

In Vivo Chondroprotection and Metabolic Synergy of Glucosamine and Chondroitin Sulfate

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Supplements of glucosamine hydrochloride, low molecular weight chondroitin sulfate, and manganese ascorbate were tested separately and in combination for their ability to retard progression of cartilage degeneration in a rabbit instability model of osteoarthritis. Computerized quantitative histologic evaluation of safranin O stained sections of the medial femoral condyles measured the grade and extent of tissue involvement of lesions. Severe lesions (Mankin grade greater than 7) were absent in all animals supplemented with a dietary mixture of glucosamine, chondroitin sulfate, and manganese ascorbate. Total linear involvement (mm of lesioned surface) and total grade (mean grade \times number of lesions per animal) were reduced significantly in animals given the combination compared with controls (59% and 74% respectively). Animals supplemented with glucosamine, chondroitin sulfate, or manganese ascorbate alone had less moderate and severe tissue involvement than controls but not to

the extent of the combined group. In vitro, a combination of glucosamine hydrochloride and chondroitin sulfate acted synergistically in stimulating glycosaminoglycan synthesis (96.6%). Chondroitin sulfate and manganese ascorbate but not glucosamine were effective in inhibiting degradative enzyme activity. These data suggest that the disease modifying effect (the ability to retard progression of cartilage degeneration) of a mixture of glucosamine, chondroitin sulfate, and manganese ascorbate is more efficacious than either agent alone.

Osteoarthritis is characterized as a progressive age-related degeneration of articular cartilage in diarthroidal joints.^{24,25,28} Therapy for medical management of osteoarthritis has been largely palliative focusing on alleviation of symptoms. A new class of therapeutic agents focusing on slowing the degenerative process and promotion of cartilage matrix synthesis has received considerable publicity. Originally termed chondroprotective, these agents now are referred to as disease or structure-modifying, referring to effects that counter the destructive processes and encourage normalization of the cartilage matrix. The most notable examples are Arteparon[®] (Luitpold-Werk, Munich, Germany), a mixture of sulfated glycosaminoglycans prepared from bovine trachea and lung¹⁵;

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Rumalon® (Robapharm, Basel, Switzerland), a mixture of glycosaminoglycan-peptide complexes prepared from bovine cartilage and bone marrow¹²; Cartrophen® (Biopharm Ltd, Bon Di Junction, Australia), a pentosan polysulfate²⁰; Chondrosulf® (IBSA, Institut Biochimique SA, Lugano, Switzerland) a low molecular weight chondroitin sulfate; Cosamin® DS (Nutramax Laboratories Inc, Edgewood, MD), a mixture of low molecular weight chondroitin sulfate, glucosamine hydrochloride, and manganese ascorbate²¹; and Hylagan® (Fidia Pharmaceutical Corp, Washington, DC), a high molecular weight hyaluronic acid preparation.²³ Administration of these agents varies from intramuscular or intraarticular injection to dietary supplementation. Studies on the mode of action are inconclusive but generally include one or more of the following: enhancement of cartilage matrix synthesis, enhanced synthesis of hyaluronan by synoviocytes, inhibition of enzymes that degrade cartilage, and reduction of joint pain and synovitis.^{3,7,16,20,31}

Complex sugars including chondroitin sulfate and amino sugars such as glucosamine have been shown to have a biologic effect on articular cartilage. There is substantial literature on their symptomatic efficacy in clinical trials^{8,10,21,31,41,43} and in animal models.^{6,16,42} These structure modifying agents also are considered to be symptomatic slow acting drugs for the treatment of osteoarthritis. Glucosamine is an amino monosaccharide nutrient and precursor of the disaccharide unit of glycosaminoglycan which is the building block of the proteoglycan component of the cartilage matrix. Glucosamine has been proposed in the Congress of Rheumatology as a slow acting agent in osteoarthritis based on its pharmacologic and clinical profile.³⁶ Its mechanism of action may involve inhibition of protease activity and stimulation of glycosaminoglycan synthesis.³ The physiochemical properties of glucosamine account for its favorable pharmacokinetic oral bioavailability and cartilage tropism.³⁷ However, there is limited *in vivo* documentation of its disease modification effects.¹¹ Chondroitin sulfate is a long-chain polymer of repeating di-

saccharide units containing galactosamine sulfate and glucuronic acid and constitutes the majority of glycosaminoglycans in articular cartilage.²⁷ Its bioavailability is well documented with up to 70% absorption after oral administration in animals and in humans.^{7,24} Published data indicate that chondroitin sulfate has variable effects including contributing to a pool of substrate available for cartilage matrix deposition, inhibition of proteases, stimulation of glycosaminoglycan (GAG) and collagen synthesis.^{42,43}

