where $\Delta C'_T = C_{T(treatment)} - C_{T(serum\ free\ control)}$ (Livak *et al* 2001), with the fold change $[2(-\Delta C'_T)]$ expressed as an average fold change from the mean and the standard deviation as the maximum fold change (maximum variability) (Dheda *et al* 2004). Absolute C_T values were used as an indicator of the level of gene expression, and the overall median expression level (C_T) and deviation from median for each housekeeping gene used as an additional measure of gene stability.

To determine gene amplification efficiency, cDNA was serially diluted (10-100 ηg/ml), and average C_T calculated for each gene of interest and each housekeeping gene. The

ΔC_T ($C_{T(\text{gene of interest})} - C_{T\ (\text{housekeeping gene})}$) was determined and plotted as a function of log cDNA dilution. The value of the slope of the regression is a measure of the difference in amplification efficiencies between the gene of interest and the housekeeping gene. A difference in efficiency of <0.1 was determined for normalization of each gene of interest to a particular housekeeping gene (Livak *et al* 2001). Selection of the 3 endogenous controls to be used for normalization was based on amplification efficiency, least variability, and level of expression.

Effect Of Glucosamine and Chondroitin Sulfate on Gene Expression

Pellet cultures were placed in fresh supplemented serum free media described above. After a 5-7 day period to establish a stable metabolism, GLN[k] (2.5, 5 and 10.0 μg/ml) or CS[l] (5.0, 10.0, 20.0 and 50.0 μg/ml) were added to all pellet cultures except positive and negative controls. One hour later, re IL-1 was added at a final concentration of 500 ρg/ml to all pellet cultures except negative controls. After a 12-hour incubation under standard cell culture conditions RNA was isolated for Q-RT-PCR. Genes studied included MMP 1, MMP 2, MMP 3, MMP 9, MMP 13, Agg 1, Agg 2, iNOS, COX 2, NFκB, and JNK. This was repeated using tissue from 8 different horses.

Statistical Analysis

Means for relative expression for each gene of interest included only those horses for which the sub-saturating dose of reIL-1β resulted in a 2-fold or greater up-regulation of expression from resting (control) levels. Distributions were tested for normality using the method of Kolmogorov-Smirnov. When required, means were $\log_{10}$ transformed

followed by analysis using a two-way ANOVA (blocked by horse). Post hoc testing was conducted using the Duncan's multiple range test. A P value < 0.05 was considered significant. A P value < 0.10 was regarded as a statistical trend.

Results

Determination of a Sub-saturating Dose of reIL-1β

Exposure of pellet cultures to graduated concentrations of reIL-1β resulted in dose dependent saturable induction of expression of Agg 1, MMP 13, iNOS and COX 2 (Figure 1). The dose of cytokine corresponding to approximately half maximal induction of expression of these genes varied from 100-1000 ρg/ml. For this reason, the intermediate dose of 500 ρg/ml was selected for use in subsequent experiments. This concentration of reIL-1β resulted in approximately 2 to 5-fold increase in expression of the 4 genes. Based on the sizeable variability in data in the subsequent experiments, an analogous dose-response protocol was repeated using cartilage from 4 different horses, with an incubation of 12 hours prior to RNA isolation and amplification with primers for COX 2 with comparable results (Figure 2).

Selection of housekeeping genes for use as endogenous controls in subsequent studies

Levels of gene expression for available housekeeping genes varied substantially and there were substantial differences in individual housekeeping gene stability between horses. Amplification efficiency varied for each housekeeping gene, with GAPDH the most suitable for comparison for 55% (6/11) of the genes studied. Three housekeeping genes (18s, GAPDH and β_2-microglobulin) were subsequently chosen based on expression

level, amplification efficiency, and the lowest level of variability, both overall and for each horse.

Effect Of Glucosamine and Chondroitin Sulfate on Gene Expression

Glucosamine significantly reduced reIL-1β induced mRNA expression of MMP 13, and Agg 1, at 10.0 μg/ml (Figures 3-4). A trend for reduction in cytokine-induced expression was also observed for iNOS (P = 0.06), COX 2 (P = 0.08) (Figures 5-6). Pellets treated with 10.0 μg/ml of GS had significantly reduced expression of JNK compared to positive (reIL-1β) controls, however, for this gene, the expression of positive and negative controls was comparable. Glucosamine at a concentration of 10 μg/ml reduced IL-1 induced stimulation of Agg 2 and NFκB, however the effect was not significant (P = 0.13 and 0.11 respectively). The dose of reIL-1β employed failed to significantly up-regulate MMP 9 expression. Chondroitin sulfate had no significant effect on gene expression at the concentrations tested.

Discussion

While the potential cartilage-sparing role of glucosamine and chondroitin sulfate has been studied by a variety of means, the specific mechanism(s) of action and the minimally effective concentrations remain to be established. We conducted a Q-RT-PCR based study to characterize the effects on gene expression of GLN and CS that corresponds to concentrations that approximate those achieved by oral administration in monogastrics (Liang *et al* 1999, Adebowale *et al* 2002, Du *et al* 2002, 2004). Our data support the hypothesis that GLN is capable of pre-translational regulation of reIL-1β-induced

stimulation of at least some of the proteins implicated in the process of cartilage degradation at a concentration of 10 μg/ml. Specifically, glucosamine led to a significant reduction of cytokine-stimulated expression of MMP 13 and Agg 1 and a statistical trend ($P < 0.1$) for iNOS and COX 2. These findings supplement those of previous experiments in our and other laboratories that were conducted with concentrations higher than those found in the plasma of animals administered GLN and CS (Orth *et al* 2002, Byron *et al* 2003, Sandy *et al* 1998, Dodge *et al* 2003). These observations parallel those of previous publications and provide further evidence supporting a cartilage-sparing effect of this aminomonosaccharide.

In contrast to our findings for MMP 13 and Agg 1, while recombinant equine IL-1 significantly up-regulated the expression of MMP 1 and MMP 3, and Agg 2 there was no significant influence of GLN treatment. For MMPs 1 and 3, there was no effect whatsoever. Although not significant, GS at 10ug/ml reduced expression of Agg 2 to levels approximately 25% those of the cytokine control. The lack of an effect of GLN for MMP 1 and 3 is in contrast to previous reports utilizing higher doses of GLN (Fenton *et al* 2000a, Byron *et al* 2003, Nakamura *et al* 2004); however, our results parallel another report in which GS at 10 μg/ml failed to significantly repress IL-1 induced MMP production (Nakamura *et al* 2004). Differences in responses among the MMPs may be due to experimental design and/or the variability inherent in these particular experiments. For example, pre-incubation of rat chondrocytes with GLN, albeit at a higher dose, down regulated MMP 3 expression (Gouze *et al* 2001). The potential for differential regulation of MMPs by GLN should be considered as has been documented in other studies (Orth *et*

al 2002, Dodge *et al* 2003, Fenton *et al* 2002). For example, Dodge *et al* showed that GLN was able to modulate MMP 3 but not MMP1 activity (Dodge *et al* 2003). Another potential reason for this observation is divergence in the regulation of these proteins. *In vitro,* both temporal and dose dependent differential regulation has been documented (Mengshol *et al* 2000, Salminen *et al* 2002, Thompson *et al* 2001, Koshy *et al* 2002). This may be further complicated by the coordinate regulation of MMPs and aggrecanases (Kevorkian *et al* 2004)

