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DIETARY SUPPLEMENTS AND THEIR EFFECT ON
STRESSED CARTILAGE EXPLANTS

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DIETARY SUPPLEMENTS AND THEIR EFFECT ON STRESSED CARTILAGE EXPLANTS

By

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ABSTRACT

DIETARY SUPPLEMENTS AND THEIR EFFECT ON STRESSED CARTILAGE EXPLANTS

By Robert Harlan

We performed two experiments: for the first experiment, three culture schemes: 1) Interleukin-1 (IL-1) (50 ng/ml) treatment with and without GLN + CS for 48 h (short-term non-impact), 2) IL-1 plus mechanical trauma (15 mega pascal) with GLN + CS for 48 h (short-term impact), 3) two-week cultures were cultured with GLN + CS and were exposed to IL-1 (50 ng/ml) on day 2 and day 10 (long-term non-impact). For the second experiment, SAMe was tested at concentrations of 1.0, 0.1, and 0.01 ug/ml (high, medium, and low dose) and ASU was tested at concentrations 10.0, 1.0, and 0.1 ug/ml (high, medium, and low dose). Interleukin-1 (IL-1) at 15 ng/ml was used to initiate inflammatory stress. Nitric oxide (NO) and prostaglandin E_2 (PGE_2) were measured as indicators of inflammatory response for both experiments. Glycosaminoglycans (GAG) were measured as an indicator of cartilage turnover for both experiments.

For the first experiment, GLN + CS did not affect NO, PGE_2, or GAG concentrations in the non-impact short-term model. When mechanical impact was combined with IL-1, the NO concentration in the GLN + CS treatment was lower than the IL-1 treatment and did not differ from control. The GAG release was also lower in the GLN + CS treatment than in the IL-1 treatment. The non-impact long-term cultures demonstrated that GLN + CS treatment did not affect NO or PGE_2 concentration or GAG content in the explants at termination. The second experiment showed no significant treatment effects for either SAMe or ASU.

I would like to dedicate this thesis to my parents for all their unconditional love and support.

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LIST OF ABBREVIATIONS

A disintegrin and metalloproteinase with thrombospondin motifs.....ADAMTS

Avocado and Soy Unsaponifiables.....ASU

Chondroitin Sulfate.....CS

Cyclooxygenase.....COX

Cyclooxygenase-1.....COX-1

Cyclooxygenase-2.....COX-2

Dimethylene blue .....DMB

Enzyme-Linked Immunosorbent Assay.....ELISA

Fetal bovine serum.....FBS

Glucosamine.....GLN

Glycosaminoglycan.....GAG

Inducible nitric oxide synthase.....iNOS

Insulin transferrin selenite.....ITS

Interleukin-1.....IL-1

Microsomal prostaglandin E synthase-1.....mPGEs1

Mega pascal.....MPa

Matrix metalloproteinase.....MMP

Nitric Oxide.....NO

Nonsteroidal anti-inflammatory drugs.....NSAIDs

Osteoarthritis.....OA

Prostaglandin E_2.....PGE_2

S-Adenosyl-L-Methionine.....SAMe

Tumor necrosis factor-alpha.....TNFα

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease marked by progressive cartilage tissue loss, pain, inflammation, and eventual loss of joint function (Felson *et al.* 1998; Hashimoto *et al.* 1998). The significance of OA to the horse racing industry is that it results in lameness. The onset of OA can be initiated by normal exercise of the animal (Stashak 2002). Since no known way to cure or prevent OA exists, better therapies are needed. In recent years, dietary supplements (now known as nutraceuticals) have been used to treat OA in both humans and animals. Glucosamine (GLN) and chondroitin sulfate (CS), S-adenosyl-L-methionine (SAMe), and avocado soy unsaponifiables (ASU) have been among some of the dietary supplements taken for joint pain.

This project consisted of two objectives: 1) our first objective was to test the anti-inflammatory effects of biologically relevant concentrations of GLN plus CS in three different equine cartilage cultures that included conditions for inducing stress; 2) The second objective was to test concentrations of ASU and SAMe in bovine cartilage explant cultures for their ability to mitigate cytokine-induced stress. Explant tissue culture provides an excellent system for testing chondroprotective agents because of the convenience and accuracy of the data collection. Mechanical loading is known to be a risk factor for OA in horses and the combination of GLN and CS at biologically relevant concentrations have never been tested in a mechanical loading tissue culture system.

In the first series of experiments, we provide evidence that biologically relevant concentrations of GLN + CS may be effective against harmful inflammatory responses to trauma in equine cartilage tissue. We exposed equine cartilage explant cultures to three

different types of conditions: 1) a short-term non-impact culture system utilizing (interleukin-1) IL-1 (50 ng/ml) as the inducer of inflammatory stress with and without GLN + CS, 2) a short-term impact utilizing IL-1 plus mechanical trauma to induce inflammatory stress with and without GLN + CS 3) a two-week culture (long term) with continuous exposure to GLN + CS and introduction of IL-1 (50 ng/ml) on day 2 and day 10.

In the second series of experiments, we used the short-term culture system mentioned above to test varying concentrations of SAMe and ASU in bovine explants. Both experiments measure tissue inflammatory and breakdown products as the response variables. Similar types of culture systems have been used in our lab previously (Chan *et al.* 2005a; Chan *et al.* 2005b; Chan *et al.* 2005c), and in this study we use an *in vitro* cartilage explant approach to test GLN and CS, SAMe, and ASU and their ability to mitigate inflammatory responses.

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Chapter 1

Literature Review

Importance of dietary supplements to the horse industry

The American Horse Council reported that the equine industry provides 1.4 million full-time jobs in the U.S. and that there are 9.2 million horses nation-wide (2005). The same report stated that the direct contribution of the horse industry to the U.S. economy is $39.9 billion annually. Also, they found that 4.6 million people are involved in the equine industry in a professional or volunteer capacity.

The significance of osteoarthritis (OA) to the racing industry is that OA results in lameness, reduced performance, and wastage (Murray *et al.* 2005); and one of the main factors that determines the value and usefulness of a race horse is soundness of the legs (Jeffcott *et al.* 1982; Rossdale *et al.* 1985; Fubini *et al.* 1999). Lameness is the most significant limiting factor for race horses; the greatest numbers of days lost to training are caused by lameness (Rossdale *et al.* 1985), and failure to race is most commonly due to lameness (Jeffcott *et al.* 1982).

The presence of OA is pervasive in the American horse population. A survey of veterinary schools found that at veterinary schools, 33% of equine patients had OA lesions (Rose 1977). In another survey of 140 2-year-old Thoroughbred horses, only 23% were sound (Jeffcott *et al.* 1982). The onset of OA can be initiated by injuries sustained during normal exercise normal exercise of the animal and has been linked to activities such as barrel racing, pole bending, dressage, and other types of competions (Stashak 2002). Since no known way to cure or prevent OA exists, better therapies are needed.

Articular Cartilage

Chondrocytes

Articular cartilage tissue at the ends of long bones provides a frictionless surface that allows for joint mobility. Cartilage also provides a shock-absorbing cushion that protects the ends of bones. The tissue is composed of chondrocytes, which are surrounded by an extracellular matrix. These cells only constitute 1% of the tissue volume (Buckwalter and Lane 1997). In healthy cartilage, chondrocytes are capable of breaking down and synthesizing extracellular matrix to maintain a stable equilibrium of catabolism and anabolism (Bullough 1992a). Chondrocytes are also capable of responding to mechanical stimuli, which cause these cells to modulate their metabolism and matrix production to meet the demands of external forces (Buschmann and Grodzinsky 1995). Extracellular matrix mediates cell signaling and maintenance of phenotype (Bullough 1992a; Blanco *et al.* 1995). This is substantiated when chondrocytes are removed from the extracellular matrix and cultured in a monolayer, the cells begin to lose their spherical morphology and chondrocyte-specific markers (Domm *et al.* 2004; Marlovits *et al.* 2004). Adult cartilage tissue is avascular and therefore chondrocytes absorb nutrients from the synovial fluid, which flows through the matrix as a result of mechanical forces on the joint (Mankin 1984). Chondrocytes absorb oxygen solely from the synovial fluid and carry on a combination of aerobic and glycolytic metabolism (Clegg *et al.* 2006).

Chondrocytes are organized into four distinct zones within the cartilage: superficial, transitional, radial, and calcified zones. Within the superficial zone,

chondrocytes have a flattened morphology and are arranged randomly within the matrix (Buckwalter and Mankin 1997). In the transitional zone, they are spherical and are also arranged in a random pattern in the matrix (Buckwalter and Mankin 1997). Chondrocytes in the radial zone are arranged in columns and also demonstrate a spherical morphology (Buckwalter and Mankin 1997). Chondrocytes in the calcified zone have a spherical shape and are arranged in a random pattern in the matrix (Bullough 1992a).

Extracellular matrix

The extracellular matrix of cartilage consists largely of collagen surrounded by proteoglycans. Collagen gives cartilage its tensile strength and provides a rigid framework, which prevents the tissue from swelling to its fullest extent (Maroudas 1976). Collagen fibrils have characteristics appropriate for the each zone of cartilage tissue (Bullough 1992b). Collagen fibrils are arranged parallel to the surface in the superficial zone while in the transitional zone the fibrils appear to be randomly arranged (Bullough 1992b). In the radial zone, the collagen fibrils are arranged perpendicular to the articular surface (Bullough 1992b). The collagen fibrils in the calcified zone however, are calcified by the surrounding chondrocytes (Bullough 1992b). Most of the collagen in the matrix is type II, but type, VI, IX, and XI are also present (Bruckner 1994).