The efficacy of these agents in protecting articular cartilage has been examined in many animal models. For example, Uebelhart et al⁴² examined oral and intramuscular delivered agents in a chymopapain induced acute model of osteoarthritis in the rabbit. Oral delivery of a mixture of low molecular weight chondroitin sulfate resulted in retardation of cartilage degeneration monitored by assay of serum levels of keratan sulfate and cartilage proteoglycan content. Hanson et al¹⁶ treated equine osteoarthritis with a combination of glucosamine and low molecular weight chondroitin sulfate with substantial improvement in clinical symptoms, and Canapp et al¹⁶ reported the same combination had a significant antiinflammatory effect against chemically-induced inflammatory synovitis in canines. Clinical trials in humans are numerous, more so in Europe than the United States. The remarkable acceptance of these agents and recent positive clinical trials in the United States attest to their efficacy in treatment of osteoarthritis. Recent reviews suggest that they potentially may become a basic drug for osteoarthritis therapy.^{11,18} However, because these compounds are dietary supplements, purity is dependent on the manufacturer and varies greatly.¹¹ Independent analysis of the preparations used in this study verified the concentration and purity of each agent.

In contrast to the proliferation and success of clinical trials using one or another of the structure modifying agents, little is known of any interaction between them. Glucosamine salt and chondroitin sulfate are considered to have overlapping functions in the management of dam-

aged articular cartilage.^{5,11,18} Additionally, a combination of chondroitin sulfate and glucosamine has been used by veterinarians for years with efficacious results.^{6,16} To examine the possibility of such an interaction, the current authors explored whether combining two or more agents would be synergistic in protecting articular cartilage in vivo in an established animal model of osteoarthritis. Similarly, in vitro studies examined the interaction by monitoring synthetic and degradative processes in cultured chondrocytes.

MATERIALS AND METHODS

Surgical instability was created in 42 New Zealand White rabbits weighing 2–3 kg using a modification of the technique of Hulth et al.¹⁷ Animals were sedated with ketamine (50mg/kg) and xylazine (10mg/kg) and placed under inhalation anesthesia (2% isoflurane in 2 L oxygen). The right hindleg was shaved and the skin prepared using Betadine (Purdue Frederick Co, Norwalk, CT) surgical solution and 70% alcohol. An incision centered over the joint line was made in the right knee with bleeding controlled by cautery. Dissection was taken down to the anteromedial joint capsule with knee flexion and extension helping to identify landmarks. The joint capsule was incised obliquely between the patellar tendon and medial collateral ligament and care was taken not to damage the tibial insertion of the lateral meniscus. An anterior drawer confirmed translation and better exposed the posterior cruciate ligament. With valgus and anterior drawer forces on the flexed knee, the anterior and posterior cruciate ligaments were visualized and transected. Removing the medial meniscus completed the procedure. After irrigation, the capsule was closed with 3-0 vicryl absorbable suture (Ethicon, Somerville, NJ) in an interrupted figure of eight fashion. The skin was closed with 4-0 polysorb absorbable suture in a subcuticular fashion with a buried knot. The wound was covered with nitrofurazone soluble dressing as no bandage was applied. Postoperatively, each rabbit was given procaine penicillin G (30,000 U per day, intramuscular) for 3 days and buprenorphine hydrochloride (0.04 mg/kg subcutaneous, twice daily) for 3 days. Two weeks postoperatively, the rabbits were allowed exercise in a 6 × 4-foot pen for 1 hour per day 3 days a week.

Dietary Regimen

Three days after surgery, Groups 1 and 2, consisting of 12 animals each, were fed the following diets: Group 1 (control) Harlan-Teklad (Madison, WI) standard rabbit chow; Group 2: a similar diet supplemented with 2% by weight Cosamin[®]DS (combined group). The composition of Cosamin[®]DS was 500 mg glucosamine hydrochloride (FCHG49[®]), 400 mg low molecular weight sodium chondroitin sulfate (TRH122[®]), and 76 mg manganese ascorbate per gram. Three additional groups of six animals each were fed the control diet, which contained either glucosamine hydrochloride, chondroitin sulfate, or manganese ascorbate at a dose equal to that given in Group 2. Food intake and weight gain was monitored weekly for 16 weeks. Cosamin[®]DS is a patented oral nutritional supplement. Chondroitin sulfate is purified from bovine trachea, and glucosamine is derived from the chitin of crustacean shells.

Analytical Methods

Preoperative blood samples were taken from the marginal ear vein and samples were obtained by intracardiac puncture after euthanasia. Serum samples were frozen for later analysis of glycosaminoglycans using dimethylmethylene blue,¹⁴ hexosamines,⁴ and to test for the presence of circulating factors which may modulate cartilage metabolism. To assay serum for cartilage activity, normal rabbit cartilage explant and cell cultures were exposed to a 1:1 mixture of test serum and an equal mixture of Dulbecco's minimum Eagles medium and F-12 containing 5 μ Ci/mL 35-sulfate for 4 hours. Liquid scintillation counting monitored isotope uptake after extensive washing of unincorporated isotope and the data were expressed as counts per minute per well or per milligram dry tissue weight. For assay of degradative activity, 35-sulfate prelabeled rabbit segments were similarly cultured in a 1:1 mixture of experimental serum and media and isotope release was monitored after 18 hours.