Unlike the other MMPs examined, significant up-regulation of MMPs 2 and 9 by reIL-1β was not observed. This is in keeping with previous studies in chondrocytes, with either no change or mild increases in expression induced by IL-1 (Clegg *et al* 1999, Sasaki *et al* 1996). This may be in part related to basal levels of both MMPs in control samples, as secretion of both MMPs by unstimulated chondrocytes has been suggested (Thompson *et al* 2001). The gelatinases are proposed to have a secondary role in OA, as collagen degradation occurs only after the collagenases such as MMP 13 have cleaved the triple helix of collagen (Nagase et al 1999). The effect on MMP 2 and 9 and may also relate to the differences between normal and OA chondrocytes, reflecting the differential expression profiles of MMPs in early compared to late OA cartilage (Kevorkian *et al* 2004, Aigner *et al* 2001).

COX 2 and iNOS are both implicated in the pathophysiologic process of cartilage degeneration. For both, a statistical trend for repression of cytokine-induced synthesis was observed. The ability of GLN to regulate expression of these genes and their

respective inflammatory mediators has been demonstrated previously in equine cartilage (Fenton *et al* 2000a,b, Orth *et al* 2002, Fenton *et al* 2002). We attribute the lack of a clearly demonstrable protective effect of GLN to the inherent variability in this design, as recent reports using bovine explants have shown that GLN at 5 ug/ml resulted in significant inhibition of rhIL-1β-induction of NO and PGE_2 (Chan *et al* 2004).

The most common form of AP-1 is as a heterodimer of two proteins, c-Jun and c-Fos (Smeal *et al* 1989). JNK phosphorylates and hence activates c-Jun, which then translocates to the nucleus and dimerizes with c-fos (Firestein *et al* 1999). The expression of JNK increases in OA cartilage (Geng *et al* 1996), with elevated expression preceding the expression of degradative enzymes such as the MMPs along with clinical signs of OA (Han *et al* 1998). Due to inconsistent induction of the JNK transcript by reIL-1β in this study, the statistical analysis was limited to data from only two horses. Thus, despite what appears to be a dramatic repression of cytokine-induced expression of this protein with 10 ug/ml GLN, categorical conclusions cannot be drawn. The inconsistent induction of JNK by IL-1 in this study may be dose related, as maximal induction of JNK activity required 10 ηg/ml rhIL-1 in rabbit articular chondrocytes (Scherele *et al* 1997). In contrast to our findings, two previous studies utilizing much higher doses of GLN found no influence on AP-1 DNA binding or activity, despite a concurrent suppression of NFκB (Largo *et al* 2003, Gouze *et al* 2002). Nonetheless, the possibility that GLN influences JNK synthesis warrants further investigation and could provide an additional mechanism by which GLN can inhibit IL-1 mediated effects in OA cartilage.

While we were unable to demonstrate a significant effect of GLN on a marked induction of NFκB expression by reIL-1β (P = 0.11), it remains possible that at least some of the effects of GLN are effected via this intracellular signaling pathway. Using human osteoarthritic cartilage, Largo *et al* reported that glucosamine sulfate significantly inhibited IL-1 induced NFκB DNA binding, translocation of p50 and p65 subunits of NFκB to the nucleus, as well as preventing IL-1 mediated degradation of IκB, the natural inhibitor of NFκB (Largo *et al* 2003). However, doses used were at least 100 fold greater, and doses equivalent to those used in this study had no effect, suggesting that the influence of GLN on this transcription factor may be dose dependent.

Regulation of gene expression was observed with GLN but not for CS. The effects of GLN on pre-translational regulation of genes coding for proteins implicated in OA has been the subject of a number of studies, however the potential effects of CS at the level of gene expression have been less frequently examined. Nonetheless, our results of finding no significant effect of CS on cytokine-stimulated equine chondrocytes contradict a number of previous reports utilizing higher doses of CS (Orth *et al* 2002, Bassleer *et al* 1998a). Specifically, using bovine cartilage explants, CS at 20 μg/ml significantly reduced the production of PGE_2 and NO in 24-hour cultures (Chan *et al* 2004). The specific reasons for this difference remain to be elucidated but may reflect differences in species (bovine vs. equine), experimental design (pellets vs. explants), the form of CS employed (source, purity, molecular weight), the magnitude of the arthritogenic stimulus (50 ηg/ml rhIL-1β vs 0.05 ηg/ml reIL-1β) and/or the influence of timing of supplementation (pre-incubation vs simultaneous to IL-1). Conversely, the beneficial

effects of CS may be principally related to anabolic processes, rather than preventing catabolic ones. For example, CS enhances the expression of genes such as type II collagen, suggesting that its chondroprotective effects may be in part due to stimulation of collagen and proteoglycan/glycosaminoglycan synthesis (Bassleer *et al* 1998a, Nishikawa *et al* 1985, Conte *et al* 1991, Ronca *et al* 1998).

Osteoarthritis research using *in vitro* models of cartilage degradation incorporating lipopolysaccharide, crude mixtures of inflammatory mediators contained in conditioned media, or recombinant cytokines to induce cartilage degradation represents a popular and cost-effective means of investigating pathophysiologic events and the effects of putative therapeutic agents on the disease process. Both glucosamine and chondroitin sulfate have been investigated in this manner; however, many of the studies to date have used relatively large quantities of both arthritogenic stimulus and nutraceuticals (Fenton *et al* 2000a,b, Orth *et al* 2002, Byron *et al* 2003, Bassleer *et al* 1998a, Nishikawa *et al* 1985, Largo *et al* 2003, Gouze *et al* 2002, Fenton *et al* 2002). This series of experiments were planned to address these limitations of previous studies. The design employed both a sub-saturating dose of recombinant cytokine and physiologically relevant concentrations of the potential cartilage sparing preparations. Though arguably more biologically relevant than some previously conducted work, the experiments were complicated by considerable variability from several sources. We endeavored to utilize a dose of reIL-1β to produce a half-maximal response in our genes of interest. The dose of 500 ρg/ml was determined by preliminary experiments employing 4 of our genes of interest using tissue from 5 different horses in an attempt to address the substantial intrinsic variability among horses.

Despite these efforts, the response to reIL-1β in the subsequent experiments varied widely at both the gene and animal level. As such, the pre-requisite 2-fold up-regulation of most genes was not uniformly achieved, resulting in inferential analyses disadvantaged by relatively small numbers.