Proteoglycans consist of a protein core with glycosaminoglycan (GAG) side chains in a radial arrangement giving the molecule a "bottle brush" type structure (Iozzo 1998). The specific proteoglycan that is produced by chondrocytes is called aggrecan, which contains chondroitin sulfate (CS) as the predominant GAG side chain. This GAG is composed of repeating disaccharide units of N-acetyl galactosamine and glucuronic

acid residues. The negatively charged sulfur groups of this disaccharide allow hydrogen bonding with water molecules. The resulting hydration endows aggrecan with the cushioning properties that are typical of articular cartilage (Prydz and Dalen 2000). As mechanical forces are applied to the cartilage tissue, fluid is expelled from between the aggrecan side chains and from the tissue. As the force is removed, fluid returns to the matrix and re-hydrates the negatively charged aggrecan side chains, thus yielding the compressive elasticity and shock absorbing properties of the cartilage matrix.

Osteoarthritis

Onset of disease

Osteoarthritis is a degenerative disease marked by progressive cartilage tissue loss, pain, inflammation, and eventual loss of joint function (Felson *et al.* 1998; Hashimoto *et al.* 1998). When injury or disruption to a joint occurs, enzymes are synthesized that break down the proteoglycan component of the matrix (Spiers *et al.* 1994a; Spiers *et al.* 1994b). Chondrocytes respond to this stress by proliferating and increasing proteoglycan synthesis; but this response is sometimes insufficient to overcome the degradation. As a result, the equilibrium is shifted toward a gradual loss of total proteoglycan, leading to cartilage destruction (Morales and Roberts 1988; Hedbom and Hauselmann 2002).

Onset of OA may be initiated by trauma or chronic mechanical stress (Quinn *et al.* 2001; Patwari *et al.* 2003). OA in horses is believed to usually be the result of trauma resulting from over use of the joint (Mackay-Smith 1962). Factors that can induce OA include intense exercise and fatigue (Hodgson and Rose 1989). The pressure on a joint

that a race horse can generate during a race can be millions of foot-pounds per mile (Mackay-Smith 1962). Another risk factor in horses is subchondral bone sclerosis, which results from excessive bone remodeling in response to mechanical forces on the bone (Kawcak 2000). Cartilage that is covering areas of the bone affected by sclerosis is vulnerable to the development of OA because the subchondral bone changes shape and increases in density (Kawcak *et al.* 2001).

Acute trauma to a joint can also lead to OA. Also injury to the anterior cruciate ligament can cause OA (Roos *et al.* 1995). For example, damage to intra-articular ligaments can lead to OA. This has been demonstrated in dogs, rabbits, and in horses by transection of the cranial cruciate ligament and lateral collateral and lateral collateral sesamoidean ligaments in horses, which leads to destabilization of the joint and cartilage wear (Pond and Nuki 1973; Troyer 1982; Simmons *et al.* 1999; Clegg *et al.* 2006). Injury to the meniscus is also a common cause for joint destabilization leading to OA in humans (Roos *et al.* 1998; Clegg *et al.* 2006). Increased concentrations of proteoglycan fragments accumulate in the synovial fluid after injury to the anterior cruciate ligament or meniscus (Lohmander *et al.* 1989). Stromelysin, an enzyme that breaks down cartilage, can increase up to 40-fold over time in an injured joint in (Lohmander *et al.* 1993).

Inflammatory mediators

Joint injury is often accompanied by an inflammatory response that includes the production of pro-inflammatory cytokines such as interleukin-1 beta (IL-1). This cytokine is secreted by macrophages, monocytes, and synoviocytes (Kirker-Head 2000; Fernandes *et al.* 2002) and is implicated in the inflammatory symptoms of OA. Another

example of an inflammatory cytokine that is implicated in OA is tumor necrosis factor-alpha (TNFα) (Hardingham *et al.* 1992; Fernandes *et al.* 2002; Lopez-Armada *et al.* 2006), which is produced by macrophages and synoviocytes. The inflammatory activity of IL-1 is mediated by signaling molecules such as nitric oxide (NO) and prostaglandin E_2 (PGE_2) (Smalley *et al.* 1995; Grabowski *et al.* 1997). IL-1 inhibits proteoglycan synthesis and increases proteoglycan breakdown. When inflammation in the joint is prolonged, it leads to a chronic imbalance in tissue turnover, which results in the eventual destruction of the cartilage tissue (Hardingham *et al.* 1992; Goldring *et al.* 1994). The imbalance is mediated by enzymes in the joint called matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMTS), which are responsible for breakdown of matrix components such as aggrecan and collagen (Struglics *et al.* 2006). Some examples of enzymes of these types that have been implicated in OA include, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 (Patwari *et al.* 2003; Neil *et al.* 2005; Struglics *et al.* 2006).

Treatment

Conventional treatments

Conventional treatments for OA include nonsteroidal anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroids (Hochberg *et al.* 1995b, 1995a). These treatments are effective for decreasing joint pain, but they do not affect the progression of the disease. They also have significant side effects (Hochberg *et al.* 1995a). Nonsteroidal anti-inflammatory drugs, amoung other things, inhibit two enzymes known as cyclooxygenase-1 and -2 (COX-1 and COX-2) (Harkins *et al.* 1993). Both of these

enzymes produce PGE_2, which mediates pain and inflammation in the joint. However, COX-1 is also an important enzyme for maintaining the gastrointestinal lining. As a result of COX-1 inhibition, NSAIDs eventually cause gastrointestinal ulceration if they are taken long-term, which results in thousands of deaths each year (Hochberg *et al.* 1995a; Griffin 1998). A common NSAID used in horses, called phenylbutazone, is associated with gastric ulcers, weight loss, and diarrhea (Traub *et al.* 1983). The development of a COX-2-specific inhibitor seemed to be a significant advance promising to become a NSAIDs treatment without significant side effects. Inhibiting COX-2 exclusively, however, resulted in cardiovascular side effects in patients. Clinical trials of some of these drugs demonstrated that adverse cardiovascular events were 3 times more frequent after coronary bypass surgery when COX-2 inhibitors were administered (Ott *et al.* 2003; Nussmeier *et al.* 2005). Reports also detail increased risk of cardiovascular events and serious skin reactions (Sibbald 2005; Talhari *et al.* 2005). This cardiovascular effect may be because COX-2 produces prostaglandin I_2 in vascular epithelial tissue, which inhibits platelet aggregation, neutrophil adhesion, and dilatation of bronchial and vascular smooth muscles (Lin 2005). Modulation of such physiological processes could cause thromboembolic events, which is a risk especially to patients with a history of cardiovascular disease (Fitzgerald 2004; Warner *et al.* 2004).

Synthetic corticosteroids can be injected intra-articularly for treatment of OA and have shown efficacy in dramatically decreasing inflammation in horses and in humans (Harkins *et al.* 1993; Bellamy *et al.* 2005). These treatments also decrease the production of prostaglandins by COX-2 enzymes (Moses *et al.* 2001; Frean *et al.* 2002; Tung *et al.* 2002). The side effects are eventually damaging to the cartilage, which may include the

formation of calcium deposits on the surface of the cartilage, fissuring of the cartilage, and decreased cartilage elasticity (Harkins *et al.* 1993).

Glucosamine and chondroitin sulfate

In recent years, dietary supplements (now known as nutraceuticals) have been used to treat OA in both humans and animals. Glucosamine (GLN) and CS have been among the most popular supplements. GLN is a precursor molecule of various components of the cartilage matrix (Hamerman 1989; Muller-Fassbender *et al.* 1994). Chondroitin sulfate is a component of the cartilage proteoglycan called aggrecan (Conte *et al.* 1991). Some evidence suggests that glucosamine is as effective as ibuprofen at relieving pain (Muller-Fassbender *et al.* 1994) and that the combination of GLN and CS is effective at relieving moderate to severe joint pain in OA patients (Clegg *et al.* 2006). GLN has an excellent safety profile, when taken orally, based on studies done in humans, dogs, and rats (Pujalte *et al.* 1980; Tapadinhas *et al.* 1982; Muller-Fassbender *et al.* 1994). CS also has an excellent safety profile in humans, dogs, and rats (Conte *et al.* 1995; Bucsi and Poor 1998; Ronca *et al.* 1998; Volpi 2002). Dosing in humans is usually 1500 mg of GLN and 1200 mg of CS daily (Clegg *et al.* 2006) and in horses the dosage is 9 g of GLN and 3 g of CS (Du *et al.* 2004).

Work has been done in animal models to show the effectiveness of these agents. For instance, work done in cultures systems with IL-1 stimulated cartilage explants from Holstein steers show that GLN and CS will decrease cartilage NO and PGE_2 synthesis at concentrations found in the blood (Chan *et al.* 2005b). Another study used fibronectin fragments to initiate cartilage degradation in bovine cartilage explants and showed that

mixture of GLN and CS at biologically relevant concentrations was effective at decreasing expression of catabolic mediators MMP-3 and MMP-13 and promoting repair (Homandberg *et al.* 2006). Studies in equine cartilage explant cultures have shown that GLN and CS are effective at decreasing NO, PGE_2 , MMP-9, and MMP-13, though the concentrations used here were higher than what is normally found *in vivo* (Orth *et al.* 2002). Studies in equine chondrocyte cell culture (pellet culture) have shown that relevant concentrations of GLN decrease expression of catabolic mediators such as MMP-13, aggrecanase-1, inducible nitric oxide synthase (iNOS), and COX-2 (Neil *et al.* 2005).