The effect of the dietary supplements on normal rabbit articular cartilage was examined by removing humeral cartilages from each animal at sacrifice and culturing ex vivo in equal amounts of Dulbecco's minimum Eagles medium and F-12 for 4 hours in the presence of 10 μ Ci/mL 35-sulfate. The tissues were chased with cold 1 mmol/L sodium sulfate for 24 hours and fixed for 48 hours in 10% phosphate buffered formalin. After dehydration in alcohol, lyophilization and weighing, each of five

samples per animal was dissolved in formic acid and radioisotope incorporation monitored by liquid scintillation counting. Radiolabeled uptake was expressed as counts per minute per milligram dry tissue weight.

Histologic and Histochemical Analysis

Multiple organs were resected for standard pathologic examination. A portion of diaphyseal bone from each femur was dried, extracted with acetone, weighed, and subsequently ashed at 700° C for 4 hours to determine bone mineral density. The distal femurs of all animals were removed and a 2 to 3 mm thick sagittal section was taken through the center weightbearing portion of the medial condyle with a diamond saw. Analysis of the progression of cartilage lesions was quantitated using a modification of the method of Mankin et al²⁶ after staining 5 to 8 μ sections with safranin O, fast green, and iron hematoxylin. This widely used method of defining lesion severity focuses on quantitative evaluation of focal degenerative changes. The system totals the assigned scores from each of four criteria including structure (0-6), cells (0-3), safranin O staining (0-4), and tidemark integrity (0-1). Measuring the linear extent of each lesion provided a better index of degeneration because many condyles had extensive lesions with varying degrees of severity. This was accomplished using Image Pro software (Media Cybernetics, Silver Spring, MD) and a SprintScan 35 Plus slide scanner (Polaroid Inc, Cambridge, MA) (Fig 1). The results are presented as the numerical values within the following grades of lesion severity: mild (1-3); moderate (4-7); and severe (> 7). Evaluation was done in blind fashion by two of the investigators.

In Vitro Proteoglycan Synthesis

Chondrocytes isolated from the metacarpophalangeal joints of adult dairy cows were isolated with collagenase digestion.²⁰ Cells were plated into 96-well polylysine-coated plates at a density of 10^5 cells per well. Plated cells were allowed to reach a metabolic steady state by maintenance in culture for 5 days in daily changes of 250 μ L Dulbecco's minimum Eagles and F-12 medium containing 10% fetal calf serum, 50 μ g/mL vitamin C sulfate, and antibiotics. Two hours before adding the experimental agents, the media was changed to plain Dulbecco's minimum Eagles and F-12 medium to diminish the metabolic effects of fetal calf serum. Twenty-four replicates of each agent and combination were

tested by adding each in a total volume of 250 μ L of Dulbecco's minimum Eagles and F-12 medium containing antibiotics, vitamin C sulfate, and 5 μ Ci/mL 35-sulfate. The concentrations tested in vitro were determined by preliminary studies. The final range tested bracketed the dose calculated by determining the distribution of the recommended human daily dose of the combination drugs (1500 mg glucosamine hydrochloride, 1200 mg chondroitin sulfate, and 228 mg manganese ascorbate) into total body water of a 70 kg individual. The final concentrations tested in vitro were 150 μ g/mL glucosamine, 140 μ g/mL chondroitin sulfate and 200 ng/mL manganese ascorbate. After 4 hours exposure the cultures were washed thoroughly with plain Dulbecco's minimum Eagles medium and isotope uptake into the cell layer assayed with a Wallac MicroBeta TriLux liquid scintillation counter (Wallac Inc, Gaithersburg, MD). The data were expressed as counts per minute per well and statistical significance determined by the Student's t test and analysis of variance (ANOVA).

In Vitro Proteoglycan Degradation

Glycosaminoglycan degradation was monitored by the release of 35-sulfate from prelabeled chondrocyte cell layers and assay of interleukin-1 (IL-1) induced collagenase activity using a BindazymeTM ProMMP-1 enzyme immunoassay kit (The Binding Site Ltd, Birmingham, England). In the first approach, bovine cells were plated into 96-well plates as described above. After an 18-hour exposure to 5 μ Ci/mL 35-sulfate and 2 hours washout, the experimental agents at doses given above were added, and release of isotope as a percentage of total incorporated isotope was determined after 18 hours exposure. Interleukin-1 induced collagenase activity was measured on aliquots of medium taken from 24-well culture plates seeded with 4×10^5 cells and cultured for 24 hours in the presence of 40 ng/mL IL-1 using equivalent doses of each chondroprotective agent.

Statistical Methods

The mean and standard deviation and standard error of the mean were calculated for each sample group. Fisher's F test was used to test homogeneity of the variances of all groups. Means were compared using ANOVA and Student's t test using multiple group comparisons. Histologic data were evaluated using the Mann-Whitney rank sum test.

