Effects of Chondroitin and Glucosamine Sulfate in a Dietary Bar Formulation on Inflammation, Interleukin-1β, Matrix Metalloprotease-9, and Cartilage Damage in Arthritis

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This study examined the effects of chondroitin sulfate (CS) alone and CS plus glucosamine sulfate (GS) in a dietary bar formulation on inflammatory parameters of adjuvant-induced arthritis and on the synthesis of interleukin-1β (IL-1β) and matrix metalloprotease-9 (MMP-9). Following 25 days pretreatment with dietary bars containing either CS alone, CS plus GS, or neither CS nor GS, rats were either sham injected or injected with Freund's complete adjuvant into the tail vein. Rats were fed their respective bars for another 17 days after inoculation. Parameters of disease examined included clinical score (combination of joint temperature, edema, hyperalgesia, and standing and walking limb function), incidence of disease, levels of IL-1β in the serum and paw joints, levels of MMP-9 in the paw joints, paw joint histology, and joint cartilage thickness. Treatment with CS plus GS, but not CS alone, significantly reduced clinical scores, incidences of disease, joint temperatures, and joint and serum IL-1β levels. Treatment with CS alone and CS plus GS inhibited the production of edema and prevented raised levels of joint MMP-9 associated with arthritis. Similarly, CS alone and CS plus GS treatment also prevented the development of cartilage damage associated with arthritis. Combination CS plus GS treatment in a dietary bar formulation ameliorates clinical, **inflammatory, and histologic parameters of adjuvant-induced arthritis. The benefits of CS and GS in combination are more pronounced than those of CS alone. The reduction of arthritic disease by CS plus GS is associated with a reduction of IL-1β and MMP-9 synthesis.** Exp Biol Med 230:255–262, 2005

Key words: chondroitin; glucosamine; MMP-9; IL-1β; cartilage

Introduction

Rheumatoid arthritis is an autoimmune disease characterized by chronic polyarticular inflammation, resulting in a number of pathologic changes such as joint swelling, pannus formation, cartilage damage, and loss of function (1, 2). A variety of inflammatory mediators have been implicated in the pathogenesis of joint disease, and a number of them are common to both rheumatoid arthritis and osteoarthritis. For example, interleukin-1β (IL-1β) promotes adhesion molecule (intercellular adhesion molecule-1 [ICAM-1] and vascular cell adhesion molecule-1 [VCAM-1]) production, prostaglandin synthesis, bone resorption, and matrix metalloprotease (MMP) synthesis (3–7). IL-1β actively increases MMP-9 levels in synovial membranes (8). Release and activation of MMPs in the joint, including MMP-9, trigger bone and cartilage degradation, which can exacerbate joint degeneration and lead to increased pain (7, 9–11). Recent findings also suggest that MMP-12 plays an important role in the pathogenesis of rheumatoid arthritis (12). Yet controversy remains as to whether elevation of these proteases is a cause or consequence of arthritic disease, and intense research activities currently investigate whether inhibition of these enzymes may confer key therapeutic benefits (13, 14, 15). As MMPs become increasingly appealing treatment targets, and in view of

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the common toxic side effects and/or lack of enzyme specificity of most synthetic MMP inhibitors available to date, the development of alternative treatment methods involving such inhibition is of great interest.

In addition to the variety of pharmacologic therapies available for arthritis treatment, including nonsteroidal anti-inflammatory drugs (NSAIDs), biologic response modifiers, and disease-modifying antirheumatic drugs, nonpharmacologic management of arthritis can also help improve the quality of life for arthritis sufferers. Nonpharmacologic treatments include patient education, exercise, and weight management. In light of the associated gastrointestinal toxicity of some NSAIDs, as well as the side effects related to the use of other pharmacologic treatments (16–19), the use of dietary supplements such as chondroitin sulfate (CS) or glucosamine sulfate (GS) (17, 18) has become increasingly popular. To date, no adverse drug reactions or toxicity have been reported in association with the use of these products (20).