The analysis of relative gene expression used herein was adapted from the $2(-\Delta\Delta C_T)$ method (Livak *et al* 2001). Validation of this technique is necessary for each experimental model as certain requirements need to be adhered to, including similarity in expression levels and amplification efficiencies of the target and reference (housekeeping) genes, along with stability of housekeeping gene expression. Variation in housekeeping gene stability has been documented in a number of studies, prompting recommendations to use more than one gene for normalization (Vandesompele *et al* 2002, Suzuki *et al* 2000). As housekeeping genes have not been previously exhaustively compared using equine cartilage, and considerable variability was observed during initial amplifications using RNA isolated from GLN and CS treated pellets, we conducted a number of ancillary experiments to more fully characterize both amplification efficiencies and stability of a variety of standard reference genes including 18s, β-actin, β_2-microglobulin, GAPDH, RPL19, and ubiquitin. We observed that the suitability of each housekeeping gene varied depending on the gene of interest and between horses. Similar sizeable inter-individual differences have been documented in studies conducted in other species (Dheda *et al* 2004, Tricarico *et al* 2002). A geometric mean (Vandesompele *et al* 2002) of 18s, β_2-microglobulin and GAPDH was used in an attempt to incorporate requirements for expression levels, amplification efficiency and gene

stability for particular genes of interest and each individual horse. However, inconsistent and irregular amplification behavior may have hampered our attempts to detect subtle treatment effects using this model. Despite attempts to limit the effects of an observed lack of constitutive expression on the part of the housekeeping genes, there remained important dispersion in the data set. It would appear that for experiments of this type provisions need to be made for greater replication than has been typical of experiments using more potent stimulation and pharmacologic doses of therapeutic compounds.

Footnotes

[a] Gibco, Grand Island, New York

[b] Sigma, St. Louis, MO

[c] Roche, Indianapolis, Indiana

[d] TRIzol®, Invitrogen, Carlsbad, CA

[e] Rneasy mini kit, Qiagen, Valencia, CA

[f] Nanodrop,

[g] Invitrogen, Carlsbad, CA

[h] Perkin-Elmer Applied Biosystems, Foster City, CA

[i] Operon Technologies, Alameda, CA; and Macromolecular Structure, Sequencing and Synthesis Facility, East Lansing, MI

[j] Genbank Database, National Centre Biotechnology Information

[k] Glucosamine Hydrochloride, Sigma, St. Louis, MO

[l] Chondroitin Sulfate A, Sigma, St. Louis, MO

[m] SigmaStat version 2.0

Table I (chapter 3): Forward and reverse primer sequences (5'→3') of housekeeping genes used for quantitative real-time polymerase chain reaction.

Gene	Genbank Accession Number[j]	Forward primer	Reverse Primer
18s	AJ311673	CGCCGCTAGAGGTGAAATTC	CATTCTTGGCAAATGCTTTCG
β-actin	AF035774	TCACGGAGCGTGGCTACAG	CCTTGATGTCACGCACGATT
β_2-microglobulin	X69083	CCGCGTGTTCCGAAGGT	GCAGTTCAGGAAATTTGGCTTT
GAPDH	AF157626	CCCACCCCTAACGTGTCAGT	TCTCATCGTATTTGGCAGCTTTC
RPL19	AY246727	CGAAAGCGAAGGGCAATGT	TGCCTTCGGCTTGTGGAT
Ubiquitin	AF506969	CGAGGCCTTTTGGTGTGGTA	ACGGACACGAAGAAACTGCTAAT

Table II (chapter 3): Forward and reverse primer sequences (5'→3') of genes of interest used for quantitative real-time polymerase chain reaction.

Gene	Genbank Accession number	Forward primer	Reverse Primer
Agg 1	AF368321	TTTCCCTGGCAAGGACTATGA	GCGGACAATGGCGTGAGT
Agg 2	AF368322	AAATGCACCTCAGCCACCAT	TGCGTGGGAGGTCTAGCAA
COX 2	AB041771	GGAGCTGTATCCTGCCCTTCT	CCATGGTCTCCCCGAAGAT
iNOS	AY027883	GGCCTTGGCTCCAGCAT	GGTGAGACAACTTCTGGTCAATGT
JNK	BM414648 (91% homology to human sequence)	TTCCAACTGGGCATCATAAATTT	CCCTGCGTCACCCCTACA
MMP 1	AF148882	GGGAGATCATCGTGACAATTCT C	TACGTGGGCCTGGCTGAA
MMP 2	AJ010314	CCCTGAGACGGTGGATGATG	GGATGCGAGAAAACCGTAGTG
MMP 3	U62529	TGTGGAGGTGATGCACAAATC	GCATGCCAGGAAATGTAGTGAA
MMP 9	BM734900 (86% homology to bovine sequence)	CGGCGTGCCCTTGGA	CGGTCCTGGGAGAAGTAAGCT
MMP13	AF034087	TGAAATCATACTACTATCCCCTT AATCCT	TCTCGGAGCCTGTCAACCA
NFκB	BI960845 (84% homology to mouse sequence)	CATCGAGCAGATAGCTCACGTT	AGGTGGTTGGTGAGATTGACAA

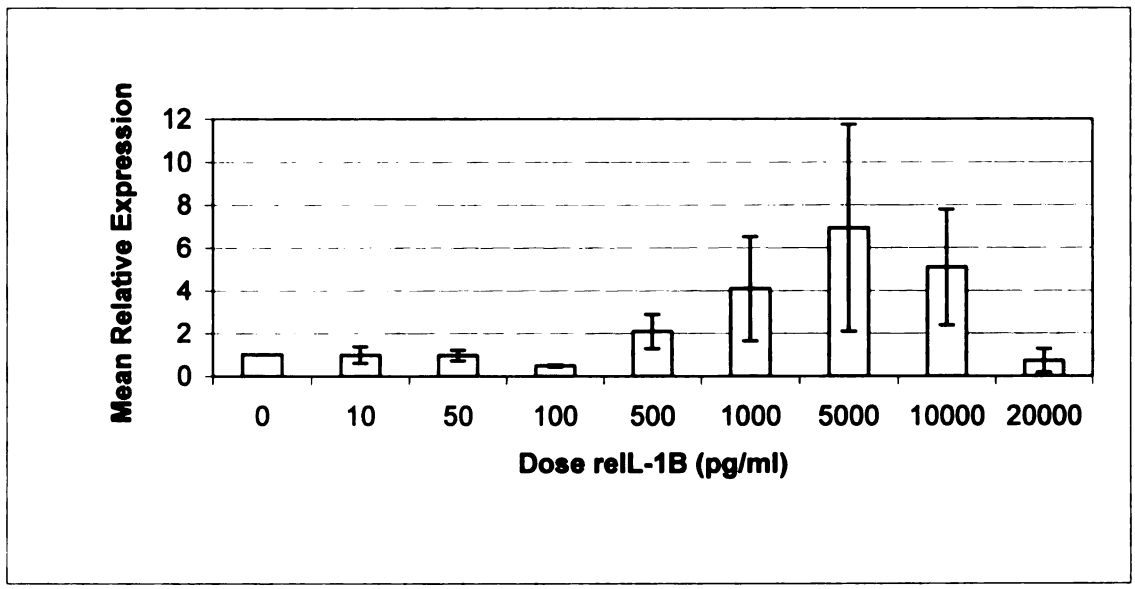

Figure 1 (chapter 3): Relative expression of COX 2 to graduated doses of recombinant interleukin-1β (reIL-1β). Chondrocyte pellet cultures (3 x 10^6 cells/pellet) were exposed to graduated concentrations (0, 10, 50, 100, 500, 1000, 5000, 10000, and 20000 pg/ml) of reIL-1β for 6 hours followed by RNA isolation and quantitative PCR using specific primers for COX 2. An approximately half-maximal stimulation was evident at 500 pg/ml. A qualitatively similar response was observed for Agg 1, iNOS and MMP 13.