The mechanism of action of these two substances has received significant attention lately. Based on the biochemistry of GLN and CS and GAG synthesis is examined, it appears feasible that these molecules could increase the synthesis of GAG (McCarty *et al.* 2000). McCarty *et al.* proposed that GLN supplementation provides the rate-limiting substrate for GAG (McCarty *et al.* 2000). Evidence for this theory was demonstrated by a study in which over-expression of glutamine fructose-6-phosphate aminotransferase, an important rate limiting enzyme in the synthesis of GLN, caused resistance to IL-1 inhibition of GAG synthesis (Gouze *et al.* 2001). In a study involving dogs with a transected cranial cruciate ligament, a combination of GLN and CS increased GAG synthesis (Johnson *et al.* 2001). One of the mechanisms for the preservation of cartilage tissue by GLN maybe the inhibition of aggrecanase (Patwari *et al.* 2003; Chan *et al.* 2005a). GLN alone decreases IL-1-induced production of NO and PGE_2 and IL-1-induced proteoglycan breakdown (Fenton *et al.* 2000; Gouze *et al.* 2001; Fenton *et al.* 2002; Chan *et al.* 2005b). The mechanism of decreasing proteoglycan breakdown is in

part due to the inhibition of MMPs, aggrecanases, and collagenase (Fenton *et al.* 2000; Piperno *et al.* 2000; Fenton *et al.* 2002; Dodge and Jimenez 2003; Chan *et al.* 2005a). GLN can also decrease the gene expression of MMP-1, MMP-3, and MMP-13 (Byron *et al.* 2003; Dodge and Jimenez 2003; Chan *et al.* 2005a). CS May stimulate GAG production by binding to membrane proteins of synovial cells (McCarty *et al.* 2000). CS increase proteoglycan synthesis and decreases proteoglycan breakdown (Bassleer *et al.* 1992; Nerucci *et al.* 2000). CS reduces proteoglycan loss from articular cartilage in humans (Uebelhart *et al.* 1998) and rats (Omata *et al.* 1999) when taken orally. CS may reduce proteoglycan loss in part by decreasing aggrecanase-1 synthesis (Chan *et al.* 2005a). CS can also decrease IL-1-induced PGE_2 and NO production (Bassleer *et al.* 1992; Chan *et al.* 2005b). The mechanism for this appears to be that CS decreases the expression of iNOS and microsomal prostaglandin E synthase-1 (Chan *et al.* 2005b).

If GLN and CS are taken together, they appear to have a synergistic effect in mitigating the clinical signs of OA. An *in vivo* study that used a rabbit instability model of osteoarthrosis showed that animals given GLN and CS did not develop OA lesions as severe as rabbits fed GLN or CS alone (Lippiello *et al.* 2000). In the *in vitro* portion of the study, the combination of glucosamine hydrochloride and chondroitin sulfate acted synergistically in stimulating GAG synthesis (Lippiello *et al.* 2000). GLN and CS also both increase chondrocyte response to mechanical stress and prevent the loss of GAG (Lippiello 2003). The fibronectin fragments study also showed a synergistic relationship between GLN and CS for reversing cartilage damage and promoting repair (Homandberg *et al.* 2006). GLN and CS have the ability to modify expression of genes that regulate inflammatory mediators such as iNOS, COX-2, and mPGEs1 (Chan *et al.* 2005b; Neil *et*

al. 2005). The concentrations used in these studies (Chan *et al.* 2005b; Neil *et al.* 2005) were within range of those found in the blood of horses after oral administration (Du *et al.* 2004), and within GLN levels achieved in the synovial fluid of horses when administed by intravenous infusion (Laverty *et al.* 2005).

S-adenosylmethionine

S-Adenosyl-L-methionine (SAMe) is synthesized by the body from the essential the amino acid L-methionine and the nucleoside adenosine triphosphate. SAMe acts as a methyl donor for reactions important in the synthesis of proteins, phospholipids, and hormones. It has a variety of roles and is involved in membrane function, gene regulation, and brain function (Chiang *et al.* 1996). SAMe was first sold over the counter in 1999 for treatment of joint pain. The common human dosage is usually between 400 and 1600 mg daily. SAMe has a good safety record, with few side effects and an absence of generation of toxic metabolites (Kagan *et al.* 1990; Gören *et al.* 2004). The concentration attained in the synovial fluid in OA patients given SAMe at 400 mg daily for 7 days is 30 to 80 ng/ml (Stramentinoli 1987). Clinical trials show that SAMe supplementation decreases pain and inflammation (Polli *et al.* 1975; Caruso and Pietrogrande 1987; Soeken *et al.* 2002). A randomized, double-blind, cross-over study, comparing SAMe with celecoxib (Celebrex) showed that SAMe, over the long term, is just as effective at relieving pain as Celecoxib (Najm *et al.* 2004).

The exact mechanism of action is not known. SAMe also upregulates the proteoglycan synthesis of chondrocytes, and some believe that it may function as a signal of sulfur availability (McCarty and Russell 1999). A study used human chondrocytes in

a thick-layer culture model and showed that SAMe increases proteoglycan synthesis (Harmand *et al.* 1987). SAMe has also demonstrated anti-inflammatory effects by suppressing the effects of TNFα and transcriptional activation of iNOS and COX-2 (Hevia *et al.* 2004). IL-1 suppresses the production of SAMe in the joint. Therefore, since IL-1 production in the joint is upregulated in the disease state of OA, a supplementation of SAMe may compensate for this deficit (McCarty and Russell 1999). SAMe also decreases with aging in human and rats (di Padova 1987; Stramentinoli 1987). Since aging is a risk factor for OA, the two may be related and supplementation with age may be a benefit.

Avocado/soybean unsaponifiables

Avocado soybean unsaponifiables (ASU) are a mixture of vegetable extracts obtained from avocado and soy bean oils that are normally taken orally. Clinical trials with ASU show that it has a good safety record (Lequesne *et al.* 2002; Little and Parsons 2002), though more study is needed to confirm this. These extracts are obtained when the oil is saponified (heated and broken down by alkaline hydrolysis). The small fraction that is not broken down is known as the "unsaponifiable" fraction (Bassleer *et al.* 1992). It relieves pain when taken orally, and is usually taken at 300 mg daily (Reginster *et al.* 2000; Walker-Bone 2003; Soeken 2004).

This decrease in pain is attributed to the ability of ASU to decrease inflammatory mediators such as IL-1, NO, and PGE_2 as seen in human chondrocyte studies (Bassleer *et al.* 1992). A study using human chondrocytes in an alginate bead tissue culture system showed that ASU increased the accumulation of GAG in the alginate beads in a dose

dependent manner (Bassleer *et al.* 1992). Avocado soy unsaponifiables also prevent collagen breakdown and thus potentially save cartilage from degradation (Mauviel *et al.* 1989). This is partially mediated by the ability of ASU to inhibit MMP production, such as MMP-3, as it did in a study of human chondrocytes in culture (Bassleer *et al.* 1992). There has been no pharmacokinetic studies done on this supplement and the exact mechanism of action is unknown.

Rationale for experiments

One objective is to test biologically relevant concentrations of GLN and CS in various equine cultures: 1) a short-term (2 d) *in vitro* culture model designed for cartilage explants in a 24-wellplate format, 2) a similar short-term culture model including mechanical loading, 3) a long-term culture system (without loading) designed to test the effects of GLN and CS over a 2-week period. Explant tissue culture provides an excellent system for testing chondroprotective agents because of the convenience and accuracy of the data collection. Mechanical loading is known to be a risk factor for OA in horses and the combination of GLN and CS at biologically relevant concentrations have never been tested in a mechanical loading tissue culture system. IL-1 will be used to model inflammatory stress in each model and a hydraulic stressor with IL-1 will be used to model mechanical loading in the short-term impact culture. The response variables will consist of concentrations of inflammatory mediators NO and PGE_2 in the media. Glycosaminoglycan concentration in the media and within cartilage explants will be measured as an indicator of cartilage breakdown. The concentrations of GLN and CS

that will be used in these cultures will be in the range of what is measured in the blood of dogs and horses after oral administration (Adebowale *et al.* 2002; Du *et al.* 2004).

Another objective is to test varying concentrations of SAMe and ASU in bovine cartilage explant cultures. The system will be a short term 2 d culture with IL-1 used to model inflammatory stress. The response variables will consist of NO, PGE_2, and GAG concentrations in the media and concentration of GAG in cartilage explants at termination of the experiment. Bovine tissue culture is used here because cartilage tissue from bovine species is readily available. Bovine tissue culture is often used as a model for testing agents that have relevance to human treatment.

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Chapter 2

The Effect of Biologically Relevant Concentrations of Glucosamine and Chondroitin Sulfate on Stressed Equine Cartilage Explants

Summary

Reasons for performing study: In the past decade, dietary supplements such as glucosamine (GLN) and chondroitin sulfate (CS) have gained popularity in treating joint pain and inflammation due to osteoarthritis (OA) in humans and animals. Although GLN and CS have established anti-inflammatory properties, to our knowledge, no study has tested biologically relevant concentrations of these molecules in equine cartilage explants.

Hypothesis: Glucosamine and CS have anti-inflammatory properties at biologically relevant concentrations when tested in equine cartilage explant cultures.

