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Inhibition of articular cartilage degradation by glucosamine-HCl and chondroitin sulphate

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Summary

Glucosamine and chondroitin sulphate in many animal and human trials has improved joint health. *In vitro* studies are beginning to clarify their mode of action. The objective of this research was to: 1) determine at what concentrations glucosamine-HCl (GLN) and/or chondroitin sulphate (CS) would inhibit the cytokine-induced catabolic response in equine articular cartilage explants and 2) to determine if a combination of the 2 was more effective at inhibiting the catabolic response than the individual compounds. Articular cartilage was obtained from carpal joints of horses (age 1–4 years). Cartilage discs (3.5 mm) were biopsied and cultured. Explants were incubated with lipopolysaccharide (LPS) in the presence of varying concentrations of GLN, CS, or both. Control treatments included explants with no LPS and LPS without GLN or CS. Media were analysed for nitric oxide (NO), prostaglandin E_2 (PGE_2) and keratan sulphate. Cartilage was extracted for analysis of metalloproteinases (MMP). Four experiments were conducted. In all experiments, GLN at concentrations as low as 1 mg/ml decreased NO production relative to LPS stimulated cartilage without GLN over the 4 day period. In general, CS at either 0.25 or 0.5 mg/ml did not inhibit NO production. The addition of CS to GLN containing media did not further inhibit NO production. GLN at concentrations as low as 0.5 mg/ml decreased PGE_2 production, whereas CS did not effect on PGE_2. The combination of GLN/CS decreased MMP-9 gelatinolytic activity but had no effect on MMP-2 activity. The combination in 2 experiments tended to decrease MMP-13 protein concentrations and decreased keratan sulphate levels in media. Overall, the combination of GLN (1 mg/ml) and CS (0.25 mg/ml) inhibited the synthesis of several mediators of cartilage degradation. These results further support the effort to understand the role of GLN and CS in preserving articular cartilage in athletic horses.

Introduction

Lameness in horses significantly impacts the racing industry. A random evaluation of 72 equine joints at necropsy revealed that 35% showed obvious cartilage damage (Morris and Treadwell 1994). Joint disease and, specifically, osteoarthritis (OA), is the most common cause of lameness. In OA, progressive and permanent degeneration of articular cartilage develops in previously normal joints damaged by a variety of factors, including developmental disorders such as osteochondrosis, sudden or repetitive injury to a joint or cartilage surface, joint infections and medications that alter joint integrity. Specifically, in racing horses, many problems in the joint are caused by either acute traumatic lesions or chronic loading to a joint that eventually results in the clinical signs of OA (Pool and Meagher 1990).

Since OA is a chronic disease, prevention and management probably requires a long-term solution. One important area of research is the use of nutraceuticals (nutritional compounds added to a diet that have pharmacological-like effects). Glucosamine and chondroitin sulphate are 2 compounds for which evidence of a chondroprotective effect exists. In studies in man, oral administration of glucosamine and chondroitin sulphate decreases pain and improves mobility in osteoarthritic joints (McAlindon *et al.* 2000). Patients with knee OA taking glucosamine sulphate for 3 years had a significant improvement in signs and a cessation of joint space narrowing (Reginster *et al.* 2001). Chondroitin sulphate has anti-inflammatory effects comparable to NSAIDs in patients with OA (Morreale *et al.* 1996). Some studies have also used a combination; in one with military personnel, the combination relieved symptoms (Leffler *et al.* 1999) and a clinical trial indicated that glucosamine in combination with chondroitin sulphate was beneficial in the treatment of radiographically mild to moderate knee OA (Das and Hammad 2000). In a rabbit instability model of cartilage degeneration, the 2 had a synergistic effect relative to the individual molecules in protecting against cartilage damage (Lippiello *et al.* 2000). In horses, the combination of the same ingredients used in the present study have been found to be bioavailable, safe and effective in alleviating symptoms of OA (Hanson *et al.* 1997, 2001; Kirkerhead and Kirkerhead 2001).