The histologic data were analyzed for three vari-

Fig 1. Photomicrograph documenting method of measurement. Site 1 graded as mild (Mankin score 1-3); site 2 graded as moderate (Mankin score 4-7); sites 3 and 4 graded as severe (Mankin score > 7). The extent of each lesion was taken by measuring the length in millimeters at each defined site and automatic calibration of the computer scans with a slide micrometer.



ables: number of lesions; grade of each lesion, and linear involvement of lesions. For these data two new variables were created, total linear involvement and total grade. These variables were calculated to sum all lesions in the same rabbit and to incorporate the number of lesions and the prospective variable. For example, the total grade was calculated by multiplying the mean grade and the number of lesions for every rabbit. For total grade and total linear involvement, group comparisons were made using Scheffe's multiple comparison test.³⁵ The proportion of animals in each group with one or more severe lesions was assessed using a chi squared test.

RESULTS

The rate of weight gain during the 16-week period was similar between groups and among an-

imals within each group. The average weight of all animals at 16 weeks ranged from 4.0 to 4.5 kg. Animals were ingesting an average of 150 g food per day during the 16-week interval. At 16 weeks this provided Group 2 with 375 mg glucosamine hydrochloride, 300 mg sodium chondroitin sulfate, and 57 mg manganese ascorbate per day per kilogram. Groups 3, 4, and 5 ob-

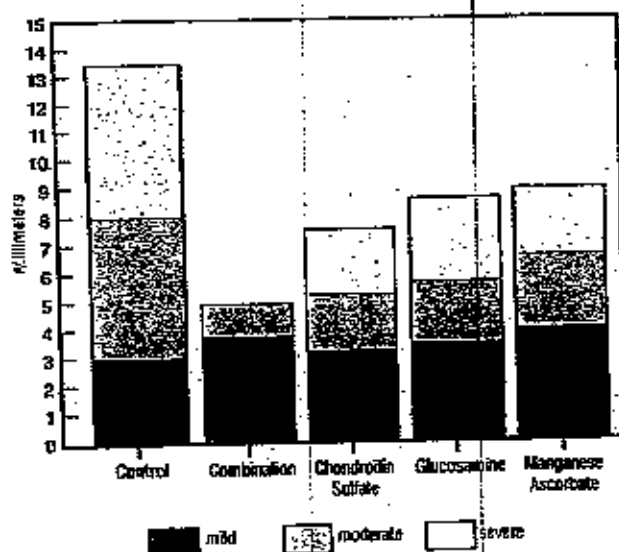


Fig 2. Distribution profile of cartilage lesions measured as millimeters surface involvement



Fig 3A-B. Representative photomicrographs of safranin O stained histologic sections of (A) the nonsupplemented control articular cartilage from an unstable joint and (B) the contralateral normal joint from the same group.

TABLE 1. Histologic Analysis: Total Grade and Millimeters of Linear Involvement

Group	(N)	Total Grade	Value	Total Linear Involvement	Value
Control	(11)	16.6 ± 2.2	—	12.3 ± 1.3	—
Chondroitin sulfate	(6)	11.0 ± 2.2	NS	7.2 ± 1.2	NS
Glucosamine	(6)	12.2 ± 3.0	NS	8.6 ± 1.0	NS
Manganese ascorbate	(6)	8.8 ± 1.4	NS	8.8 ± 1.8	NS
Combined group	(11)	4.4 ± 1.0	0.05*	5.1 ± 1.2	0.05*

Values represent the mean ± standard error; NS = not significant; *p < 0.05 by Scheffé test.³⁴

The analysis compared the four study groups with the control group and the results are presented in Table 2. Animals supplemented with either glucosamine or chondroitin sulfate alone had the same incidence of severe lesions as the unsupplemented Group 1 (67%–83%). Manganese ascorbate supplementation reduced this further (not significantly different) whereas the combination group had no severe lesions. Ex

TABLE 2. Histologic Analysis: Proportions of Animals Having Severe Lesions

Group	Severe Lesions	Value
Control	8/11 (83%)	—
Chondroitin sulfate	4/6 (67%)	0.80
Glucosamine	4/6 (67%)	0.80
Manganese ascorbate	2/6 (33%)	0.30
Combined group	0/11 (0%)	0.002

Values given as percentages; P value calculations based on a chi square test comparing each group with control.

vivo incorporation of radiolabeled sulfate by the noninvolved humeral cartilage revealed no significant difference in cartilage proteoglycan synthesis between supplemented groups and controls (Table 3). Analysis of serum hexosamines ranged from 1.112 to 1.322 mg/mL and glycosaminoglycans detected by dimethylmethylene blue was 18.4–19.2 µg/mL. There were no significant differences between supplemented groups and controls or between samples taken at 0 time and after 16 weeks. Glycosaminoglycan synthetic activity of normal rabbit cartilage exposed to a 1:1 solution of 16 week serum samples and Dulbecco's minimum Eagles medium also revealed no significant difference between supplemented groups and control (Table 4).