The biologic mechanisms underlying the apparent benefits of CS and/or GS remain obscure. CS is a glycosaminoglycan (GAG) that is part of the proteoglycan structure in the joint cartilage (20, 21). Glucosamine is an amino sugar that acts as a precursor in the biosynthesis of GAGs and proteoglycan aggregates (20, 21). Given that one of the characteristics of rheumatoid arthritis and osteoarthritis is an imbalance in the synthesis and degradation of cartilage components, one of the possible physiologic roles of exogenous glucosamine is to function as an additional source of hexosamine and sulfate precursors for GAG synthesis (20, 22). Indeed, oral glucosamine hydrochloride (HCl) prevents the loss of GAG in joint cartilage damaged by intra-articular injection of chymopapain (23).

In addition to their constitutive roles in the joint, both CS and glucosamine also possess anti-inflammatory properties. *In vitro* studies have shown that CS reverses the detrimental effects of IL-1β on proteoglycan and prostaglandin synthesis in chondrocytes (4, 24) and inhibits cytolytic complement function (20). Glucosamine inhibits cartilage catabolic responses and prevents IL-1β–induced increases in nitric oxide synthesis (4, 25). Studies *in vitro* suggest that combined glucosamine and chondroitin sulfate may decrease the gelatinolytic activity of MMP-9 (26). Yet the combined effects of these compounds on inflammatory mediators like IL-1β and MMP-9 *in vivo* are less clear.

Previous studies using a model of rheumatoid arthritis have shown that severity of disease is decreased in rats given an oral combination of CS, glucosamine HCl, and manganese ascorbate (27). Moreover, combined CS and glucosamine ameliorates signs of arthritic disease in dogs (16, 28–30), rabbits (31), and horses (26, 32). While both glucosamine hydrochloride and GS have demonstrated efficacy in treating joint disease (21, 30, 32–35), the sulfated form of glucosamine is commonly used as a nutritional supplement in the United States and throughout Europe (34–36), and a therapeutic effect of sulfate in glucosamine sulfate has been speculated (37). Therefore, the sulfated form of glucosamine—glucosamine sulfate (GS)—was used throughout the present study.

Preliminary experiments done in our laboratory indicated that treatment of experimental rheumatoid arthritis in rats with a new dietary bar formulation containing only CS could have a marginal benefit over CS administered in drinking water (data not shown). In light of these results, the aims of the present study were (i) to assess the effects of dietary modulation through CS in a novel dietary bar formulation on inflammatory parameters using an adjuvant-induced model of rheumatoid arthritis, (ii) to determine whether addition of GS to a dietary bar formulation could potentiate the effects of CS alone, and (iii) to assess the effects of CS and GS in bar formulation on production of the potent inflammatory mediators IL-1β and MMP-9. By using a new dietary formulation to deliver CS and GS to arthritic rats, results from this study will help establish a rational basis for the development of nonpharmacologic products to control arthritic disease in humans and possibly in companion animals.

Materials and Methods

Experimental Design. Male Wistar rats (150–175 g) (Charles River Laboratories, Montreal, Canada) were weight ranked and randomly assigned to four groups (n = 12 for each group). Preliminary experiments in our laboratory established that rats fed *ad libitum* exclusively with the dietary bar used herein grow normally and show no ill effects. The present study used the model of Freund's adjuvant arthritis as described previously (38). Rats in Group 1 (healthy) were fed placebo dietary bars (New Era Nutrition Inc., Edmonton, Canada) and sham injected with phosphate-buffered saline (PBS). Group 2 rats (control) were also fed placebo bars (New Era Nutrition), but were immunized with Freund's complete adjuvant. Rats in Group 3 (CS) were fed bars containing only CS (New Era Nutrition) and immunized with Freund's complete adjuvant, while rats in Group 4 (CS plus GS) were fed bars containing both CS and GS (New Era Nutrition) and immunized with Freund's complete adjuvant (38). Specific injection procedures and bar formulations are detailed below. All rats were housed individually and given tap water *ad libitum*. Rats were housed in the Life and Environmental Sciences Animal Resource Center at the University of Calgary, in 12:12-hr light:dark photoperiods at 20°C. All animal handling protocols complied with the Canadian Council of Animal Care guidelines and were approved by the University of Calgary Animal Care Committee.