The biochemical mechanisms by which glucosamine and chondroitin sulphate protect articular cartilage have not been clearly elucidated. We have shown that in equine articular cartilage explants that glucosamine inhibits nitric oxide production and matrix metalloproteinase activity (Fenton *et al.* 2000). Chondroitin sulphate also can decrease collagenase activity and improve synovial fluid viscosity by increasing hyaluronic acid concentrations (Ronca *et al.* 1998). One drawback of our studies was the use of relatively high concentrations of glucosamine that may not be pharmacologically relevant. Also, until now we have not evaluated the chondroprotective properties of chondroitin sulphate. Therefore, the objectives of our research were to determine 1) the concentrations of glucosamine or chondroitin sulphate or both that would inhibit cytokine-induced cartilage degradation and 2) whether a combination of the 2 molecules would be more effective in inhibiting cartilage degradation than the individual molecules.

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TABLE 1: Description of treatment groups in equine articular cartilage explant experiments

Experiment	Age (years)	Treatments*	Analyses performed
1	2	1.0 mg/ml GLN, 0.25 mg/ml CS, 0.5 mg/ml CS, 1.0 mg/ml GLN + 0.25 mg/ml CS, or 1.0 mg/ml GLN + 0.5 mg/ml CS	NO, KS, Zymograms
2	1	1.0 mg/ml GLN, 0.25 mg/ml CS, 0.5 mg/ml CS, 1.0 mg/ml GLN + 0.25 mg/ml CS, or 1.0 mg/ml GLN + 0.5 mg/ml CS	NO, MMP-13 Western, KS, Zymograms, PGE_2
3	1	0.5 mg/ml GLN, 0.125 mg/ml CS, 0.5 mg/ml GLN + 0.125 mg/ml CS, or 1.0 mg/ml GLN + 0.25 mg/ml CS	NO, MMP-13 Western, Zymograms, PGE_2
4	4	0.5 mg/ml GLN + 0.25 mg/ml CS, 0.75 mg/ml+0.25 mg/ml CS, 1.0 mg/ml GLN + 0.25 mg/ml CS	NO

*All experiments included control groups: 10% fetal bovine serum and 10% fetal bovine serum + 10 µg/ml of lipopolysaccharide. All treatments consisted of 6 wells. CS = chondroitin sulphate, GLN = glucosamine HCL, KS = Keratan sulphate, NO = Nitric oxide, PGE_2 = Prostaglandin E_2.

Materials and methods

Experimental design

Articular cartilage was obtained from the antebrachiocarpal and middle carpal joints of horses (age 1–4 years) sacrificed for reasons other than joint problems. Each of the experiments outlined in Table 1 used cartilage from one horse, since the cartilage was collected at various times during the spring and summer of 2001. Cartilage discs (3.5 mm) were biopsied and 3 discs cultured per well of a 24-well Falcon culture plate[1] with a modified version of Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham) (1:1)[2] (Rosselot *et al.* 1992). The media were supplemented with 50 µg/ml ascorbate and 100 units/ml penicillin/streptomycin[2]. The explants were maintained in culture in a humidified incubator with 7% CO_2 at 37°C. After 2 days of equilibration, explants were incubated with 10% fetal bovine serum (FBS)[2] and 10 µg/ml of lipopolysaccharides (LPS) in the presence of varying concentrations of glucosamine-HCl (FCHG49)[3] (GLN), or chondroitin sulphate (TRH122)[3] (CS), or both (Table 1). Control treatments included explants with no LPS and LPS without GLN or CS. Treatments include 6 wells. Conditioned media were collected daily and stored at 4°C until analysis.

Biochemical analyses

Nitric oxide: Nitric oxide (NO) was measured indirectly in the conditioned media as described previously (Blanco *et al.* 1995). Nitrite, a stable end-product of nitric oxide metabolism, was quantified using the Greiss reaction and a standard curve of sodium nitrite. Briefly, 75 µl conditioned medium was incubated with 75 µl 1.0% sulphanilamide, 0.1% N-1-naphthylethylenediamide dihydrochloride in 25% phosphoric acid at room temperature for 5 min. Due to some precipitation of reagents with CS, plates were spun at 3250 rpm for 4ºC. The supernatant was transferred to a new 96-well plate. Optical density was measured at 540 nm. Results are expressed as nmol NO/well.

Prostaglandin E_2: Prostaglandin E_2 (PGE_2) was measured using a competitive enzyme immunoassay kit[4]. Indomethacin (10 µg/ml) was added to conditioned media samples after 1 day of the experimental trial and stored at -20°C until analysis. Samples were diluted 20-fold in provided assay buffer and run in duplicate. Briefly, the sample competes with a fixed amount of alkaline phosphatase-labelled PGE_2 for sites on a mouse monoclonal antibody. The mouse monoclonal antibody becomes bound to the goat anti-mouse antibody coating the microplate during the incubation time. Washes were performed to remove excess conjugate and unbound samples. Substrate solution was added to determine bound enzyme activity and the absorbance read at 405 nm with a wavelength correction set at 590 nm. A 4 parameter logistic curve ranging from 39–5000 pg/ml PGE_2 was used to determine sample concentrations. Total activity, nonspecific binding, maximum binding and substrate blanks of each plate were acceptable.