In Vitro Studies

Four outliers were revealed in the analysis of 35-sulfate uptake into the cell layer fraction of cultured bovine chondrocytes exposed to combinations of each agent. With these four values removed, the data appear to be normally distrib-

TABLE 3. Ex Vivo 35-Sulfate Incorporation into Humeral Articular Cartilage Of Rabbits Fed Dietary Supplements

Group (Number of Animals)	Mean*	% Change	Value
Control	(11) 2284 (720)	—	—
Glucosamine	(6) 1724 (405)	-25%	NS
Chondroitin sulfate	(6) 2122 (360)	-7%	NS
Manganese ascorbate	(6) 2265 (678)	-1%	NS
Combined group	(11) 1890 (527)	-17%	NS

Data expressed as mean (standard deviation) counts per minute per mg dry tissue weight; Significance determined by Mann-Whitney test.

TABLE 4. Effect of 16-Week Experimental Rabbit Serum on Normal Bovine Cartilage Synthesis and Degradation

Group	Synthesis	Synthesis	Degradation*
	Explants	Cell Culture	Explants
Control	3368 (727)	508 (37)	5.29 (2.0)
Glucosamine	3163 (556)	450 (77)	3.24 (0.6)
Chondroitin sulfate	2426 (678)	388 (35)	6.56 (2.4)
Manganese ascorbate	2106 (418)	460 (42)	7.92 (1.3)
Combined group	3224 (940)	541 (96)	6.14 (1.8)

Values represent mean (\pm standard deviation) of counts per minute 35-sulfate per mg dry tissue weight; *Data given as counts per minute 35-sulfate released as a % of total counts per minute; Significance determined by Mann-Whitney test; Values not significantly different from control.

uted and the variances were more comparable among groups. Analysis by ANOVA gave significant differences (Table 5) in all groups compared with the control (glucosamine, +33%; chondroitin sulfate, +33%; a combination of

TABLE 5. Synergistic Effect of Chondroprotective Agents on In Vitro Incorporation of 35-Sulfate Into Glycosaminoglycans

Group (n = 24)	Mean*	% Change
Control	1226 (141)	—
Glucosamine	1629 (273)	+32%
Chondroitin sulfate	1628 (344)	+32%
Glucosamine + Chondroitin sulfate	2374 (336)	+96.6%**

Data presented as mean (\pm standard error) of counts per minute per 10^6 cells; **P value = 0.003 in test for synergy defined as a significant difference between the additive effect of each component and the value for the combination.

glucosamine and chondroitin sulfate, +94%) and a definitive p value less than 0.003 in the test of synergy between glucosamine and chondroitin sulfate. Synergy is defined as a significant difference between the additive effect of each component and the values obtained with the combination. The most striking results were obtained with the higher doses but a dose response was obtained with all three agents down to levels of 5 μ g/mL (data not shown).

Data on the release of radioactivity from 35-sulfate prelabeled cell cultures as an index of degradative activity are presented in Table 6. The values were calculated as the percent change from control cultures and show significant inhibition of release in the presence of a mixture of glucosamine, chondroitin sulfate, and manganese ascorbate (25% less than control; $p < 0.001$). Exposure of cells to each agent alone resulted in significant inhibition with chondroitin sulfate (21%; $p < 0.003$) but

TABLE 6. Effect of Chondroprotective Agents on Chondrocyte Cultures Assay of 35-Sulfate Released From Prelabeled Cells and IL-1 Induced Collagenase Activity

Group	% 35-Sulfate Released	% Change	Value	ng	% Change	Value
				Collagenase		
Control	7.81 (0.4)	—	—	3.6	—	—
Chondroitin sulfate	6.14 (0.6)	-21%	0.003	2.0	-45%	0.001
Glucosamine	7.08 (1.09)	-10%	NS	3.6	0%	NS
Manganese ascorbate	7.71 (1.4)	-1%	NS	1.8	-50%	0.001
Combined group	5.82 (0.97)	-25%	0.001	2.4	-34%	0.001

Values given as mean (\pm standard deviation); Significance determined by Mann-Whitney test.

not with glucosamine or manganese ascorbate. The greater inhibition of release was seen using the combination of agents. Similarly, antiprotease effects monitored by direct assay of IL-1-induced collagenase activity revealed significant inhibition with chondroitin sulfate (34%–50%; $p < 0.001$), manganese ascorbate (50%; $p < 0.001$) and a combination of all three agents (34%; $p < 0.001$) but not with glucosamine alone (Table 6).

DISCUSSION

Combinations of low molecular weight chondroitin sulfate and glucosamine HCl were more efficacious than either agent alone in retarding progression of articular cartilage lesions in a rabbit instability model of osteoarthritis. It was the authors' observation that quantifying the degree of cartilage degeneration based on a system designed for focal lesions did not lend itself in this model because many specimens had lengthy lesions with marked gradations of severity. The authors' modification takes this into consideration by measuring the millimeters of linear involvement of each grade of severity. This provided for a more accurate picture of the extent and progression of the degenerative process. The Polaroid slide scanner permitted captures of the entire tissue section image which made it easy to directly measure the linear involvement of tissue destruction. Similar techniques were adapted by Shimizu et al³⁸ using a gray scaling method for quantitating glycosaminoglycans in histologic sections.