Methods: Three culture schemes were used: 1) IL-1 (50 ng/ml) treatment with and without GLN + CS (short-term non-impact) 2) IL-1 plus mechanical trauma with and without GLN + CS (short-term impact) 3) two-week cultures were cultured with and without GLN + CS and were exposed to IL-1 (50 ng/ml) on day 2 and day 10 (long-term non-impact). Nitric oxide (NO) and prostaglandin E_2 (PGE_2) were measured as indicators of inflammatory response. Glycosaminoglycans (GAG) were measured as an indicator of cartilage turnover.

Results: Glucosamine + CS did not affect NO, PGE_2, or GAG concentrations in the non-impact short-term model. When mechanical impact was combined with IL-1 the NO production in the GLN + CS treatment was lower than the IL-1 treatment and did not

differ from control. The GAG release was also lower in the GLN + CS treatment than the IL-1 treatment, although the PGE_2 concentration was not affected by GLN + CS. The non-impact long-term cultures demonstrated that GLN + CS treatment decreased GAG release and did not affect NO or PGE_2 concentration or GAG content in the explants at termination.

Conclusions: This study suggests that biologically relevant concentrations of GLN + CS may have protective effects against some indicators of mechanically-induced trauma in explants.

Potential relevance: These *in vitro* data provide support that GLN and CS may be beneficial for equine joint health.

Introduction

In recent years, dietary supplements (now known as nutraceuticals) have been used to treat osteoarthritis (OA) in both humans and animals. Glucosamine (GLN) and chondroitin sulfate (CS) have been among the most popular dietary supplements taken for joint pain. Glucosamine is a precursor of N-acetyl galactosamine, which is a component of glycosaminoglycans (GAG). Glycosaminoglycans, such as CS, are components of a proteoglycan called aggrecan, which gives cartilage its distinctive properties. Both of these molecules increase synthesis (Bassleer *et al.* 1992; Lippiello *et al.* 2000) and decrease breakdown (Orth *et al.* 2002; Lippiello 2003) of GAG in cartilage tissue, and they may be an effective treatment for decreasing joint pain in clinical trials (Clegg *et al.* 2006; Qui *et al.* 2005).

We use three different culture systems with equine cartilage explants: short-term non-impact used previously in our lab (Chan *et al.* 2005b), short-term impact system (Ewers *et al.* 2001), and a long-term non-impact system. Our objective was to test the anti-inflammatory effects of biologically relevant concentrations of GLN and CS in these three different equine cartilage cultures that. We provide evidence that biologically relevant concentrations of GLN and CS may be effective against harmful inflammatory responses to trauma in equine cartilage tissue.

Materials and methods

Explant Cultures

Articular cartilage was isolated from antebrachial-carpal and middle carpal joints of 2- to 9- year-old horses, of several breeds (Thoroughbred, Appaloosa, Tennessee Walking Horse, Arabian, and Paint). Horses were euthanized at The Diagnostic Center for Population and Animal Health at Michigan State University for reasons other than lameness. Cartilage discs (6 mm in diameter) were isolated and randomly distributed to be cultured in 24-well Falcon plates[b] with two discs per well. Each well contained 1 ml of media. The medium (Ham media: 1:1 Dulbecco's Modified Eagles Medium: nutrient mixture F-12)[a] was supplemented as previously described (Fenton *et al.* 2000). Medium was also supplemented with all 20 amino acids[c] at 25% the concentration as previously described (Rosselot *et al.* 1992).

Human recombinant interleukin-1 beta[e] (IL-1) at a concentration of 50 ng/ml was used to induce inflammatory stress. Glucosamine HCl[f] (1, 5, or 10 μg/ml) and low

molecular weight (16.9 kilodaltons) chondroitin sulfate[f] (5, 20, or 50 μg/ml) were all within the range of concentrations known to occur in equine blood after oral administration (Adebowale *et al.* 2002; Du *et al.* 2004).

Short-Term Non-Impact Model

Explants for short-term experiments were harvested from six different horses and equilibrated for 2 d in media without fetal bovine serum[a] (FBS). Media was removed and replaced with fresh media 1 d after harvesting. Cultures were then incubated in media supplemented with 10% FBS[a] and treatments 1-4 in Table 1 for 2 d. There were 6 to 12 wells per treatment depending on the availability of cartilage from a given horse. Treatments were non-impact treatments were assigned as listed in Table I. Media were replaced daily until termination of the experiment at 4 d after harvest.

Short-term Impact Model

Explants from three horses were mechanically impacted as a single acute load of 15 mega pascal (MPa) for 50 msec using a Servo-Hydraulic Testing Machine (Model 1331).[g] The unconfined explants were compressed between highly polished stainless steel plates and peak load was recorded electronically. There were 6 wells per treatment and the impact treatments were assigned as listed in Table I.

Long-Term Model

Explants in long-term cultures were equilibrated as above for 2 d and the media was changed at 24 h after harvesting. Cultures were then incubated in serum-free media supplemented with 1 µl/ml insulin transferring selenite[h] (ITS) to termination. There were 9 wells per treatment assigned as listed in Table II. Media were replaced every 2 d until termination of the experiment at 14 d. The cartilage explants were then stored at -20° C for later papain digestion and GAG analysis.

All conditioned media samples were stored at 4° C for later assay. All cultures were maintained at 37° C in a humidified incubator with 7% CO_2. For the short-term non-impact system, there were six horses used (n = 6) for all treatments except the low dose, which only had four horses (n = 4). For both the short-term impact and the long-term non-impact there were three horses (n = 3).

Biochemical Analyses:

Nitrite levels in the media were assayed as previously described (Blanco *et al.* 1995; Chan *et al.* 2005b). Absorbance was detected at 540 nm by a Spectromax 300 plate reader.[i] Results are expressed as nmol NO/ml.

GAG release into the media was assayed by dimethylene blue[j] (DMB) assay as previously described (Chandrasekhar 1987). The total amount of GAG in the explant was obtained from papain[c]-digested cartilage. Results of explant DMB assays are expressed as µg GAG/mg wet weight of cartilage.

Prostaglandin E_2 was assayed using a commercially available kit[e] according to the manufacturer's instructions concentrations are reported in pg/ml. Indomethacin[c] (10

μg/ml) was added to the media at collection and samples were stored at -20° C until analysis. To bring the samples within range of the standard curve, samples were diluted 1:10 or 1:20 depending on the PGE_2 level of the sample, and PGE_2 was detected at 405 nm with a wavelength correction set at 590 nm with a spectrometer.

Statistical Analysis

Data were analyzed using Fisher's Least Significant Difference in the PROC MIXED procedure of SAS software.[k] The data from all wells were pooled according to treatment, with individual horses as replicates. The random effects included horse, treatment*horse, and horse*day. The long-term data had the same random effects and were analyzed for each day using Fisher's Least Significant Difference in the PROC MIXED procedure by using daily average and the repeated measures option. We also analyzed the long-term cumulative total for GAG and NO for all days of the long term using Fisher's Least Significant Difference, except that the horse*day effect and the repeated measure options were omitted since only one measurement was being analyzed for a total time period. GAG content in explants were also measured with Fisher Least Significant Difference with no horse*day effect and no repeated measures option. A p value of <0.05 was considered significant and p values <0.1 were considered a trend.

Results

Short-Term Non-Impact Model

The explants in the IL-1-only treatment released significantly more NO, PGE_2, and GAG compared with the control. The NO concentration in the media in the 5 μg/ml GLN + 20 μg/ml CS (medium dose), and 1 μg/ml GLN + 5 μg/ml CS (low dose) did not differ from the IL-1-only treatment (Fig 1A). The low dose did not differ from the control (Fig 1A). The nutraceutical treatments had no effect on PGE_2 or GAG release into the media. (Fig 1B, C).

Short-term Impact Model

The IL-1 + impact explants released more NO ($p = 0.03$) and GAG ($p = 0.02$) than the control (Fig 2A-C). The impact model included only the medium dose of GLN + CS because of lack of available tissue. The NO concentration in the IL-1 + impact treatment containing GLN + CS was lower than in the IL-1 + impact treatment ($p = 0.013$) and did not differ from the control (Fig 2A). Neither the IL-1 nor the GLN + CS treatment had a significant effect on PGE_2 release into the media (Fig 2B). GAG release in the GLN + CS treatment was lower than in the IL-1 + impact treatment ($p = 0.023$) (Fig 2C).

Long-Term Model

The IL-1 treatment resulted in increased NO release in all treatments with IL-1 on d 4, d 12, and total for the two week accumulation (Table 3). The GLN + CS treatments did not affect the IL-1-induced NO release (Table 3).

The GAG release was higher in all treatments with IL-1 than those without IL-1 on d 4, but the IL-1 did not effect the GAG release on d 12 (Table 4). The high dose

GLN and CS treatment had lower GAG release than the IL-1 treatment on d 4 ($p < 0.001$) (Table 4). For the cumulative totals, all treatments containing IL-1 were lower than the IL-1-only, except for the control, which tended to be lower ($p = 0.093$) (Table 4). The high dose GLN and CS treatment had lower GAG release over the two-week period ($p = 0.001$) and the low dose tended to be lower ($p = 0.055$).

The assay for GAG content in explants did not show any difference between the control and the IL-1-only treatment (Fig 3). Furthermore, the IL-1-only treatment did not differ significantly from the control or any other treatment.