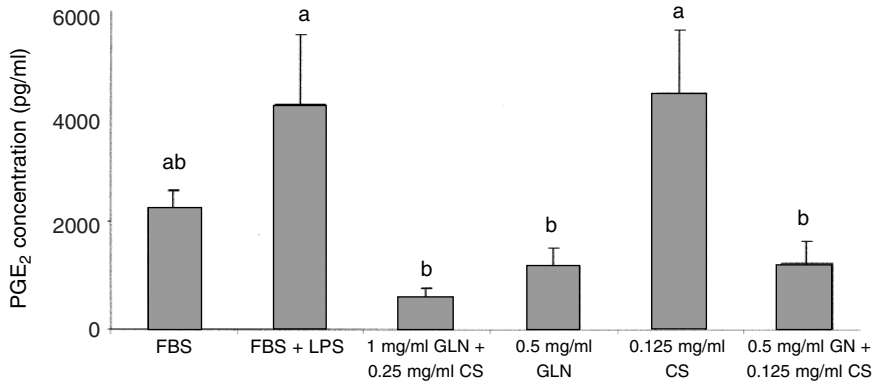

Fig 1: The effect of glucosamine and/or chondroitin sulphate on prostaglandin E_2 production one day after stimulation of cartilage explants by LPS. Plotted values are means ± s.e. of 6 wells/treatment. Different superscripts indicate significant differences at P≤0.05. The results shown are from experiment 3.

TABLE 2: The effect of glucosamine and/or chondroitin sulphate on nitric oxide production

Treatment	Days in culture after LPS addition			
	1	2	3	4
FBS	5.6 ± 0.8^{a}	2.4 ± 0.5^{a}	0.4 ± 0.2^{a}	0.0 ± 0.0^{a}
LPS	15.2 ± 0.7^{c}	25.9 ± 1.3^{b}	15.8 ± 0.7^{b}	10.1 ± 0.5^{bc}
1 mg/ml GLN	10.2 ± 0.8^{be}	15.2 ± 1.3^{c}	8.1 ± 0.9^{c}	6.0 ± 0.8^{c}
0.25 mg/ml CS	13.8 ± 1.2^{bc}	24.8 ± 2.1^{b}	17.7 ± 1.4^{b}	13.2 ± 1.4^{b}
0.5 mg/CS	11.8 ± 0.9^{bcd}	22.3 ± 3.7^{bc}	15.6 ± 2.5^{bd}	11.8 ± 1.6^{b}
1 mg/ml GLN and 0.25 mg/ml CS	9.4 ± 1.1^{ade}	14.6 ± 1.5^{c}	10.0 ± 1.2^{cd}	11.5 ± 1.4^{b}
1 mg/ml GLN and 0.5 mg/ml CS	7.7 ± 0.9^{ae}	13.4 ± 1.3^{c}	7.9 ± 0.8^{c}	8.6 ± 0.7^{bc}

Values are mean ± s.e. for 6 wells per treatment. For each day, different superscripts are significantly different at P≤0.05. Values are expressed as nmol/well.

Keratan sulphate: Quantification of keratan sulphate (KS) in conditioned media was measured using a previously described enzyme-linked immunosorbent assay (ELISA) with an inhibition step using a monoclonal antibody specific for KS (Thonar *et al.* 1985). Incubation of the media with anti-KS monoclonal antibody[6] was performed to bind antigen to antibody: the more KS antigen present the less free antibody will remain. The detection process required incubation with the secondary antibody, goat anti-mouse IgG HRP-conjugated[5], on an antigen coated plate. Colour development was initiated using 0-phenylenediamine[6] and stopped with 2 mol/l sulphuric acid and read at 490 nm. Intensity is inversely proportional to the amount of KS antigen present in the sample. Samples are compared to a KS standard (kindly provided by the laboratory of Dr Eugene Thonar, Rush University, Chicago, Illinois) and are expressed as ng/ml.