In the method of Hulth et al¹⁷ for inducing experimental osteoarthritis in the rabbit, an unstable joint leads to cartilage degeneration with the first evidence appearing approximately 2 weeks after surgery. The development of osteophytes typical of osteoarthritis was a prominent feature in the current study and has been described previously in this model.³² No doubt the exercise regimen that was used facilitated osteophyte development as a result of functional demand under conditions of altered joint mechanics.⁴⁰ The dietary supplements used did not appear to inhibit or

stimulate osteophyte formation. Unlike glucocorticoids, structure-modifying drugs for osteoarthritis previously have been found to have no effect on osteophyte formation.⁴⁷

Glucosamine and chondroitin sulfate are considered to have overlapping functions in the management of damaged articular cartilage. Synergy between these two structure-modifying agents has been postulated previously in the context of clinical studies with chondroitin sulfate having antiinflammatory activity and glucosamine influencing cellular metabolism.^{3,6,31,33} The current study is the first showing that glucosamine and chondroitin sulfate synergistically act to stimulate glycosaminoglycan synthesis in chondrocytes. It is apparent that this phenomenon in addition to the antiprotease action of chondroitin sulfate provides greater efficacy in retardation of the degenerative process. Although glucosamine stimulated glycosaminoglycan synthesis, it had little effect on inhibition of aggrecanase (labeled sulfate release) or collagenase. Chondroitin sulfate, however stimulated glycosaminoglycan synthesis and showed significant antiprotease activity against both enzymes. Manganese and ascorbate also are known to be active agents on cartilaginous tissue^{9,29,45} but generally are not classified as structure modifying. Dietary supplements of manganese ascorbate alone resulted in a 47% reduction in total mean grade, and a 28% reduction in linear involvement with only 1/2, two of six animals having severe lesions. Statistically these reductions were not significant but this may be a function of the limited number of animals used. In vitro, manganese ascorbate exposure was seen to cause an inhibition of IL-1-induced collagenase activity which may partly explain the *in vivo* reduction of lesion severity. The data presented in the *in vitro* study were generated using tissues from adult dairy cows (greater than 5 years of age). In other experiments (data not shown), tissues from fetal or newborn animals had minimal responses.

Animals in this study received a daily dose approximately 20 times the human dose based on a total body weight of 70 kg (300 mg chondroitin sulfate and 375 mg glucosamine per

kilogram per day versus the 17 mg and 21 mg/kg per day for humans respectively). The use of 2% dietary supplements was based on an estimate of daily food consumption by rabbits and compensation for the more than twofold difference in basal metabolic rate.¹³ Additionally, the goal in the current study was to maximize the therapeutic effect and test for safety in other organs. Even at these high doses, a comparison of serum levels of dimethylmethylene blue positive glycosaminoglycan in samples taken preoperatively and 16 weeks later revealed no increase in circulating levels of either component in any animal group. Similar findings were reported by Baici et al² in human volunteers after receiving oral dosing. In rabbits, negative results also were obtained using uronic acid assays of cetylpyridinium chloride precipitates¹ and uronic acid and hexosamine analysis.¹⁹

There are three possible explanations for the lack of elevations in serum levels of glycosaminoglycans. The first is that no serum elevation occurred because chondroitin sulfate, like the glycosaminoglycan heparin, is not absorbed in the gastrointestinal tract presumably because of its size and ionic repulsion from negatively charged epithelial tissue.³⁹ Distribution of these negatively charged polymers may occur via the lymphatic system when given orally. The second explanation is the timing of serum sampling at the termination of the study relative to ingestion of the diet. In a report by Yamanashi et al,⁴⁶ chondroitin sulfate levels in serum returned to normal within 6 hours after intravenous administration. Rabbits are nocturnal feeders and chondroitin sulfate levels would have returned to normal by midmorning when the study was terminated. The third explanation can be attributed to the lack of specificity of the dimethylmethylene blue reaction for byproducts of glycosaminoglycan metabolism, disaccharides or desulfation and lack of sensitivity of the method. However, Volpi et al⁴⁴ recently reported glycosaminoglycan elevations detected by densitometric scanning and agarose-gel electrophoresis after a single oral dose of the same chondroitin sulfate in volunteers. Their

data suggest that glycosaminoglycans degraded by intestinal enzymes to disaccharides are not detected by dimethylmethylene blue. In the case of glucosamine, testing was done for protein-bound and free hexosamine. The relatively rapid turnover of hexosamines and first pass effect in the liver may explain the lack of detectable increases for this amino sugar. A recent study documented disappearance of glucosamine 2 hours after an oral dose of 2 g in dogs.²²

The lack of an effect of any of the dietary supplements on normal cartilage metabolism (humerus) alternatively may suggest a tropism of these agents for cartilage in the reactive state because of stress. These results are in accord with recent recommendations by the European Group for the Respect of Ethics and Excellence in Science which state that drugs used in the treatment of osteoarthritis not have a deleterious effect on normal cartilage.³⁴ It would appear that one mechanism of action of glucosamine and chondroitin sulfate is to synergistically enhance the natural hypermetabolic repair response of chondrocytes and in this way affect a stabilization of cartilage matrix which retards the progression of cartilage degeneration.