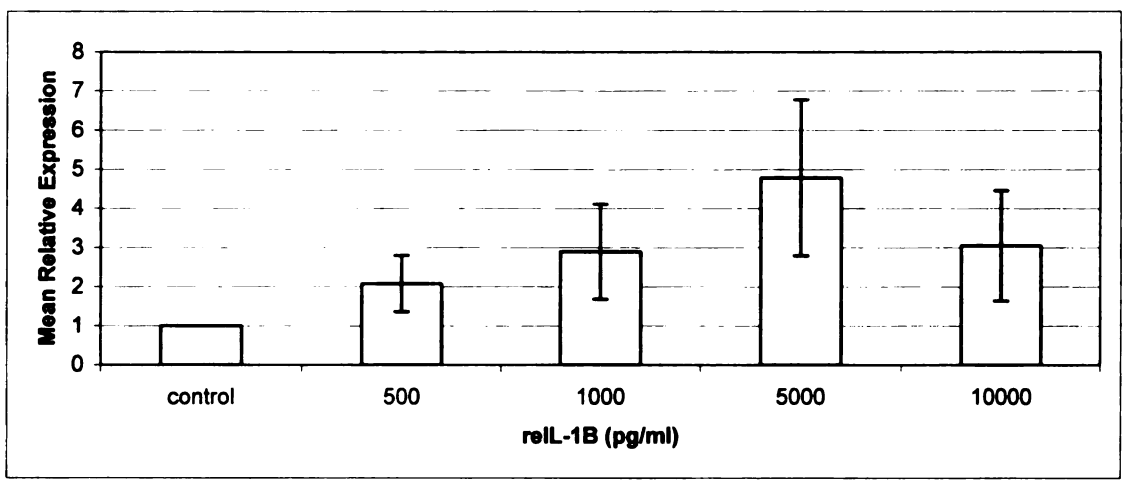

Figure 2 (chapter 3): Relative expression of COX 2 to graduated doses of recombinant interleukin-1β (reIL-1β). Chondrocyte pellet cultures (6 x 10^6 cells/pellet) were exposed to graduated concentrations (0, 500, 1000, 5000, and 10000 pg/ml) of reIL-1β for 12 hours followed by RNA isolation and quantitative PCR using specific primers for COX 2. A qualitatively similar response was observed for iNOS.

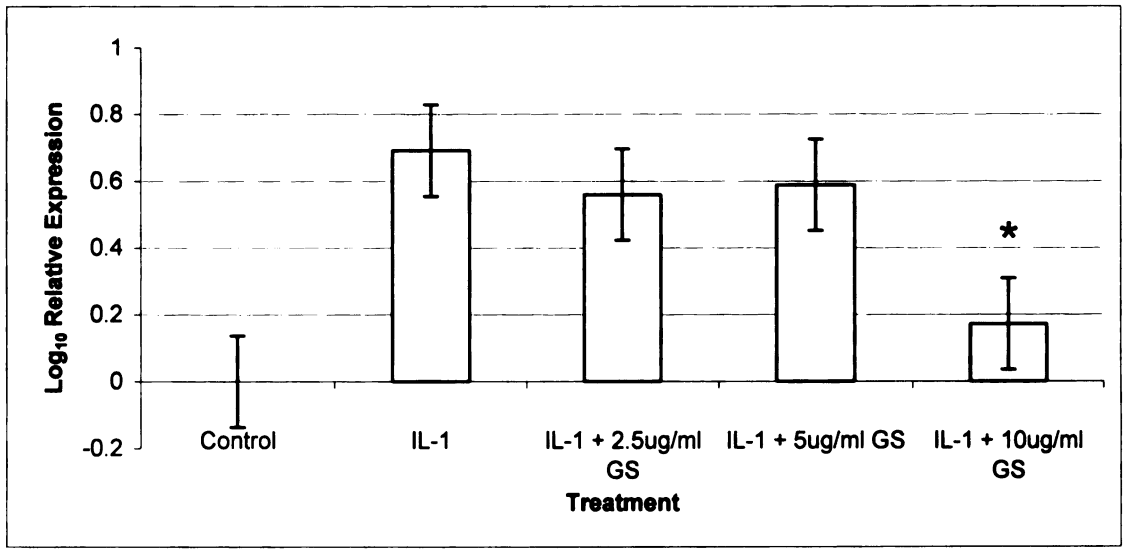

Figure 3 (chapter 3): Influence of graduated concentrations of glucosamine hydrochloride on recombinant interleukin-1β-induced MMP 13 expression. Values are mean (+/- SEM) from experiments conducted with tissue from 3 different horses. Relative expressions were calculated using the $2(-\Delta\Delta C_T)$ method with 18s, GAPDH and $\beta_2$microglobulin as the reference genes. * Indicates statistical significance at $p < 0.05$ compared to positive control.