Cartilage extraction: Matrix metalloproteinases were extracted from articular cartilage using a modified protocol (Pelletier *et al.* 2000). Articular cartilage explants were rinsed with sterile phosphate buffer solution. All 3 explants in a well were placed in a cold stainless steel mortar apparatus and snap froze with liquid nitrogen. Explants were powdered immediately using a stainless steel pestle and hammer. Powdered explants were placed in microcentifuge tubes with stir bars and 10 µl extraction buffer (50 mmol/l Tris HCl, 10 mmol/l $CaCl_2$, 2 mol/l guanidine HCl and 0.05% Brij-35: pH 7.5) per mg tissue. Samples were stirred overnight at 4°C and then centrifuged at 18000 ***g*** for 30 min at 4°C. Supernatant was dialysed (24 h) against assay buffer (50 mmol/l Tris HCl, 10 mmol/l $CaCl_2$, 0.2 mol/l NaCl, 0.05% Brij-35: pH 7.5) using Spectrapor 2 dialysis tubing with a 12-kd cutoff[7]. Dialysis was continued for 48 h with distilled water. The amount of protein in the extracts was determined using the Pierce Micro BCA Protein Assay[8] with bovine serum albumen as the standard. Extractions were aliquoted and stored at -20°C until analysis.

Gel zymography: Extracts of articular cartilage samples containing 8 µg of protein were applied without reduction to an 8% polyacrylamide gel with 1 mg/ml gelatin incorporated as the substrate. Samples were diluted appropriately with 4x sample buffer and gel electrophoresis was performed at room temperature. After electrophoresis, gels were incubated in 2.5% (v/v) Triton X-100 for 1 h and then overnight at 37°C in 50 mmol/l Tris (pH 7.5) containing 200 mmol/l NaCl, 10 mmol/l $CaCl_2$, 10 µmol/l $ZnCl_2$ and 0.02% Brij-35. The gels were then stained with Coomassie Blue R250 for 1 h at room temperature. Enzyme activity was quantified using scanning densitometry (Gel Doc 2000)[9], using Quantity One 4.0.1 software.

Western blot analysis: Cartilage extracts were heat-denatured with 2-mercaptoethanol and subjected to an 8% SDS-PAGE. Samples were loaded on an equal protein basis. Proteins were transferred onto a polyvinylidene difluoride membrane and blocked with BLOT-QuickBlocker in 1X femtoTBST[10]. The membrane was then incubated with 5 µg/ml mouse anti-human MMP-13 monoclonal antibody[5]. The antibody recognises the pro and active form of human MMP-13. Equine MMP-13 has over 90% homology with human MMP-13. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies[11] at a dilution of 1:1000, followed by protein detection using the femtoLUCENT chemiluminescent detection system[11] according to the manufacturer's instructions. Only one major band was detected on the blot and it corresponded to the human MMP-13 standard.

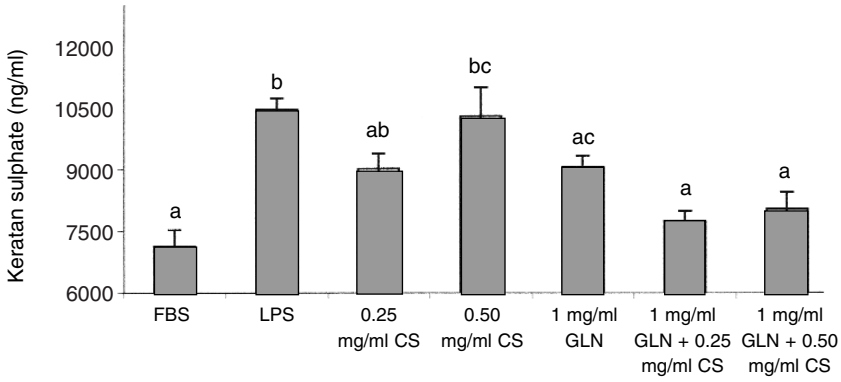

Fig 2: The effect of glucosamine and/or chondroitin sulphate on keratan sulphate accumulation in conditioned media one day after stimulation of cartilage explants by LPS. The plotted values are means ± s.e. of 6 wells per treatment. Different superscripts indicate significant differences at P≤0.05. The results shown are from experiment 2.