Formulation of Dietary Bars. Three different types of dietary bar formulations were used in this study: placebo bars, CS bars, and CS plus GS bars, all provided by New Era Nutrition Inc. Placebo bars (New Era's bar formulation) contained neither CS nor GS and consisted of approximately 10% total fat, 62% total carbohydrate, and 10% protein.

In bars containing only CS, bovine tracheal CS (44 kDa,

greater than 90% pure dry weight; OptaFlex, Cargill Health and Food Technologies, High River, Canada) was mixed in New Era's bar formulation to a final concentration of 18 mg CS/g bar. In bars containing CS and GS together, GS (H&A, Toronto, Canada), at a final concentration of 22.5 mg GS/g bar, in addition to CS (18 mg CS/g bar) (Cargill), was mixed into New Era's bar formulation. In this study, the GS used was a glucosamine preparation containing sulfate cocrystallized with potassium (D-glucosamine sulfate, potassium salt). Based on preliminary experiments, calculations of the amount of CS and GS in bars were based on an average consumption of 20 g of bar per rat per day and an average rat body weight of 300 g. Since the body weight of the rats ranged from 150 to 500 g over the course of the study, the doses of CS and GS were calculated based on average body weight throughout the whole study. Selected doses of CS (1.2 g/kg/day) and GS (1.5 g/kg/day) were consistent with those previously established for experiments in rats (27, 38–41). Rats were fed bars for 25 days before sham injection or immunization with Freund's complete adjuvant, and then for an additional 17 days after inoculation.

Arthritis Induction. Rats from Groups 2–4 were immunized in the base of the tail with 150 µl of 7.5 mg/ml attenuated *Mycobacterium butyricum* (Difco, Mississauga, Canada) emulsified in Freund's complete adjuvant (Difco), as previously established (38, 42, 43). Rats from Group 1 were injected with 150 µl PBS intradermally through each side of the base of the tail. Rats were sacrificed with an intraperitoneal overdose of sodium pentobarbital 17 days following immunization, and samples were collected as outlined below.

Clinical Score and Incidence of Arthritic Disease. Clinical symptoms of arthritis were assessed Day 17 after sham or Freund's complete adjuvant injection and scored on a scale from 0 to 4 based on four criteria, each worth one point. In an attempt to closely reflect the cardinal signs of inflammation, the four criteria consisted of (i) heat, joint temperature >32.0°C; (ii) edema, paw volume >2.70 ml; (iii) hyperalgesia, withdrawal latency <5.0 secs; and (iv) standing and walking limb function, paw lifting, limping. Criteria were assessed according to procedures outlined below, using protocols that have been previously validated (38, 44–47). Rats exhibiting one or more of the four above criteria for clinical scores were considered to have a positive clinical score. Incidence of arthritic disease was calculated and expressed as the percentage of rats in each treatment group with a positive clinical score over time.

Joint Temperature. Rats were lightly anesthetized with 5% halothane, and a blunt needle thermocouple digital thermometer (Doric Scientific, San Diego, CA) was applied topically and nestled onto the right ankle joint of each rat. Joint temperatures were recorded on Day 17 after injection. Ambient temperature was maintained at 22°C.

Paw Volume. Paw volume was measured using a fluid displacement plethysmometer (Kent Scientific, Torrington, CT) as previously described (38, 39). The right hind paw of each rat was immersed in the plethysmometer, and the paw volume was recorded on Day 17 after injection.

Hyperalgesia. Hyperalgesia in response to a radiant heat stimulus in each rat's paw was assessed using the plantar test apparatus (Harvard Apparatus, Holliston, MA) as previously described (46, 47). A light source was placed beneath the plantar surface of the right hind paw of each rat and turned on. As the intensity of the light increased over time, the rat would withdraw its paw away from the source of light. The withdrawal latency time of the rat in response to the thermal stimulus (i.e., the time between when the light was turned on and the time when the rat withdrew its paw) was automatically recorded. Withdrawal latency times were recorded on Day 17 after injection.