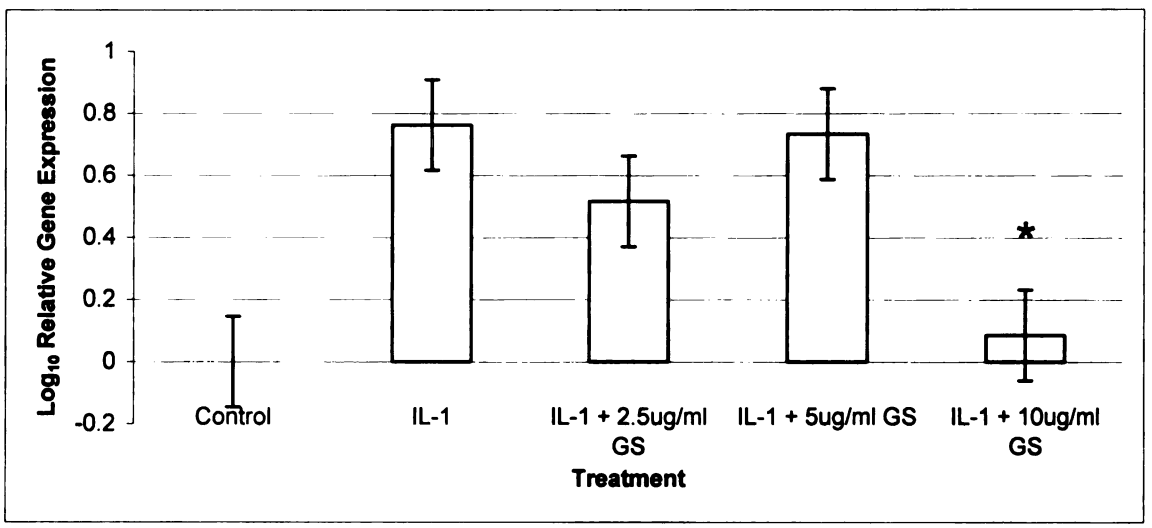

Figure 4 (chapter 3): Influence of graduated concentrations of glucosamine hydrochloride on recombinant interleukin-1β-induced Agg 1 expression. Values are mean (+/- SEM) from experiments conducted with tissue from 4 different horses. Relative expressions were calculated using the $2(-\Delta\Delta C_T)$ method with 18s, GAPDH and $\beta_2$microglobulin as the reference genes. * Indicates statistical significance at $p < 0.05$ compared to positive control.

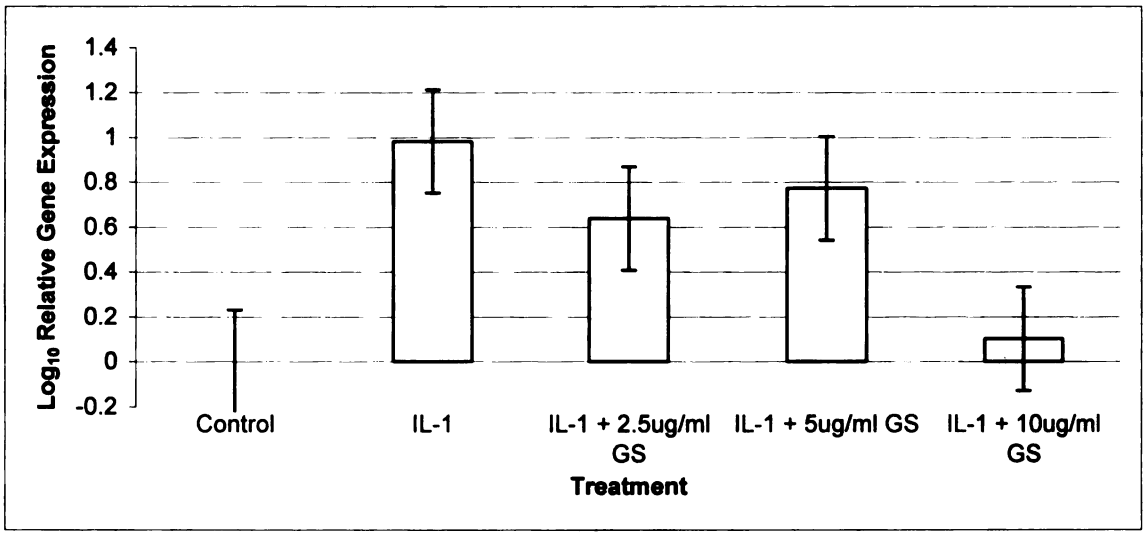

Figure 5 (chapter 3): Influence of graduated concentrations of glucosamine hydrochloride on recombinant interleukin-1β-induced iNOS expression. Values are mean (+/- SEM) from experiments conducted with tissue from 3 different horses. Relative expressions were calculated using the $2(-\Delta\Delta C_T)$ method with 18s, GAPDH and $\beta_2$microglobulin as the reference genes. There is a statistical trend for a treatment effect ($P = 0.06$).

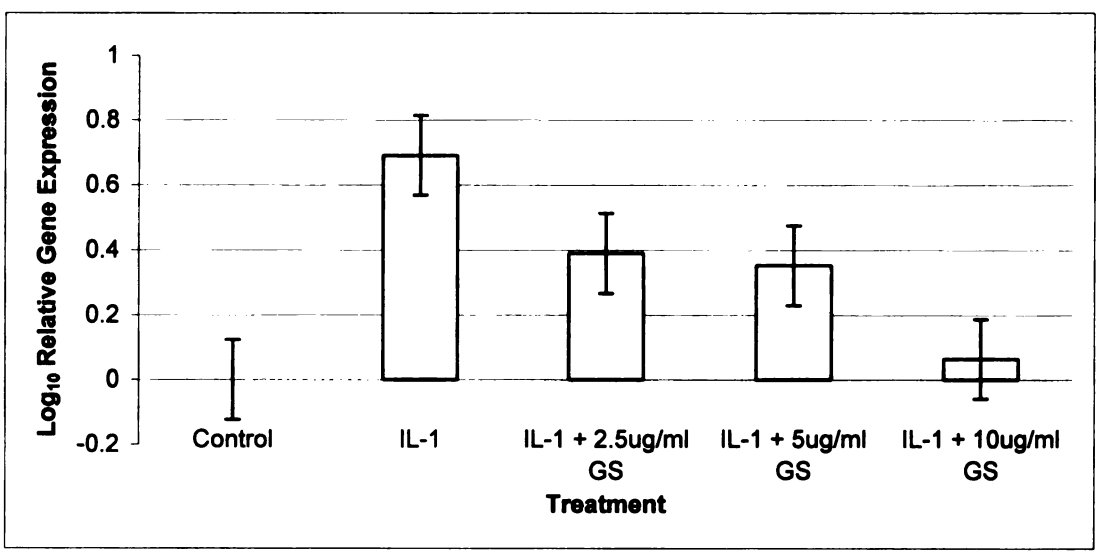

Figure 6 (chapter 3): Influence of graduated concentrations of glucosamine hydrochloride on recombinant interleukin-1β-induced COX 2 expression. Values are mean (+/- SEM) from experiments conducted with tissue from 3 different horses. Relative expressions were calculated using the $2(-\Delta\Delta C_T)$ method with 18s, GAPDH and $\beta_2$microglobulin as the reference genes. There is a statistical trend for a treatment effect ($P = 0.08$).

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CHAPTER 4: QUANTIFICATION OF GENE EXPRESSION USING A SUBSATURATING MODEL OF OSTEOARTHRITIS: CONSIDERATIONS FOR ENDOGENOUS CONTROLS AND EXPERIMENTAL DESIGN

Quantitative real time polymerase chain reaction (Q-RT-PCR) is a powerful tool for determining gene expression. This technique is inherently more sensitive than reverse transcriptase polymerase chain reaction (PCR) and Northern hybridization, as gene expression is quantified as the reaction proceeds rather than at the end point. An additional advantage is the ability to measure gene expression in smaller samples (Fehr *et al* 2000). Problems with end point analysis reside from the influence that factors other than the initial amount of template may have on the end point of the reaction. Such factors include consumption of nucleotides, product degradation and depletion of reagents as the reaction nears completion, which can compromise precision, sensitivity and resolution. Furthermore, the end point of a PCR reaction varies between samples, and quantification with gel electrophoresis may not enable resolution of such variability in yield. In contrast, Q-RT-PCR uses fluorescence detection methods to measure the amount of PCR product at each cycle, creating an amplification plot of accumulated product over the reaction. This enables quantification during the exponential phase when reagents are not depleted and reaction kinetics favor doubling of the PCR product with every cycle.