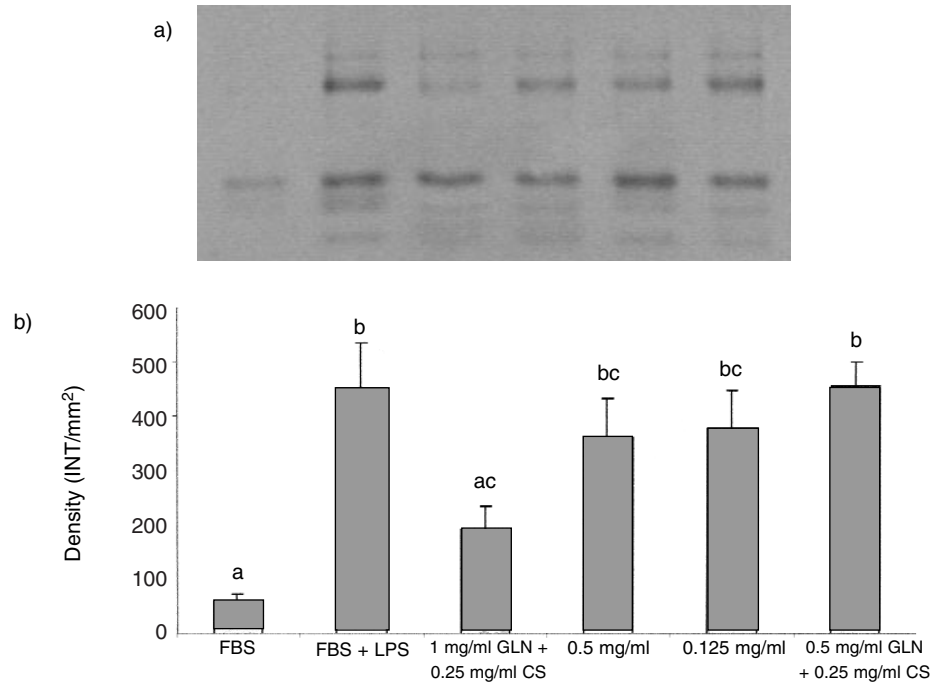

Fig 3: The effect of glucosamine and/or chondroitin sulphate on gelatinolytic activity in experiment 3. a) A representative zymogram of one of the 6 gels is shown as an inverted image. The top bands correspond to MMP-9 and bottom to MMP-2, which was not affected. b) Scanning densitometry was used to quantify the intensity of MMP-9 bands. Plotted values are mean ± s.e. of 6 treatment wells. Different superscripts indicate significant differences (P≤0.05). The gel lanes are in the same order as the graph.

Statistical analysis

Data from each experiment were analysed independently by the generalised least-square means of fixed effects using JMP Software[11]. Treatment effects were analysed within day. Significance was determined at P≤0.05 using the Student's *t* test. Additionally, we pooled data and analysed NO inhibition (4 experiments), PGE_2 production (2 experiments) and KS media concentrations (2 experiments) to determine if there was a horse by treatment effect in the FBS, FBS + LPS and FBS + LPS + 1 mg/ml GLN and 0.25 mg/ml CS treatments. The GLN and CS treatment was chosen due to its consistent ability to inhibit catabolic parameters.

Results

Nitric oxide and prostaglandin E_2

Glucosamine in the media at 1 mg/ml decreased NO production in experiments 1 and 2 (see Table 2 for results of experiment 2). Chondroitin sulphate at 0.25 and 0.5 mg/ml decreased NO production, on Days 1 and 2 in experiment 1, but had no effect on Days 3 and 4. In experiments 2 and 3, the addition of CS at either 0.125, 0.25 or 0.5 mg/ml did not decrease NO production. The addition of CS at any concentration to media with 1 mg/ml GLN did not further inhibit NO production in any experiment. However, in experiment 4, GLN at 0.5 mg/ml and CS at 0.25 mg/ml inhibited NO production, even though in experiment 3 GLN at 0.5 mg/ml had no effect. Prostaglandin E_2 was analysed in experiments 2 and 3 one day after the addition of LPS to cultures. Glucosamine at 1 mg/ml, in experiment 2, and 0.5 mg/ml, in experiment 3, decreased PGE_2 production (Fig 1). Chondroitin sulphate at any concentration tested had no effect on PGE_2 production in the presence or absence of GLN.