Cytokine (IL-1β) and Protease (MMP-9) Levels. On Day 17 after injection, right ankle joints were collected (adjacent to both sides of the joint capsule), skinned, stripped of the soft tissue, snap frozen in liquid nitrogen, and stored at −80°C. Frozen joints were milled with bone cutters, homogenized in 2 ml of PBS (in an attempt to maximize cytokine and MMP recovery), and centrifuged at 800 *g* for 20 mins. The supernatants (excluding the top fat layer) were collected and frozen at −80°C for measurements of IL-1β and MMP-9 levels. Levels of IL-1β were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) per manufacturer's instructions. This assay is specific for both recombinant and natural rat IL-1β and has a minimum detectable dose of less than 5 pg/ml IL-1β. Levels of MMP-9 activity were measured using a commercially available ELISA kit that recognizes both proenzyme and active forms of MMP-9 (Amersham Biosciences, Baie d'Urfe, Canada), per manufacturer's instructions. This assay cross-reacts with rabbit and mouse MMP-9 but does not cross-react with other MMPs. The effective range of this assay was 0.5–16 ng/ml MMP-9. IL-1β and MMP-9 were detected against purified standards of the mediators and expressed as level (picogram or nanogram, respectively) per gram of joint tissue. In addition, systemic IL-1β levels were measured from serum samples collected *via* cardiac puncture on Day 17 and are expressed as picograms per milliliter of serum.

Histology. Left ankle joints of each rat were fixed in 10% formalin (VWR, Edmonton, Canada) for 14 days, decalcified with CalEx decalcification solution (Fisher Scientific, Nepean, Canada) for 35 days, and processed in paraffin (50:50 Paraplast Plus and Paraplast Xtraplus) (VWR). Sections (7 µm) were stained with hematoxylin (Fisher Scientific), saffranin-O (Fisher Scientific), and fast green (Fisher Scientific). Average thickness of the cartilage, including the tangential, transitional, radial, and calcified cartilage layers, was calculated from three different measurements in the apical condyle of the tibial bone and three different measurements in the apical condyle of the tarsal bone in the tibiotarsal joint, using Openlab 3.0.3

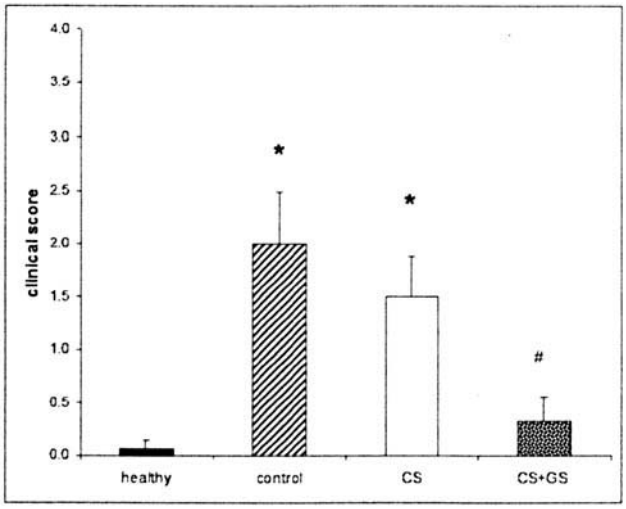

Figure 1. Clinical scores (based on joint temperature, edema, hyperalgesia, and standing and walking limb function) of rats 17 days after sham or Freund's complete adjuvant injection. $n = 12$ per group. Rats either were placebo-treated healthy rats or were given bars containing CS, CS plus GS, or no active compound (control) before injection. Values are mean plus or minus SEM. * $P < 0.05$ versus healthy, # $P < 0.05$ versus control.