However, inherent requirements of Q-RT-PCR need to be adhered to. Endogenous control genes, or housekeeping genes, are used for normalization of quantity of input RNA, loading error, variation in RNA integrity, and efficiency of cDNA conversion and

PCR amplification. Validity of the delta delta Ct method (Livak *et al* 2001), commonly used for quantification of gene expression by Q-RT-PCR, relies on certain assumptions. The expression of the endogenous control gene should remain constant, and the amplification efficiencies of the target gene and the endogenous control must be approximately equal. Notably, the control gene should maintain a consistent level of expression and not be altered by the experimental treatment.

Recently, a number of reports have documented problems with a number of frequently used endogenous control genes (Suzuki *et al* 2000, Thellin *et al* 1999). This may reflect their documented functions *in vivo*, but also may be attributed to the effect of experimental conditions or pathological processes on the expression of these genes. The expression of housekeeping genes may vary among species, tissues and cell types related to differences in overall transcriptional activity. For instance, glyceraldehyde phosphate dehydrogenase (GAPDH) is an important glycolytic enzyme, catalyzing the oxidative phosphorylation of glyceraldehydes-3-phosphate, with other roles in endocytosis, DNA replication and repair, and apoptosis documented (Sirover 1997). However, expression may be altered by a variety of *in vitro* and *in vivo* conditions such as hypoxia, insulin and dexamethasone (Oliveira *et al* 1999). Cellular proliferation is an important modulator of GAPDH levels, with cell-cycle dependent regulation apparent in quiescent cell cultures that are stimulated by addition of serum (Suzuki *et al* 2000).

B-actin, often used as a housekeeping gene, is a ubiquitous cytoskeletal protein; however, expression levels can vary following hypoxia, and exposure of cultured cells to growth

factors or serum (Elder *et al* 1984). Further, the presence of pseudogenes may interfere with mRNA quantification (Dirnhofer *et al* 1995). Ribosomal RNAs, such as 18s and 28s, constitute 85-90% of toal cellular RNA and are often used as endogenous controls. However, ribosomal RNA may not always be representative of mRNA levels as rRNA may be lost during purification of mRNA, and rRNA is not recommended for quantification of samples when oligo dT sequences are used for cDNA synthesis (Overbergh *et al* 2003). Also, expression levels of rRNA are much higher compared to mRNA (Bustin 2002).

This series of experiments were designed to investigate the effect of glucosamine and chondroitin sulfate on expression of a number of genes of interest in equine pellet cultures stimulated with a sub-saturating dose of recombinant equine interleukin-1β (reIL-1β). However, results were hampered by both variability in the response to reIL-1β at both the gene and animal level, and variabililty in housekeeping gene expression despite equalization of input template and validation procedures. In an attempt to address limitations of previous studies that had used large quantities of both interleukin and/or nutraceuticals (Fenton *et al* 2000a,b, Orth *et al* 2002, Byron *et al* 2003, Bassleer *et al* 1998, Nishikawa *et al* 1985, Largo *et al* 2003, Gouze *et al* 2002, Fenton *et al* 2002), experiments employed both a sub-saturating dose of recombinant cytokine and physiologically relevant concentrations of glucosamine and chondroitin sulfate. Other potential confounding variables in experimental design were also addressed in an attempt to more accurately mimic *in vivo* conditions. For instance, we used serum-free media as the potential influence of undefined serum factors on experimental parameters has been

identified in a number of studies (Barnes *et al* 1980, Rosselot *et al* 1992, Kawcak *et al* 1996). Further, the influence of serum on housekeeping gene expression has been quantified (Elder *et al* 1984, Schmittgen *et al* 2000, Suzuki *et al* 2000). Similarly, pellet cultures were chosen based on established phenotypic stability and a more accurate reflection of chondrocytes *in vivo* compared to other culturing techniques (Stewart *et al* 2000).

Preincubation with GS and CS prior to stimulation with reIL-1β was chosen in an attempt to identify whether these commonly used compounds could potentially prevent induction of inflammatory mediatiors and degradative enzymes and hence OA. Pretreatment with glucosamine has been beneficial in other *in vitro* studies using rat and human chondrocytes, albeit using higher doses (Largo *et al* 2003, Gouze *et al* 2002). Whilst the duration of establishment of pellet cultures prior to experimental manipulation did change during the course of the study, this was not thought to have influenced our results, as housekeeping gene expression varied in cultures of different ages. Although a number of experiments were conducted in 5-day old cultures, the extracellular matrix of pellet cultures is established by day 3 with aggrecan and collagen content approximating *in vivo* levels by day 4 (Stewart *et al* 2000).

Initially, RNA was isolated following a 6-hour incubation period, based on previous findings of temporal aspects of induction by reIL-1β in our laboratory (Tung *et al* 2002). However, considerable variability both at the gene and animal level in terms of both response to reIL-1β stimulation and response to treatment with glucosamine and

chondroitin sulfate was apparent. Normalization to GAPDH, 18s and the geometric average of both GAPDH and 18s produced a quantitatively similar result, as did normalization to the median housekeeping C_T and analysis of results using IL-1 as the calibrator. Erroneus outlier results were common, and in conjunction with the wide variability that was apparent, further hindered analysis of results with no statistically significant effect of treatment discernable. This prompted the change in experimental design to a 12-hour incubation. Despite these efforts, the variation in terms of response to reIL-1β and to treatment with glucosamine and chondroitin sulfate in the subsequent experiments remained. As such, the pre-requisite 2-fold up-regulation of most genes was not uniformly achieved, and the variability between individuals was marked.

Although limited experiments were conducted using glucosamine and chondroitin sulfate in combination, results were again hampered by considerable variability, resulting in insufficient samples for analysis. Initially, experiments were performed following a 6-hour incubation with doses of GS and CS of 100 μg/ml alone or in combination. These doses were chosen to approximate those that had been previously shown to be effective using bovine cartilage in our laboratory. Although data was only collected from 2 horses, in general both GLN and CS appeared to negatively influence expression of MMP 13, COX 2 and Agg 1, results that were not consistent with responses to these nutraceuticals demonstrated previously. This experiment was performed before the impact of variability in housekeeping gene expression was investigated. However, the combination of GLN and CS did appear to reduce NFκB expression and CS alone may have had a stimulatory effect on type II collagen production, although accuracy of the latter effect was hindered

by a comparable expression of positive and negative controls. Conversely, the combination at doses of GLN 10 μg/ml and CS 20 μg/ml following a 12-hour incubation had no effect. The dose of 20 μg/ml was chosen based on doses that had been shown to be effective in combination in bovine cartilage (Orth *et al* 2004). As previously discussed, the lack of effect of CS may relate to a number of factors inherent to the design of the experiments. In particular, the CS used in bovine cartilage experiments in our laboratory previously was obtained from a different source, and it has recently been suggested that purified low molecular weight CS may be more effective than intact CS (Cho *et al* 2004).