Keratan sulphate and gel zymography

In experiments 1 and 2, KS, an indicator of proteoglycan degradation, was quantitated in conditioned media after one day of LPS stimulation. In experiment 1, only the combination of GLN at 1 mg/ml and CS at 0.5 mg/ml decreased KS concentrations in conditioned media. In experiment 2, GLN at 1 mg/ml as well as the combination of GLN at 1 mg/ml and CS at 0.25 mg/ml also decreased KS concentrations with the latter being the most effective (Fig 2). Gelatin gel zymography, indicators of MMP-2 and -9 activity in cartilage, for experiments 1 and 2, showed some decrease in MMP-2 and MMP-9 activity in the presence of chondroitin sulphate or a combination of the 2 (data not shown). In these 2 experiments cartilage samples were pooled so that each treatment had only 2 extracts and therefore were not statistically analysed. However, in experiment 3, cartilage extractions were done on individual wells and each treatment therefore had 6 samples. Glucosamine significantly decreased MMP-9 activity but did not affect MMP-2 activity (Fig 3). Lower concentrations of GLN and CS did not affect either MMP-2 or -9 activity.

Matrix metalloproteinase-13

In experiment 2, GLN at 1mg/ml inhibited MMP-13 production relative to LPS treatment alone while CS had no effect in the

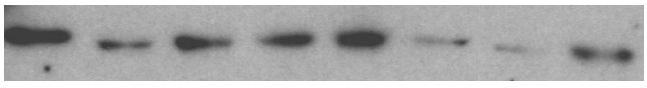

Fig 4: The effect of glucosamine and/or chondroitin sulphate on MMP-13 concentration in equine cartilage in experiment 2. Equine cartilage was extracted and 8 µg of protein/extract was loaded onto an 8% gel. Lane 1: MMP-13 standard; Lane 2: FBS; Lane 3: LPS; Lane 4: LPS + 0.25 mg/ml CS; Lane 5: LPS + 0.5 mg/ml CS; Lane 6: LPS + 1 mg/ml GLN; Lane 7: LPS + GLN + 0.25 mg/ml CS; Lane 8: LPS + GLN + 0.5 mg/ml CS.

presence or absence of GLN (Fig 4). In experiment 3, CS at 0.125 mg/ml and GLN at 0.5 mg/ml with CS at 0.125 mg/ml also inhibited MMP-13 production (data not shown).

Pooled data analysis

Nitric oxide, KS and PGE_2 levels were analysed across experiments for the 2 control treatments and GLN at 1 mg/ml and CS at 0.25 mg/ml. No horse by treatment effect was detected for NO and KS levels. For PGE_2 there was a horse by treatment effect due to one horse not responding quite as well to LPS as the other. However, the PGE_2 levels were similar in both experiments with GLN and CS treatments.

Discussion

Glucosamine and chondroitin sulphate inhibited the production or activity of several catabolic factors in LPS-stimulated cartilage obtained from 4 horses that varied in age (1–4 years) and environment (different locations in Michigan). Glucosamine at concentrations as low as 0.5 mg/ml, but routinely at 1 mg/ml, inhibit nitric oxide production. This work is in agreement with our previous results, although the concentration is at least 2.5-fold lower than previously used (Fenton *et al.* 2000). Similar results have been found using rat chondrocyte cultures (Gouze *et al.* 2001). Nitric oxide is probably a catabolic factor in articular cartilage. Patients with arthritis have higher concentrations of nitric oxide catabolites in their serum and urine than age-matched individuals with no clinical signs of arthritis (Grabowski *et al.* 1996). Articular cartilage explants from horses with moderate OA produce more nitric oxide than normal cartilage (von Rechenberg *et al.* 2000). In dogs, daily supplementation of N-iminoethyl-L-lysine, a selective inhibitor of inducible nitric oxide synthase, reduces the progression of experimentally induced arthritis (Pelletier *et al.* 1998). Therefore, GLN could protect cartilage by preventing NO production. Other than the first few days of experiment 1, CS had no effect on NO production in our system. However, CS can protect articular chondrocytes from nitric oxide dependent apoptosis *in vitro* (Conrozier 1998).

Glucosamine inhibited PGE_2 production at concentrations as low as 0.5 mg/ml. In rat chondrocytes, GLN at 1 mg/ml also inhibited PGE_2 production (Gouze *et al.* 2001). Prostaglandin E_2 is upregulated during an inflammatory response and is found in increased concentrations in the synovial fluid of patients with arthritis (Sahap Atik 1990). Horses with degenerative joint diseases have elevated concentrations of PGE_2 (May *et al.* 1994). In man, specific inhibitors of cyclooxygenase-2, the enzyme that synthesises PGE_2, are being used for the management of OA. Therefore, the ability of GLN to alleviate clinical signs of OA could be due to its ability to inhibit PGE_2 synthesis and future studies will determine the minimum concentration required.