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References

1. Andermann G, Dietz M: The influence of the route of administration on the bioavailability of an endogenous macromolecule: Chondroitin sulfate (CSA). *Eur J Drug Metab Pharm* 7:11-16, 1982.
2. Baici A, Horler D, Moser B, et al: Analysis of glycosaminoglycans in human serum after oral administration of chondroitin sulfate. *Rheumatol Int* 12:81-88, 1992.
3. Bassleer C, Rovati L, Franchimont P: Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from osteoarthritic articular cartilage in vitro. *Osteoarthritis Cartilage* 6:427-433, 1998.
4. Blumenkrantz N, Asboe-Hansen G: An assay for total hexosamine and differential assay for glucosamine and galactosamine. *Clin Biochem* 9:269-274, 1976.
5. Bucci L: Chondroprotective agents: Glucosamine salts and chondroitin sulfates. *Townsend Letter for Doctors* 1:52-54, 1994.

- Canapp SO, McLaughlin RM, Hoskinson JJ, et al: Scintigraphic evaluation of dogs with acute synovitis after treatment with glucosamine hydrochloride and chondroitin sulfate. *J Vet Res* 60:1552-1557, 1999.
7. Conte A, Volpi N, Palmieri L, et al: Biochemistry and pharmacokinetic aspects of oral treatment with chondroitin sulfate. *Arzneim Forsch/Drug Res* 45:918-925, 1995.
8. Crolle G, Este DE: Glucosamine sulphate for the management of arthrosis: A controlled clinical investigation. *Curr Med Res Opin* 7:104-109, 1980.
9. Daniel JC, Pauli BU, Kuestner KE: Synthesis of cartilage matrix by mammalian chondrocytes in vitro. III. Effects of ascorbate. *J Cell Biol* 99:1960-1969, 1984.
10. Das AK, Hammad TA: Efficacy of a combination of FCHG49[®] (glucosamine hydrochloride, TRH122[®]) low molecular weight sodium chondroitin sulfate and manganese ascorbate in the management of knee osteoarthritis. *Osteoarthritis Cartilage*, September, 2000.
11. Deal CL, Moskowitz RW: Nutraceuticals as therapeutic agents in osteoarthritis. *Rheum Disease Clin North Am* 25:379-395, 1999.
12. Dean DD, Muniz OE, Rodriguez I, et al: Amelioration of lapine osteoarthritis by treatment with glycosaminoglycan-peptide associated complex (rumalon). *Arthritis Rheum* 34:304-313, 1991.
13. Donhoffer S: Body size and metabolic rate: Exponent and coefficient of the allometric equation. The role of units. *J Theor Biol* 119:125-137, 1986.
14. Farndale RW, Buttle DJ, Barrett AJ: Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 883:173-177, 1986.
15. Goldring J, Ghosh P: Drugs for osteoarthritis. II. The effects of a glycosaminoglycan polysulphate ester (Arteparon) on proteoglycan aggregation and loss from articular cartilage of immobilized rabbit knee joints. *Curr Therapy Res* 34:67-80, 1983.
16. Hanson RR, Smalley LR, Huff GK, et al: Oral treatment with a glucosamine-chondroitin sulfate compound for degenerative joint disease in horses: 25 cases. *Equine Pract* 19:16-22, 1997.
17. Hulth A, Lindberg L, Telford H: Experimental osteoarthritis in rabbits. *Acta Orthop Scand* 41:522-530, 1970.
18. Hungerford DS: Treating osteoarthritis with chondroprotective agents. *Orthopedics Special Ed* 4:39-42, 1998.
19. Konador A, Kawiak J: Changes in chondroitin sulfate concentration in rabbit blood plasma depending on the method of its administration. *Arch Immunol Ther Exp* 25:895-903, 1977.
20. Kongtawelert P, Brooks PM, Ghosh P: Pentosan polysulfate (cartrophen) prevents the hydrocortisone injected loss of hyaluronic acid and proteoglycans from cartilage of rabbit joints. *J Rheum* 16:1454-1459, 1989.
21. Leffler CT, Philippi AF, Leffler SG, et al: Glucosamine, chondroitin and manganese ascorbate for degenerative joint disease of the knee or low back: A randomized, double-blind placebo-controlled pilot study. *Mil Med* 164:85-91, 1999.
22. Liang Z, Leslie J, Adebawale A, et al: Determination of the nutraceutical, glucosamine hydrochloride in raw materials, dosage forms and plasma using pre-column derivatization with ultraviolet HPLC. *J Pharm Biomed Analysis* 20:807-814, 1999.
23. Listrat V, Ayral X, Patarnello F, et al: Arthroscopic evaluation of potential structure modifying activity of hyaluronan (Hyalgan) in osteoarthritis of the knee. *Osteoarthritis Cartilage* 5:153-160, 1997.
24. Lualdi R: Bioavailability of oral chondroitin sulfate. *Rheumatol Int* 13:39-40, 1993.
25. Mankin HJ: Biochemical and metabolic aspects of osteoarthritis. *Orthop Clin North Am* 2:19-24, 1971.
26. Mankin HJ, Dorfman H, Lippiello L, et al: Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg* 53A:523-537, 1971.
27. Mankin HJ, Johnson M, Lippiello L: Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. III. Distribution and metabolism of amino sugar-containing macromolecules. *J Bone Joint Surg* 63A:131-139, 1981.
28. Mankin HJ, Lippiello L: Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. *J Bone Joint Surg* 52A:424-434, 1970.
29. McNatt ML, Fiser FM, Elders MI, et al: Uridine diphosphate xylosyltransferase activity in cartilage from manganese-deficient chicks. *Biochem J* 15:211-216, 1976.
30. Mitrovic D, Lippiello L, Mankin HJ: Use of enzymatically isolated chondrocytes for short term metabolic studies. *J Rheumatol* 6:124-130, 1979.
31. Morreale P, Manopulo R, Galati M, et al: Comparison of the anti-inflammatory efficacy of chondroitin sulfate and diclofenac sodium in patients with knee osteoarthritis. *J Rheumatol* 23:1385-1391, 1996.
32. Moskowitz RW, Davis W, Sammarco J, et al: Experimentally induced degenerative joint lesions following partial meniscectomy in the rabbit. *Arthritis Rheum* 16:397-405, 1973.
33. Ronca F, Palmieri L, Panicucci P, et al: Anti-inflammatory activity of chondroitin sulfate. *Osteoarthritis Cartilage* 6: (Suppl A):14-21, 1998.
34. Recommendations for the Registration of Drugs Used in the Treatment of Osteoarthritis. *Ann Rheum Dis* 55:552-557, 1996.
35. Scheffe H: A method for judging all contrasts in the analysis of variance. *Biometrika* 40:87-104, 1953.
36. Semi U: Profile of glucosamine sulfate as an example of slow acting drug in osteoarthritis. 18th Congress Rheumatology. *Rev Esp Reumatol* 20:222, 1993. Abstract.
37. Setnikar L, Palumbo R, Canali S, et al: Pharmacokinetics of glucosamine in man. *Arzneim-Forsch/Drug Res* 43:1109-1113, 1993.
38. Shimizu C, Courts RD, Healey RM, et al: Method of histomorphometric assessment of GAG in articular cartilage. *J Orthop Res* 15:670-674, 1997.
39. Sue TK, Jaques LB, Yuen E: Effects of acidity, cations and alcoholic fractionation on absorption of heparin from the gastrointestinal tract. *Can J Physiol Pharmacol* 54:613-618, 1976.