The analysis of relative gene expression used herein was adapted from the $2(-\Delta\Delta C_T)$ method (Livak *et al* 2001). Validation of this technique is necessary for each experimental model as certain requirements need to be adhered to, including similarity in expression levels and amplification efficiencies of the target and reference (housekeeping) genes, along with stability of housekeeping gene expression. Variation in housekeeping gene stability has been documented in a number of studies, prompting recommendations to use more than one gene for normalization (Hamalainen *et al* 2001, Vandesompele *et al* 2002, Suzuki *et al* 2000, Schmid *et al* 2003). In particular, the use of a ribosomal gene in conjunction with another housekeeping gene has been advocated (Ropenga *et al* 2004). Other methods proposed include normalization to total RNA content; however, this does not account for differences in reverse transcriptase or PCR amplification efficiencies (Bunner *et al* 2004). To minimize variations in reverse transcriptase efficiency, all samples from a particular horse were reverse transcribed

simultaneously. In an attempt to correct for variability, Q-RT-PCR was conducted based equalization of both RNA and cDNA concentration quantified with spectrophotometry. However, variability in both housekeeping gene expression and genes of interest remained, even when cDNA content was further quantified with Nanodrop® technology to further ensure accurate sample loading.

Levels of gene expression for available housekeeping genes varied substantially (Figure 1) and there were substantial differences in individual housekeeping gene stability between horses (Figure 2). These results were also apparent with the 6-hr incubation period, and, although only 18s and GAPDH were investigated at that time period, these genes showed greater variability than at the 12-hour period. Amplification efficiency also varied for each housekeeping gene (Table II). The effect of normalization with different housekeeping genes alone or in combination was determined for MMP 13 using tissue from 5 horses, including using the best housekeeping gene for each horse based on least variability. The effect on MMP 13 expression was marked, highlighting the potential for confounding that could be introduced if an inappropriate housekeeping gene was chosen. As the ideal choice of housekeeping gene varied not only for each gene of interest but also between horses, three housekeeping genes (18s, GAPDH and β_2-microglobulin) were subsequently chosen in an attempt to satisfy all criteria based on expression level, amplification efficiency, and the lowest level of variability, both overall and for each horse. Using the geometric average of 4 housekeeping genes did not appear to be any more beneficial than the geometric average of 3 housekeeping genes.

Six housekeeping genes (18s, β-actin, $\beta_2$microglobulin, GAPDH, RPL19, ubiquitin) were selected based on the availability of full or partial length equine sequences for commonly used endogenous controls. No equine nucleotide sequences were available for other commonly used housekeeping genes such as 28s, acidic ribosomal protein, β-glucuronidase, cyclophilin A, hypoxanthine guanine phosphorylbosyl transferase (HPRT), hypoxanthine ribosyl transferase, phosphoglycerokinase or transferrin receptor. The three housekeeping genes subsequently used in this series of experiments have been used for normalization in Q-RT-PCR, PCR and Northern hybridizarion experiments using equine cartilage conducted in our and other laboratories. However, these genes have not been previously exhaustively compared. Normalization should reduce variability associated with errors in RNA purity, RNA quantification, or the amount of RNA initially used (Balaburski *et al* 2003). We observed that the suitability of each housekeeping gene varied depending on the gene of interest and between horses, suggesting that the housekeeping genes varied independently of each other and necessitating the use of more than one gene as an endogenous control. Similar sizeable inter-individual differences have been documented in studies conducted in other species (Dheda *et al* 2004, Tricarico *et al* 2002). A geometric mean (Vandesompele *et al* 2002) of 18s, β_2-microglobulin and GAPDH was used in an attempt to incorporate requirements for expression levels, amplification efficiency and gene stability for particular genes of interest and each individual horse. However, inconsistent and irregular amplification behavior may have hampered our attempts to detect subtle treatment effects using this model, as there needs to be less than one $\Delta C'_T$ difference in order to detect differences in gene expression lower than two fold (Livak *et al* 2001, Vandesompele *et al* 2002).

Despite attempts to limit the effects of an observed lack of constitutive expression on the part of the housekeeping genes, there remained important dispersion in the data set. Interestingly, such large gene variability has been identified in other studies investigating expression of degradative enzymes and extracellular matrix proteins in OA cartilage (Aigner *et al* 2001, Martin *et al* 2001). Areas requiring further investigation include the effects of using the sub-saturating dose of IL-1 simulataneously with physiologically relevant doses of GLN and CS; using different forms of CS; and the duration of effect of GLN and CS. Further, the effect of GLN and CS on expression of other genes for which primers were also developed (aggrecan, type II collagen, ILRa, TIMP 1, 3), remains to be determined, as does the effect on other transcription factors and intermediarys in IL-1 signaling pathways and pathophysiological phenomena such as apoptosis.

Table I (chapter 4): Forward and reverse primer sequences (5'→3') of genes used for quantitative real-time polymerase chain reaction.

Gene	Genbank Accession Number[]	Forward primer	Reverse Primer
18s	AJ311673	CGCCGCTAGAGGTGAAATTC	CATTCTTGGCAAATGCTTTCG
Aggrecan	AF040637	CCGCTGGTCAGATGGACACT	GAAGAAGTTGTCGGGCTGGTT
Aggrecanase-1	AF368321	TTTCCCTGGCAAGGACTATGA	GCGGACAATGGCGTGAGT
Aggrecanase-2	AF368322	AAATGCACCTCAGCCACCAT	TGCGTGGGAGGTCTAGCAA
β-actin	AF035774	TCACGGAGCGTGGCTACAG	CCTTGATGTCACGCACGATT
$\beta_2$microglobulin	X69083	CCGCGTGTTCCGAAGGT	GCAGTTCAGGAAATTTGGCTTT
COX 2	AB041771	GGAGCTGTATCCTGCCCTTCT	CCATGGTCTCCCCGAAGAT
GAPDH	AF157626	CCCACCCCTAACGTGTCAGT	TCTCATCGTATTTGGCAGCTTTC
IL-1Ra	AF072535	TGCTCTATTCCTGGGACTCCAT	TGGTTCCTAATCTCATCACCAGACT
iNOS	AY027883	GGCCTTGGCTCCAGCAT	GGTGAGACAACTTCTGGTCAATGT
JNK	BM414648 (91% homology to human sequence)	TTCCAACTGGGCATCATAAATTT	CCCTGCGTCACCCCTACA
MMP 1	AF148882	GGGAGATCATCGTGACAATTCTC	TACGTGGGCCTGGCTGAA
MMP 2	AJ010314	CCCTGAGACGGTGGATGATG	GGATGCGAGAAAACCGTAGTG
MMP 3	U62529	TGTGGAGGTGATGCACAAATC	GCATGCCAGGAAATGTAGTGAA
MMP 9	BM734900 (86% homology to bovine sequence)	CGGCGTGCCCTTGGA	CGGTCCTGGGAGAAGTAAGCT
MMP13	AF034087	TGAAATCATACTACTATCCCCTTAATCCT	TCTCGGAGCCTGTCAACCA
NFκB	BI960845 (84% homology to mouse sequence)	CATCGAGCAGATAGCTCACGTT	AGGTGGTTGGTGAGATTGACAA
RPL19	AY246727	CGAAAGCGAAGGGCAATGT	TGCCTTCGGCTTGTGGAT
TIMP 1	U95039	AGCCTCTGCGGATACTTCCA	TTGTCCGGCGATGAGAAAC
TIMP 3	AJ243283	CCAGGACGCCTTTTGCAA	CCCTCCTTCACCAGCTTCTTC
Type II collagen	U62528	AAGAGCGGAGACTACTGGATTGAC	TCCATGTTGCAGAAAACCTTCA
Ubiquitin	AF506969	CGAGGCCTTTTGGTGTGGTA	ACGGACACGAAGAAACTGCTAAT