The combination of the 2 was able to decrease proteoglycan degradation, which agrees with previous research (Lippiello *et al.* 2000). Higher concentrations (2.5 mg/ml or higher) of GLN alone inhibit MMP activity (Fenton *et al.* 2000). Glucosamine also inhibits aggrecanases (enzymes involved in the initiation of proteoglycan degradation) *in vitro* (Sandy *et al.* 1998). Specifically, the combination inhibited MMP-9 but not MMP-2 activity. We have also seen with either glucosamine or mannosamine alone in bovine articular cartilage explants that they do not inhibit MMP-2 activity (Mello 2001). In articular cartilage, MMP-2 is constitutively expressed while MMP-9 is induced by inflammatory cytokines such as interleukin-1 (Sasaki *et al.* 1998). In equine chondrocyte cultures, transforming growth factor beta-1 differentially regulates MMP-2 and -9 (Thompson *et al.* 2001). In addition, the production of MMP-13, which is probably the main enzyme involved in the initiation of collagen *type II* degradation, was inhibited by GLN and CS. This enzyme is also induced by cytokines. At least partial inhibition of MMP activity could be due to decreased transcription of their mRNA. Preliminary work done in our laboratory has shown that GLN at 1 mg/ml and CS at 0.25 mg/ml decreases MMP-13 expression by around 50% percent in LPS stimulated bovine articular cartilage explants. Both MMP-13 and nitric oxide synthesis are upregulated via the NF-κß pathway (Mengshol *et al.* 2000). Potentially GLN or CS could block signalling pathways for new protein synthesis without effecting constitutively expressed proteins like MMP-2. The inhibition of MMP or aggrecanase activity at any point in their regulation would be quite valuable in preventing the increased cartilage degradation seen in OA and joint trauma.

Our research suggests that GLN specifically could be regulating cell signalling molecules, such as nitric oxide and PGE_2, while the combination inhibits proteolytic activity. Some have proposed that the combination have a synergistic effect in protecting cartilage from damage (Lippiello *et al.* 2000). Our data at least suggests that the 2 have complementary benefits. The concentrations we used *in vitro* are still probably higher than concentrations achieved *in vivo*. However, one limitation with our system is that the cartilage disks have no dynamic loading during the culture period. *In vivo* models of disuse show that cartilage degrades with the removal of applied loads (Ratcliffe *et al.* 1994). Our model has some chronic degradation occurring because of the removal of forces on the cartilage. Additionally, fluid flow through the disks would probably improve with dynamic loading under minimal compressive forces. Other investigators have shown that CS in particular is more effective when chondrocytes are exposed to mechanical forces (Nerucci *et al.* 2000). Therefore, *in vivo* concentrations of GLN and CS necessary to inhibit the catabolic mediators we tested could be substantially lower.

The benefits of taking oral supplements containing GLN and CS have been shown in many different types of animal models and some human clinical studies (McLaughlin 2000). Elucidating their modes of action at the cellular and molecular level is becoming an important area of study in arthritis research. Chronic biomechanically induced lesions probably contribute to a majority of the joint problems in horses and can have a major economic impact (Pool and Meagher 1990). Therefore, understanding how GLN and CS work as chondroprotective agents ultimately could translate into improved prevention or management of cartilage degeneration in athletic and performance horses.

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Manufacturers' addresses

[1]Fisher Scientific, Pittsburgh, Pennsylvania, USA.
[2]Gibco, Grand Island, New York, USA.
[3]Nutramax Laboratories, Edgewood, Maryland, USA.
[4]R&D Systems, Minneapolis, Minnesota, USA.
[5]ICN Pharmaceuticals Inc, Costa Mesa, California, USA.
[6]Sigma Chemical, St Louis, Missouri, USA.
[7]Spectrum Medial Industries, Los Angeles, California, USA.
[8]Pierce, Rockford, Illinois, USA.
[9]BioRad, Hercules, California, USA.
[10]Geno Technology, Inc., St. Louis, Missouri, USA.
[11]SAS Institute, Inc., Cary, North Carolina, USA.

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