40. Suter E, Herzog W, Leonard TR, et al: One year changes in hind limb kinematics, general reaction forces and knee stability in an experimental model of OA. *J Biomech* 31:511-517, 1998.
41. Uebelhart D, Thonar EJ, Delmas PD, et al: Effects of oral chondroitin sulfate on the progression of knee osteoarthritis: A pilot study. *Osteoarthritis Cartilage* 6:39-46, 1998.
42. Uebelhart D, Thonar EJM, Zhang J, et al: Protective effect of exogenous chondroitin 4,6-sulfate in the acute degradation of articular cartilage in the rabbit. *Osteoarthritis Cartilage* 6:6-13, 1998.
43. Verbruggen G, Goemaere S, Veys EM: Chondroitin sulfate SYSMOAD (structure/disease modifying anti-osteoarthritis drug) in the treatment of finger joint OA. *Osteoarthritis Cartilage* 6:37-38, 1998.
44. Volpi N, Roth M, Uebelhart D: Quantitative and qualitative evaluation of chondroitin sulfate in normal human plasma. 12th European League Against Rheumatism Congress, Scotland 289, 1999. Abstract.
45. Wright Jr GC, Wei XQ, McDevitt CA, et al: Stimulation of matrix formation in rabbit chondrocyte cultures by ascorbate. 1. Effect of ascorbate analogs and beta aminopropionitrile. *J Orthop Res* 6:397-407, 1988.
46. Yamanashi S, Toyoda H, Furuya N et al: Metabolic study on chondroitin sulfates in rabbits. *Yakugaku Zasshi* 111:73-76, 1991.
47. Yu LP, Smith Jr GN, Brandt KD, et al: Reduction of the severity of canine osteoarthritis by prophylactic treatment with oral doxycycline. *Arthritis Rheum* 35:1150-1159, 1992.