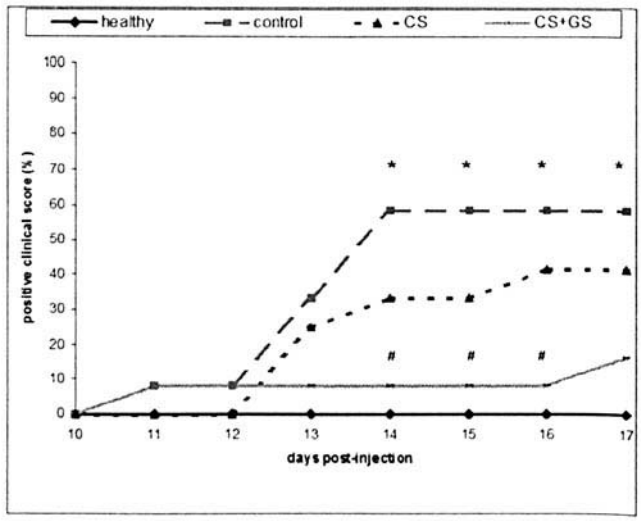

Figure 2. Incidence of arthritic disease (percentage of rats with one or more clinical score criterion [joint temperature edema, hyperalgesia, standing and walking limb function] on each day) in rats that either were placebo-treated healthy rats or were given bars containing CS, CS plus GS, or no active compound (control) before injection. $n = 12$ for each data point. Values are mean plus or minus SEM. * $P < 0.05$ versus healthy, # $P < 0.05$ versus control.

software (Improvision Ltd., Lexington, MA) at 100× magnification.

Statistical Analyses. Results were expressed as means plus or minus standard error of mean (SEM). Statistical significance was evaluated using one-way analysis of variance (ANOVA) for parametric data or the Kruskal-Wallis nonparametric test, followed by Tukey's multiple comparison analysis. All calculations were performed using SigmaStat statistical software (SPSS Inc., Chicago, IL). Values of $P < 0.05$ were considered statistically significant.

Results

Clinical Score. Arthritic control rats had a significantly higher clinical score compared with healthy rats (Fig. 1). Arthritic rats treated with CS alone also showed a significantly ($P < 0.05$) higher clinical score than healthy rats. The clinical score of arthritic rats treated with CS plus GS was not different from that of healthy rats and showed a lower clinical score than the arthritic control group ($P < 0.05$).

Incidence of Arthritic Disease. Signs of disease were first observed 11 days after injection (Fig. 2). Rats injected with Freund's complete adjuvant and treated with placebo bar (control) had a significantly ($P < 0.05$) higher incidence of arthritic disease than healthy rats 14–17 days after injection (Fig. 2). Rats treated with CS plus GS, but not those given CS alone, had a significantly ($P < 0.05$) lower incidence of disease than arthritic control rats at days 14–16. Disease incidence in both the arthritic CS-treated group and the arthritic CS plus GS–treated group was not significantly different from that of the healthy rats at any time point during the study (Fig. 2).

Joint Temperature. Both arthritic control rats and arthritic rats treated with CS had significantly ($P < 0.05$) higher joint temperatures than healthy rats (Fig. 3). Rats treated with CS plus GS had joint temperatures that were significantly ($P < 0.05$) lower than both the arthritic control group and the group treated with CS alone and were not different from healthy rats (Fig. 3).

Paw Volume. Arthritic control rats had significantly ($P < 0.05$) higher paw volume than healthy controls (Fig. 4). In contrast, paw volume of rats treated with CS or CS plus GS was not different from that of healthy rats, although

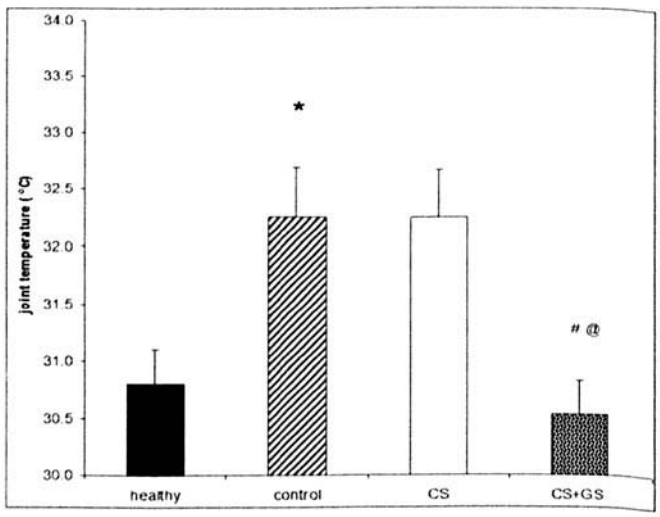

Figure 3. Joint temperatures (17 days after injection) in rats that either were placebo-treated healthy rats or were given bars containing CS, CS plus GS, or no active compound (control) before injection. $n = 12$ per group. Values are mean plus or minus SEM. * $P < 0.05$ versus healthy, # $P < 0.05$ versus control, @ $P < 0.05$ versus CS bar.