Discussion

We used equine cartilage explant cultures including short-term, long-term, and mechanical trauma (impact) culture systems. The short-term model has been used befor in out laboratory to test these agents (Chan *et al.* 2005a-c). To our knowledge, this is the first study to test physiologically relevant concentrations of GLN + CS in equine articular cartilage using trauma to stress the explants. This is a relevant way to induce stress is to apply mechanical impact, since OA often results from acute trauma to the joint and subsequent inflammation (Quinn *et al.* 2001; Patwari *et al.* 2003). We modeled acute trauma to the cartilage using a hydraulic impact concomitant with IL-1 treatment as previously reported (Quinn *et al.* 2001; Patwari *et al.* 2003). A previous study (Dvoracek-Driksna 2001) has demonstrated that impact treatment at 15 MPa pressure causes fissuring and cell death in the superficial tangential zone of bovine cartilage. We used this treatment concomitantly with IL-1, to model trauma and inflammatory stress found *in vivo*. The long-term study was used to see if there were long-term effects of

GLN and CS on cartilage explants. The advantage of the long-term system is that total release of NO and GAG can be calculated at the end of an experiment as well as the average concentration of GAG within the explant for each treatment. This cumulative effect of the treatments may reveal effects that may not be seen over the short-term, especially since these agents are known to be slow acting when compared with conventional treatments (Muller-Fassbender *et al.* 1994).

NO is an inorganic free radical released into the media by the cartilage tissue. Studies using rabbit and human cartilage have shown that NO can increase GAG loss and decrease GAG synthesis (Taskiran *et al.* 1994; Hardy *et al.* 2002). Nitric oxide promotes inflammation and cartilage breakdown by increasing cytokine production, suppressing matrix synthesis, and increasing matrix metalloproteinase (MMP) synthesis (Taskiran *et al.* 1994; Evens 1995; Murrell *et al.* 1995). We demonstrated that the NO release was lower in cartilage explants treated with GLN + CS and impact + IL-1 than in cartilage explants treated with impact + IL-1 alone, though GLN + CS were not effective without impact as they were in a previous study (Chan *et al.* 2005b).

Prostaglandin E_2 was also measured because it is implicated in the pain, inflammation, and cartilage breakdown observed in OA (Hardy *et al.* 2002; Kirker-Head *et al.* 2000; Schueter and Orth 2004). Why the GLN and CS treatments were ineffective at decreasing the PGE_2 release or why concentration of PGE_2 was so much higher in the GLN and CS low dose treatment than even in the IL-1–only treatment in the short-term non-impact model is not known (Fig 1B). In contrast, a previous study has demonstrated success in effecting a change in PGE_2 release with GLN + CS at these concentrations in explants from Holstein steers (Chan *et al.* 2005b). Other studies that used equine

cartilage demonstrated positive affects of GLN (alone) on PGE_2, but at much higher (up to 100 times) concentrations of GLN used in the present study (Orth *et al.* 2002; Schlueter and Orth 2004).

IL-1-induced MMP synthesis causes cartilage matrix breakdown, which we quantified by measuring GAG release (Chan *et al.* 2005a). GLN alone decreases IL-1-induced proteoglycan breakdown (Fenton *et al.* 2000; Gouze *et al.* 2001; Fenton *et al.* 2002). CS also may reduce proteoglycan loss in part by decreasing MMP-13 (Chan *et al.* 2005a). Another study showed a synergistic relationship between GLN and CS for reversing cartilage damage and promoting repair (Homandberg *et al.* 2006). In the current study, the high dose was most effective at protecting cartilage against the loss of GAG (Table 4), and there was also an effective protection using the medium dose in the impact system (Fig 2 C). There was no treatment effect on the GAG content in the explants at termination (Fig 4).

Chan *et al.* (2005b) have previously demonstrated that genes responsible for NO and PGE_2 synthesis are down-regulated when bovine explant cultures are exposed to physiologically relevant concentrations of GLN + CS. Metalloprotienases responsible for breakdown of the cartilage matrix are also down regulated (Chan *et al.* 2005a). The present study tested the same physiologically relevant concentrations of GLN and CS as those used by Chan *et al.* (2005a-b). There were instances in this study where the GLN + CS treatments were less effective than reported in earlier studies. It is unknown why the PGE_2 concentration was unaffected by the GLN + CS treatment in either the short-term impact experiment or the short-term non-impact experiments. It is also difficult to explain the lack of responsiveness of NO and GAG level to GLN + CS treatment in the short-

term non-impact experiment. Significant differences may not have been found because the GLN + CS concentrations may have been too low to effect a change. Also, the variation between horses, which differed with respect to breed and age, may have been too high to detect a statistically significant difference between treatments. The Chan *et al.* (2005a-b) studies used bovine species of a single breed and common age, which may have decreased the variation in these studies and made finding differences more statistically possible.

Conclusion

Our hypothesis was that GLN + CS would decrease inflammatory markers and cartilage breakdown at physiologically relevant concentrations. Our results indicate that GLN + CS are effective in mitigating harmful inflammatory responses due to mechanical impact + IL-1 in equine cartilage tissue. The advantage of this impact model is the combination of impact and IL-1. The reason for this is that OA is often initiated by trauma to the joint, which is then followed by IL-1-mediated inflammation (Quinn *et al.* 2001; Patwari *et al.* 2003). This makes the conditions in this system somewhat similar to those found *in vivo.* We demonstrated that the NO release was lower in cartilage explants treated with GLN + CS and impact + IL-1 than in cartilage explants treated with impact + IL-1 alone. We have also shown that the GAG release was lower in cartilage explants treated with GLN + CS and impact + IL-1 than in cartilage explants treated with impact + IL-1 alone. We also demonstrated that, over a two-week period, the GAG release was lower in cartilage explants treated with GLN + CS and IL-1 than in cartilage explants treated with IL-1 alone. This study provide some support these supplements are

beneficial for horses, in maintaining equine joint health, although *in vivo* research should be done to confirm the findings.

Manufacturer's address

[a]Gibco, Grand Island, New York, U.S.A.

[b]Fisher Scientific, Pittsburgh, Pennsylvania, U.S.A.

[c]Sigma Chemical, St Louis, Missouri, U.S.A.

[d]J.T. Baker, Phillipsburg, New Jersey, U.S.A.

[e]R&D Systems, Minneapolis, Minnesota, U.S.A.

[f]Nutramax Laboratories, Edgewood, Maryland, U.S.A.

[g]Instron Corrperation, Canto, Massachusetts, U.S.A.

[h]Roche Diagnostics Corporation, Indianapolis, Indiana, U.S.A.

[i]Molecular Devices, Sunnyvale, California, U.S.A.

[j]Polyscience, Inc., Warrington, Pennsylvania, U.S.A.

[k]SAS Institute, Inc., Cary, North Carolina, U.S.A.

Table I

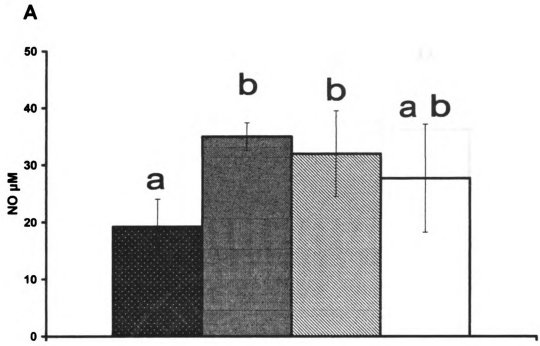
Agents added to explant cultures-experiment #1
(10% FBS added to media for all treatments)

Treatment	IL-1 (ng/ml)	SAMe (µg/ml)	ASU (µg/ml)	EthOH (%)
1) Control: No IL-1	-	-	-	-
2) IL-1-only	15	-	-	-
3) IL-1 + SAMe High Dose	15	1	-	-
4) IL-1 + SAMe Medium Dose	15	0.1	-	-
5) IL-1 + SAMe Low Dose	15	0.01	-	-
6) IL-1 + Ethanol	15	-	-	0.3
7) IL-1 + ASU High Dose	15	-	10.0	0.3
8) IL-1 + ASU Medium Dose	15	-	1.0	0.3

Table II

Agents added to explant cultures-experiment #2
(10% FBS added to media for all treatments)

Treatment	IL-1 (ng/ml)	ASU (µg/ml)	EthOH (%)
1) Control: No IL-1	-	-	-
2) IL-1-only	15	-	-
3) IL-1 + Ethanol	15	-	0.3
4) IL-1 + ASU High Dose	15	10.0	0.3
5) IL-1 + ASU Medium Dose	15	1.0	0.3
6) IL-1 + ASU Low Dose	15	0.1	0.3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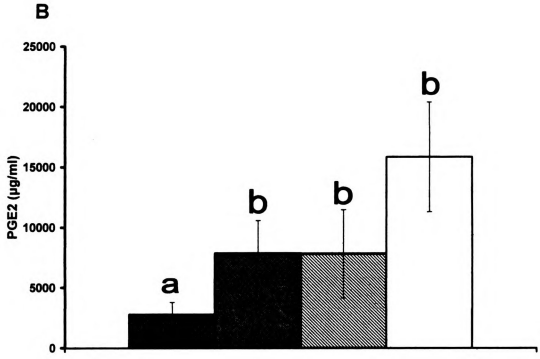
30
20
10
0
NO µM
a
b
b
a b

Fig1.A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20000
15000
10000
5000
0
PGE2 (µg/ml)
a
b
b
b

Fig1.B

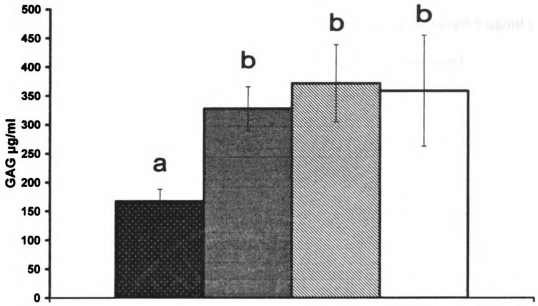

Fig1.C

Legend

Control	**IL-1**
IL-1 + Medium Dose GLN + CS	**IL-1 + Low Dose GLN + CS**

Fig 1: Mean (±SE, n=6 for medium dose, n=4 for low dose) NO, GAG, and PGE_2 concentrations in media samples at d 1 (A, C) or at d 1 (B). IL-1 (50 ng/ml) was added to each treatment except for control. The experimental treatments contained 5 μg/ml GLN + 20 μg/ml CS (medium dose) or 1 μg/ml GLN + 5 μg/ml CS (low dose).