Table II (chapter 4): Suitability of housekeeping genes for each gene of interest based on amplification efficiency. The value of the slope of the regression of the plot of ΔC_T versus log cDNA dilution was used as a measure of the difference in amplification efficiencies between the gene of interest and each housekeeping gene. A difference in efficiency of <0.1 was determined for normalization of each gene of interest to a particular housekeeping gene.

	Housekeeping Gene			
	18s	**GAPDH**	**$\beta_2$microglobulin**	**Ubiquitin**
Gene of Interest	MMP1 NFκB	Agg 1 COX 2 INOS JNK MMP 3 MMP 13	Agg 2 MMP 2	MMP 9

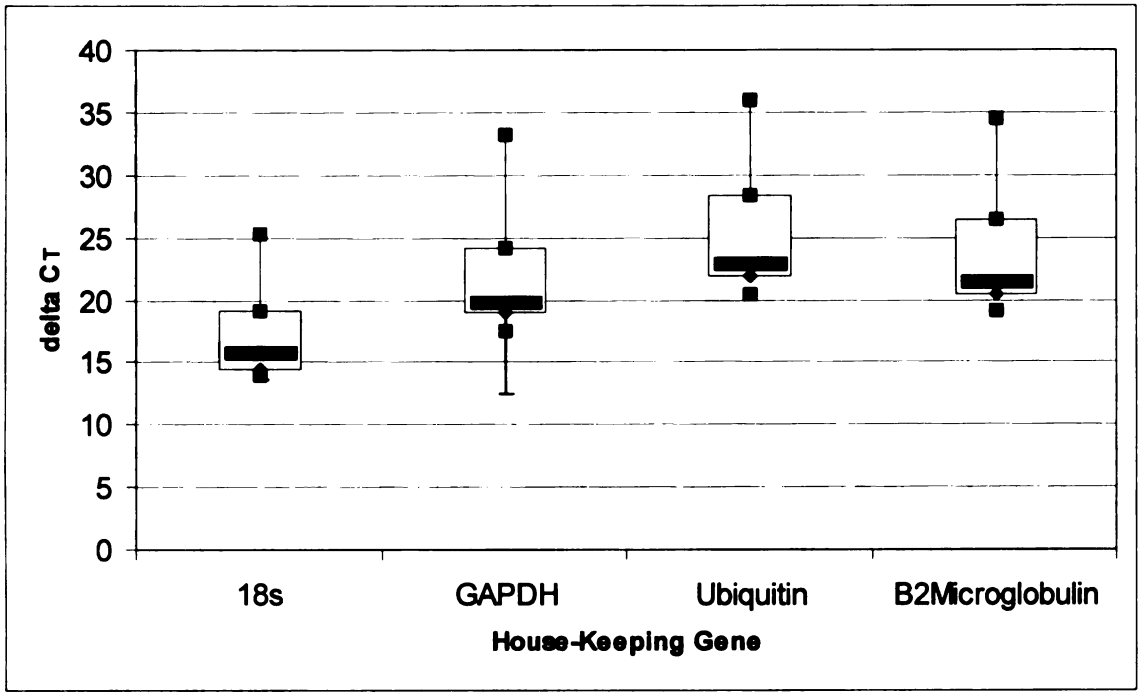

Figure 1 (chapter 4): Q-RT-PCR cycle threshold values. Expression levels are shown as medians (lines), 25th percentile to 75th percentile (boxes) and ranges (whiskers). RPL19 was not detectable in all samples and intermittent primer/dimers were evident using β-actin.

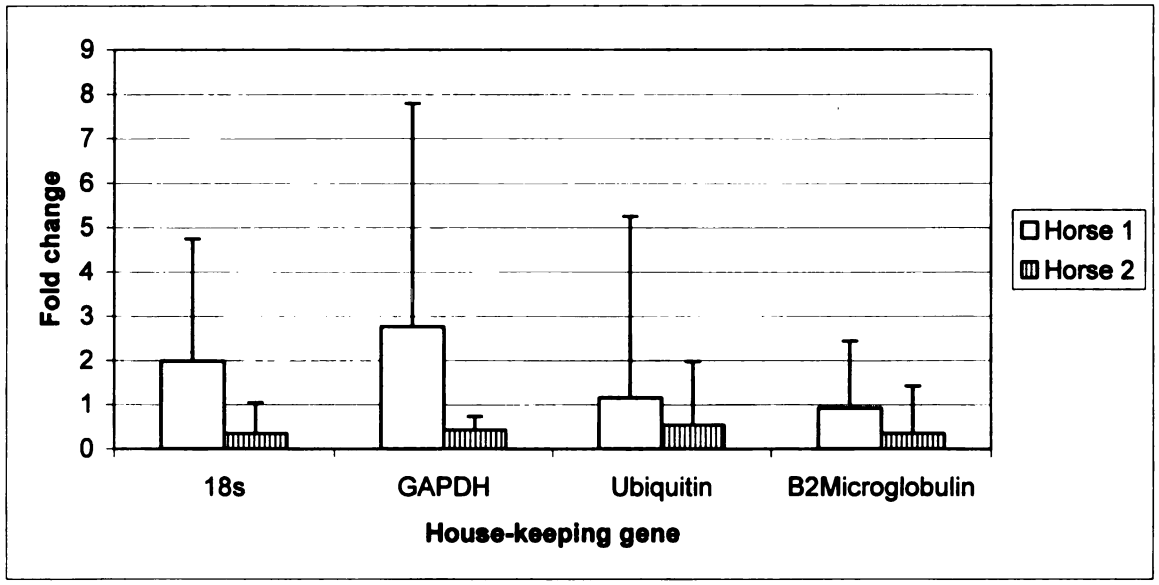

Figure 2 (chapter 4): Fold change in gene expression. Variability in housekeeping gene expression for 2 horses shown as average fold change from the mean (columns) and maximum fold change (error bars).

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