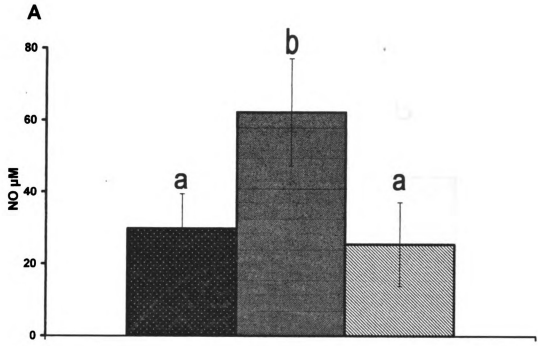

Fig2.A

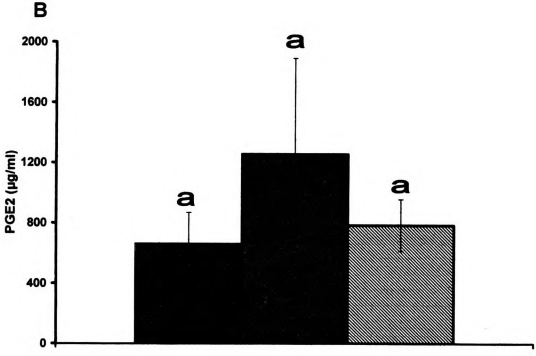

Fig2.B

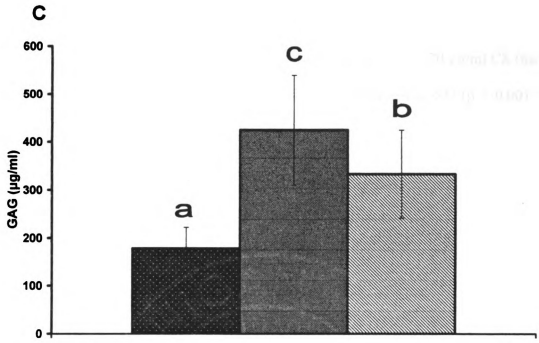

Fig2.C

Legend

Control **IL-1** **IL-1 + Medium Dose GLN + CS**

Fig 2: Mean (±SE, n=3) NO, PGE_2, and GAG concentrations in media samples at d 0 and 1 (A, C) or at d 1 (B). IL-1 (50 ng/ml) + Impact was added to each treatment except for control. The experimental treatment contained 5 μg/ml GLN + 20 μg/ml CS (medium dose). GLN + CS treatment differed from IL-1-only treatment in NO ($p = 0.0013$) and GAG ($p = 0.023$) concentrations.

Table III

Mean NO concentration (μM) ± SE with n=3

Day 4, 12, and cumulative over 14 days

Treatment	Day4	Day 12	Cumulative
1) Control: No IL-1 / No impact	15.0 ± 1.3***	11.17 ± 2.4**	102.3 ± 10.2***
2) GLN + CS Low Dose	14.4 ± 1.3***	10.81 ± 2.5***	97.7 ± 12.1***
3) GLN + CS Medium Dose	14.7 ± 1.1***	11.75 ± 2.4**	101.8 ± 11.2***
4) GLN + CS High Dose	15.4 ± 1.3***	11.15 ± 2.9***	98.9 ± 9.6***
5) IL-1	64.3 ± 12.0	25.53 ± 6.3	197.6 ± 18.8
6) IL-1 + GLN + CS Low Dose	55.1 ± 6.6	24.27 ± 7.4	182.0 ± 17.7
7) IL-1 + GLN + CS Medium Dose	57.9 ± 5.9	26.90 ± 6.3	193.3 ± 14.4
8) IL-1 + GLN + CS High Dose	59.1 ± 9.1	26.20 ± 7.4	189.5 ± 19.1

Table 3: Mean NO concentration on day d 4 and d 12 period and cumulative total of NO accumulation in media over 14 d time period (± SE with n=3). The experimental treatments contained 1 μg/ml GLN + 5 μg/ml CS (low dose), 5 μg/ml GLN + 20 μg/ml CS (medium dose), or 10 μg/ml GLN + 50 μg/ml CS (high dose). Statistical differences from the IL-1 treatment (# 5) are shown: p value of < 0.05 indicated by *, $p < 0.01$ indicated by **, $p > 0.001$ indicated by ***.

Table IV

Mean GAG concentration (μg/ml) ± SE with n=3

Day 4, 12, and cumulative over 14 days

Treatment	Day4	Day 12	Cumulative
1) Control: No IL-1 / No impact	58.1 ± 7.6***	55.8 ± 3.2	703.4 ± 107.9
2) GLN + CS Low Dose	44.0 ± 7.1***	41.9 ± 9.9	572.2 ± 158.7**
3) GLN + CS Medium Dose	41.2 ± 6.9***	48.7 ± 11.5	500.6 ± 149.6**
4) GLN + CS High Dose	4.6 ± 16.9***	67.8 ± 14.6	413.4 ± 123.9***
5) IL-1	166.0 ± 27.3	75.1 ± 18.3	868.9 ± 85.0
6) IL-1 + GLN + CS Low Dose	148.6 ± 37.3	60.1 ± 25.4	676.8 ± 117.0
7) IL-1 + GLN + CS Medium Dose	150.0 ± 51.2	64.4 ± 23.0	739.5 ± 222.4
8) IL-1 + GLN + CS High Dose	69.7 ± 39.9***	53.8 ± 27.6	489.4 ± 129.5**

Table 4: Mean GAG concentration on day d 4 and d 12 period and cumulative total of NO accumulation in media over 14 d time period (± SE with n=3). IL-1 (50 ng/ml) was added to treatments 5 to 8. The experimental treatments contained 1 μg/ml GLN + 5 μg/ml CS (low dose), 5 μg/ml GLN + 20 μg/ml CS (medium dose), or 10 μg/ml GLN + 50 μg/ml CS (high dose). Statistical differences from the IL-1 treatment (# 5) are shown: p value of < 0.05 indicated by *, $p < 0.01$ indicated by **, $p > 0.001$ indicated by ***.

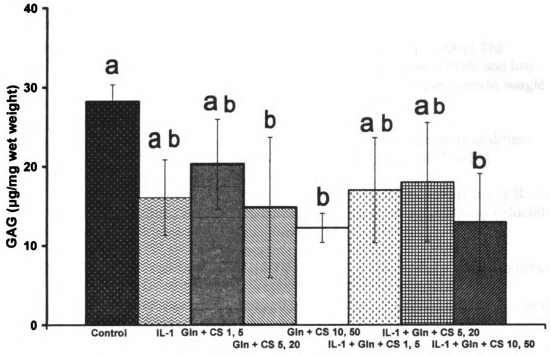

Fig 3: Mean (±SE, n=3) final GAG content in cartilage explants at day 14.

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Chapter 3

The Effect of Avocado and Soy Unsaponifiables and S-Adenosyl-L-Methionine on Inflammatory Mediators in Bovine Cartilage

Summary

Reasons for performing study: Alternative ways of treating osteoarthritis (OA) have become popular in the past decade. S-Adenosyl-L-Methionine (SAMe) and avocado soy unsaponifiables (ASU) are among the supplements that are available for controlling pain and inflammation due to OA. We used bovine cartilage explant cultures to measure the effect of both of these agents on inflammatory mediators and cartilage breakdown.

Hypothesis: SAMe and ASU have anti-inflammatory properties and will decrease inflammatory mediators and cartilage breakdown in a dose dependent manner.

Methods: Two experiments were performed: the first tested SAMe at concentrations of 1.0, 0.1, and 0.01 ug/ml (high, medium, and low dose) and ASU at concentrations 10.0 and 1.0 ug/ml (high dose and medium dose); the second tested ASU at concentrations of 10.0, 1.0, and 0.1 ug/ml (high, medium, and low dose). Interleukin-1 (IL-1) at 15 ng/ml was used to initiate inflammatory stress. Nitric oxide (NO) and Prostaglandin E2 (PGE_2) were measured as indicators of inflammatory response. Glycosaminoglycans (GAG) were measured as an indicator of cartilage turnover.

Results: The first experiment showed no significant treatment effects for either SAMe or ASU. However, the medium dose of SAMe tended to have a lower PGE_2

concentration than the IL-1-only ($p = 0.09$). In the second experiment, ASU again did not significantly decrease inflammatory mediators or cartilage breakdown.

Conclusions: This study yields little evidence that SAMe or ASU affects inflammation or cartilage breakdown at these concentrations.

Potential relevance: Further testing with different *in vitro* models is needed to determine whether these agents affect inflammation and cartilage turnover and at what concentrations.

Introduction

Osteoarthritis (OA) is a degenerative joint disease marked by progressive cartilage tissue loss, pain, inflammation, and eventual loss of joint function (Felson *et al.* 1998; Hashimoto *et al.* 1998). Onset of OA may be initiated by trauma or chronic mechanical stress and is marked by production of proinflammatory cytokines such as interleukin 1 beta (IL-1) (Quinn *et al.* 2001; Patwari *et al.* 2003). The inflammatory activity of these cytokines is mediated by signaling molecules such as nitric oxide (NO) and prostaglandin E_2 (PGE_2) (Smalley *et al.* 1995; Grabowski *et al.* 1997). IL-1 inhibits glycosaminoglycan (GAG) synthesis and increases GAG catabolism. Since GAG is an important component of cartilage extracellular matrix, this imbalance in tissue turnover leads to ultimate destruction of the cartilage tissue and joint disability (Hardingham *et al.* 1992; Goldring *et al.* 1994).

GLN and CS are the most popular nutraceuticals for management of OA; however other nutraceuticals such as S-Adenosyl-L-Methionine (SAMe) seem effective against the symptoms of OA. SAMe was first sold over the counter in 1999 for treatment of joint

pain. It is a compound that is synthesized by the body from the essential the amino acid L-methionine and adenosine triphosphate. Clinical trials show that SAMe supplementation decreases joint pain and inflammation (Polli *et al.* 1975; Caruso and Pietrogrande 1987; Soeken *et al.* 2002) and increases proteoglycan synthesis (Harmand *et al.* 1987), which is important since proteoglycan is responsible for the shock absorbing properties of cartilage tissue. The exact mechanism of these protective effects is not known.

Avocado soy unsaponifiables (ASU) are a mixture of natural vegetable extracts obtained from avocado and soy bean oils. These extracts are obtained when the oil is saponified (heated and broken down by alkaline hydrolysis). The small fraction that is not broken down is known as the "unsaponifiable" fraction (Bassleer *et al.* 1992), which may relieve pain when taken orally (Reginster *et al.* 2000; Walker-Bone 2003; Soeken 2004). This decrease in pain is attributed to the ability of ASU to decrease inflammatory mediators such as IL-1, NO, and PGE_2 seen in human chondrocyte studies (Bassleer *et al.* 1992). Avocado soy unsaponifiables also increase synthesis and decrease breakdown of GAG, thus potentially preserving cartilage from breakdown (Mauviel *et al.* 1989; Bassleer *et al.* 1992).

Our objective was to test biologically relevant concentrations of SAMe and ASU in bovine cartilage explant cultures. We have utilized this type of culture system to study GLN and CS (Chan *et al.* 2005a; Chan *et al.* 2005b; Chan *et al.* 2005c), and in this study we use a similar approach to test SAMe and ASU and there ability to mitigate inflammatory responses to IL-1.

Materials and methods

Explant Cultures

Articular cartilage was isolated from antebrachial-carpal and middle carpal joints of Holstein steers (18-24 mo old) from a local abattoir within 3 hours of slaughter. The cartilage was obtained in 6 mm discs, which were washed three times in media. The discs were cultured in 24-well Falcon plates[b] with two discs per well. Each well contained 1 ml of media (1:1 Dulbecco's Modified Eagles Medium: nutrient mixture F-12).[a] The medium was supplemented as previously described (Fenton *et al.* 2000). Media was also supplemented with all 20 amino acids[c] at the concentrations used by Chan *et al.* (2005b). All cultures were maintained at 37° C in a humidified incubator with 7% CO_2.

Human recombinant IL-1 beta[e] (IL-1) at a concentration of 15 ng/ml was used to induce inflammatory stress. S-Adenosyl-L-Methionine concentrations were 1.0, 0.1, and 0.01 μg/ml (high, medium, and low doses, respectively). These concentrations, especially the lower two, approximate those in the blood and synovial fluid when orally administered (Stramentinoli 1987). Avocado soy unsaponifiables concentrations were 10, 1, 0.1 ug/ml (high, medium, and low doses, respectively). Concentrations of ASU are achieved in the synovial fluid after oral administration are not known, but these concentrations were chosen because past studies have found effects using similar concentrations (Bassleer *et al.* 1992).

Explants were equilibrated in media without fetal bovine serum[a] (FBS) for 2 d with the media being changed at 24 hours. The media without FBS was replaced with

media with FBS and treatments at 48 h. At 72 h the media was replaced again with FBS and treatments in the media. The conditioned media was stored at 4° C until analysis for NO and GAG content. A small fraction of the media was isolated for PGE_2 analysis. The cartilage explants were stored at -20° C for later papain digestion and GAG analysis.

Two experiments were performed: (1) the first included concentrations for both SAMe and ASU and (2) in the second only ASU concentrations. There were six wells per treatment for each experiment, which were assigned to one of eight treatments for the first experiment (Table I) and one of six treatments for the second experiment (Table II). Each experiment was replicated four times with cartilage being isolated from four different Holstein steers.

Biochemical Analyses:

Nitrite levels in the media were assayed as previously described (Blanco *et al.* 1995). Absorbance was detected at 540 nm by a Spectromax 300 plate reader.[i] Results are expressed as nmol NO_2/ml. GAG release into the media was assayed by the dimethylene blue[j] (DMB) assay as previously described (Chandrasekhar 1987). The digest contained 1 μg papain[c] per mg cartilage digest. Results of explant DMB assays are expressed as μg/mg wet weight for cartilage. PGE_2 was assayed using a commercially available kit.[e] Indomethacin[c] (10 μg/ml) was added to the media at collection and samples were stored at -20° C until analysis. Media samples were assayed for PGE_2 on media samples from the first 24 h of both experiments according to the

manufacturer's instructions and are reported in pg/ml. To bring the samples within range of the standard curve, samples were diluted 1:10 or 1:20 depending on the PGE_2 level of the sample. PGE_2 was detected at 405 nm with a wavelength correction set at 590 nm.

Statistical Analysis

Data were analyzed using the PROC MIXED procedure of SAS software.[k] The data from all wells were pooled according to treatment, with individual cows as replicates. The random effects included cows, treatment*cow, and cow*day. A p value of <0.05 was considered significant and p values <0.1 were considered a trend.

Results

The explants in the IL-1-only treatment released significantly more NO, PGE_2, and GAG than control explants, as expected. The SAMe treatments did not have lower NO, PGE_2, or GAG concentration than the IL-1-only treatments (Fig 1A-C). However, the PGE_2 concentration in the medium dose of SAMe showed a trend ($p = 0.09$) of being lower compared to the IL-1-only treatment (Fig 1B). The treatments also had no effect on final GAG content in the explants (data not shown).

None of the ASU treatments decreased NO, PGE_2, or GAG concentration in the media (Fig 1 and 2). The GAG concentration was significantly affected by the ethanol since the ethanol control released more GAG than did the IL-1-only treatment. The medium concentration of ASU increased rather than decreased the GAG concentration

(Fig 2C). The ASU treatments also had no effect on final GAG content in the explants (data not shown).

Discussion

SAMe has the ability to decrease the transcriptional activation of genes that effect NO and PGE2 level (iNOS and COX-2, respectively) (Hevia *et al.* 2004). Our study did show a trend that the medium dose of SAMe was lower than the IL-1-only. Our study did not show the effects of SAMe the Hevia *et al.* 2004 study showed. However, the current study differed from the Hevia *et al.* (2004) study in that 5'-methylthioadenosine (MTA), a product of SAMe metabolism, was used instead of SAMe (Hevia *et al.* 2004). Another difference was that the Hevia *et al.* study use a murine macrophage cell line and rat hepatocytes (Hevia *et al.* 2004). They also used LPS to model inflammation rather than IL-1. It may be that the anti-inflammatory effects of SAMe are mediated by the breakdown product MTA. If this is the case, then MTA, as a downstream molecule, may be more potent than SAMe as an anti-inflammatory. SAMe has also been reported to increase proteoglycan synthesis in human chondrocytes *in vitro* at concentrations used in the current study (1 to 10 μg/ml) (Harmand *et al.* 1987). Harmand *et al.* (1987) used human articular osteoarthritic chondrocytes in a thick-layer culture model. Using chondrocytes in culture without the extracellular matrix may yield a more responsive culture system with respect to SAMe supplementation than using explants in culture. A clinical study showed that SAMe was just as effective as a known anti-inflammatory (celecoxib) at relieving pain, however SAMe took a much longer time to elicit the same effects as celecoxib (Najm *et al.* 2004). Because SAMe is involved in so many different

reactions, our experiments may not have been carried on long enough to detect the delayed nature of the SAMe.

ASU increased cartilage proteoglycan content and thickness in sheep (Cake *et al.* 2000). In that study, OA was induced by meniscectomy and ASU was administered orally. ASU also inhibited the breakdown of rat cartilage implanted beneath the skin of mice (Khayyal and el-Ghazaly 1998). The concentrations of ASU achieved in the blood or in the synovial fluid in either of these studies, which may account for the discrepancy between the results from Khayyal and el-Ghazaly and the current study was unknown. ASU also had anti-inflammatory effects in two studies using human chondrocytes: IL-1-induced PGE_2 was reduced by 40 to 50% (Bassleer *et al.* 1992), and ASU decreased basal production of NO and PGE_2 in human OA chondrocytes (Bassleer *et al.* 1992).

In the case of both the SAMe studies and the ASU studies, a species other than bovine species was used, which raises the possibility of species-specific effects. Bovine cartilage tissue may be less responsive to SAMe and ASU than cartilage tissue from other species. Another consideration is that all of the tissue culture experiments reported have used monolayers rather than explants. Cells may respond differently to these supplements when cultured without the natural extra cellular matrix surrounding the cells.

Conclusion

The results presented here using bovine explants do not support the usefulness of ASU and SAMe as chondroprotective molecules. Further work is needed to elucidate the concentration of ASU in the blood and synovial fluid upon oral administration. Further

work is also need to delineate the exact mechanism of these two supplements as the mechanism of both of these supplements remains largely unknown.

Manufacturer's address:

[a]Gibco, Grand Island, New York, U.S.A.

[b]Fisher Scientific, Pittsburgh, Pennsylvania, U.S.A.

[c]Sigma Chemical, St Louis, Missouri, U.S.A.

[d]J.T. Baker, Phillipsburg, New Jersey, U.S.A.

[e]R&D Systems, Minneapolis, Minnesota, U.S.A.

[f]Nutramax Laboratories, Edgewood, Maryland, U.S.A.

[g]Roche Diagnostics Corporation, Indianapolis, Indiana, U.S.A.

[h]Molecular Devices, Sunnyvale, California, U.S.A.

[i]Polyscience, Inc., Warrington, Pennsylvania, U.S.A.

[j]SAS Institute, Inc., Cary, North Carolina, U.S.A.

Table I

Agents added to explant cultures-experiment #1
(10% FBS added to media for all treatments)

Treatment	IL-1 (ng/ml)	SAMe (µg/ml)	ASU (µg/ml)	EthOH (%)
1) Control: No IL-1	-	-	-	-
2) IL-1-only	15	-	-	-
3) IL-1 + SAMe High Dose	15	1	-	-
4) IL-1 + SAMe Medium Dose	15	0.1	-	-
5) IL-1 + SAMe Low Dose	15	0.01	-	-
6) IL-1 + Ethanol	15	-	-	0.3
7) IL-1 + ASU High Dose	15	-	10.0	0.3
8) IL-1 + ASU Medium Dose	15	-	1.0	0.3

Table II

Agents added to explant cultures-experiment #2
(10% FBS added to media for all treatments)

Treatment	IL-1 (ng/ml)	ASU (µg/ml)	EthOH (%)
1) Control: No IL-1	-	-	-
2) IL-1-only	15	-	-
3) IL-1 + Ethanol	15	-	0.3
4) IL-1 + ASU High Dose	15	10.0	0.3
5) IL-1 + ASU Medium Dose	15	1.0	0.3
6) IL-1 + ASU Low Dose	15	0.1	0.3

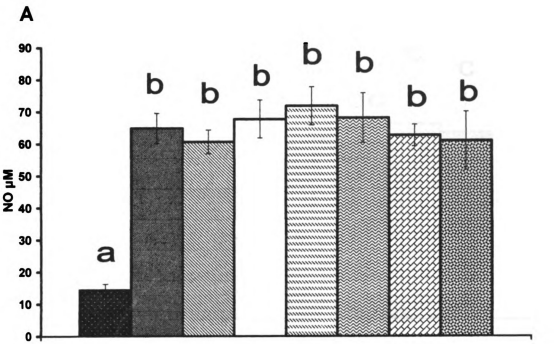
A
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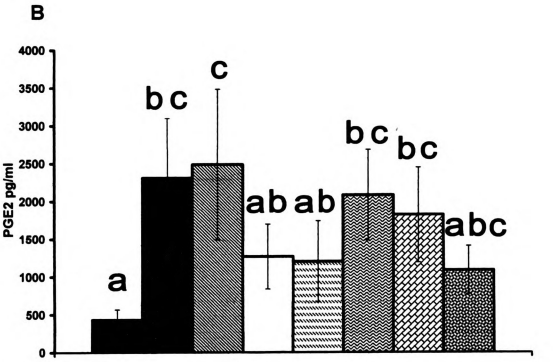
Fig1.A


B
PGE2 pg/ml
4000
3500
3000
2500
2000
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a
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Fig1.B

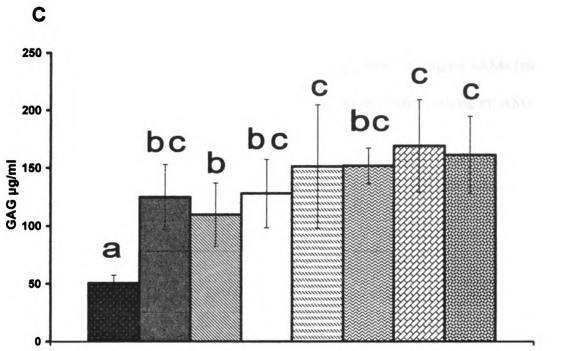
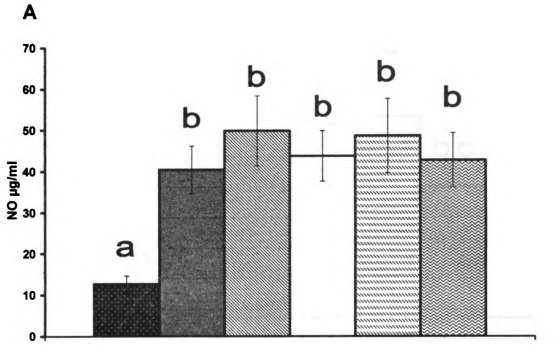


Fig1.C

Legend

Control	hrIL-1	SAMe High Dose	SAMe Medium Dose
SAMe Low Dose	0.3% Ethanol	ASU High Dose	ASU Low Dose

Fig 1: Mean (±SE, n=4) NO, PGE_2, and GAG concentrations in media samples at day 1 (A-C). IL-1 (15 ng/ml) was added to each treatment except for control. The experimental treatments contained 1 ug/ml SAMe (high dose); 0.1 ug/ml SAMe (medium dose); 0.01 ug/ml SAMe (low dose); 0.3% EthOH (EthOH control); 10 μg/ml ASU (high dose); or 1 ug/ml ASU (medium dose).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0
a
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b
b
b

Fig.2A

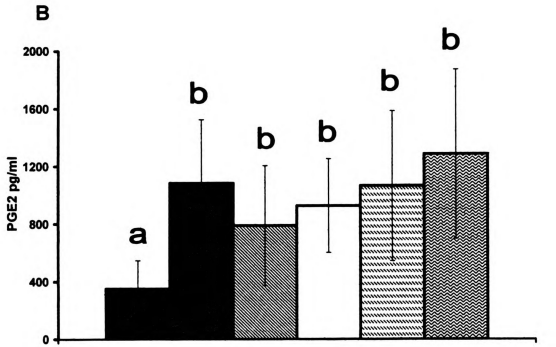
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Fig2.B

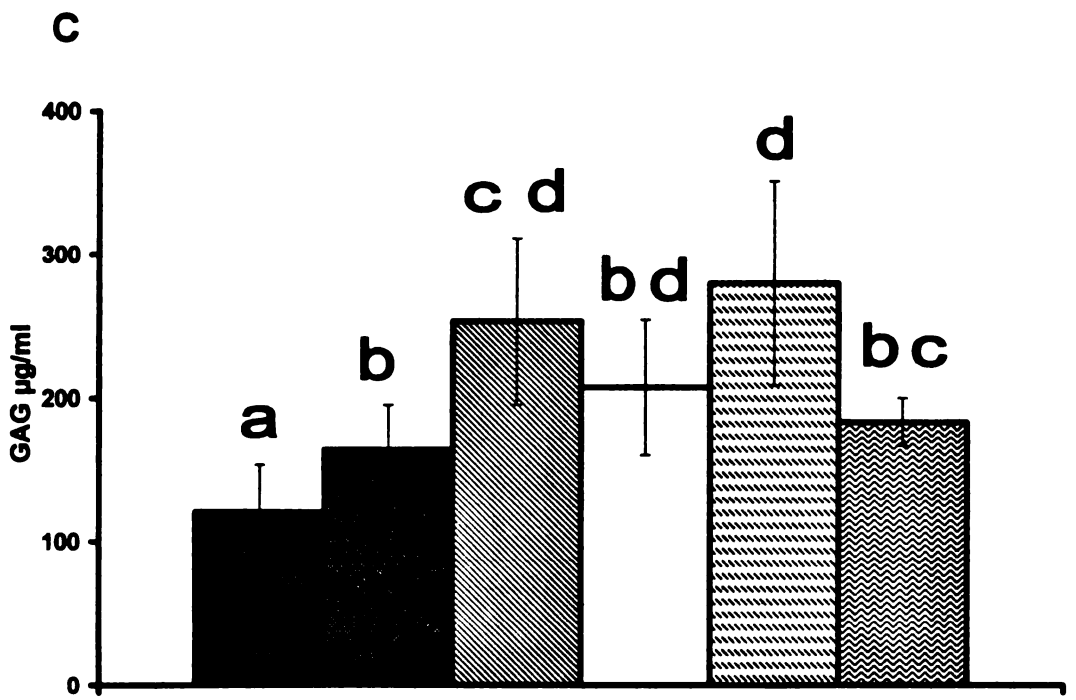

Fig2.C

Legend

Control	15 ng/ml hrIL-1	0.3% Ethanol
ASU High Dose	ASU Medium Dose	ASU Low Dose

Fig 2: Mean (±SE, n=4) NO, PGE_2, and GAG concentrations in media samples at day 1 (A-C). IL-1 (15 ng/ml) was added to each treatment except for control. The treatments contained: 0.3% EthOH (EthOH control); 10 μg/ml ASU (high dose); 1 ug/ml ASU (medium dose); 0.01 ug/ml ASU (low dose).

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