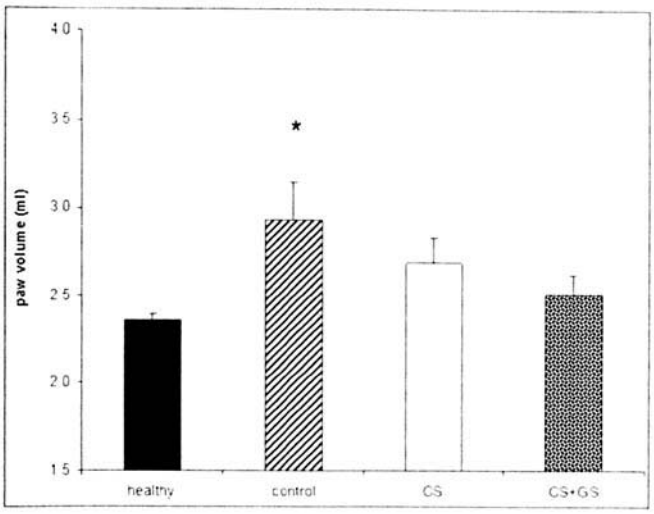

Figure 4. Paw volumes (17 days after injection) of rats that either were placebo-treated healthy rats or were given bars containing CS, CS plus GS, or no active compound (control) before injection. $n = 12$ per group. Values are mean plus or minus SEM. * $P < 0.05$ versus healthy.

the values were not statistically lower than those measured in arthritic rats either.

Hyperalgesia. Healthy rats had a withdrawal latency time of 9.04 ± 1.14 secs (mean plus or minus SE), while groups injected with Freund's complete adjuvant (control, CS, and CS plus GS groups) had withdrawal latency times of 5.98 ± 1.14 secs, 7.27 ± 0.66 secs, and 6.66 ± 0.40 secs, respectively (data not shown). There were no statistically significant differences among groups.

Joint IL-1β. Levels of IL-1β in the joints of arthritic control rats and rats treated with CS (1013.37 ± 280.46 pg/g and 431.67 ± 117.11 pg/g, respectively) were significantly ($P < 0.05$) higher than those of healthy rats (78.16 ± 18.51 pg/g) (Fig. 5a).

Bars containing combined CS and GS inhibited the increase of IL-1β in the joints (Fig. 5a).

Serum IL-1β. Rats in the arthritic control group and in the group treated with CS showed significantly ($P < 0.05$) higher serum IL-1β levels (0.437 ± 0.271 pg/ml and 0.543 ± 0.162 pg/ml, respectively) compared with healthy rats (0.000 ± 0.055 pg/ml) (Fig. 5b). In contrast, treatment with CS plus GS inhibited the increase of systemic IL-1β. Serum IL-1β levels in rats given CS plus GS (0.000 ± 0.170 pg/ml) were significantly ($P < 0.05$) lower than in rats treated with CS alone (Fig. 5b).

Joint MMP-9. Levels of MMP-9 in the joints of arthritic control rats (1.09 ± 0.14 ng/g) were significantly ($P < 0.05$) higher than those in healthy rats (0.72 ± 0.09 ng/ml) (Fig. 5c). Joint MMP-9 concentrations were not elevated in rats given CS (0.75 ± 0.12 ng/g) or CS plus GS (0.80 ± 0.08 ng/g) and were similar to MMP-9 levels in healthy rats (Fig. 5c).

Histology. Representative light micrographs of joint surfaces and cartilage thickness measurements of rats in

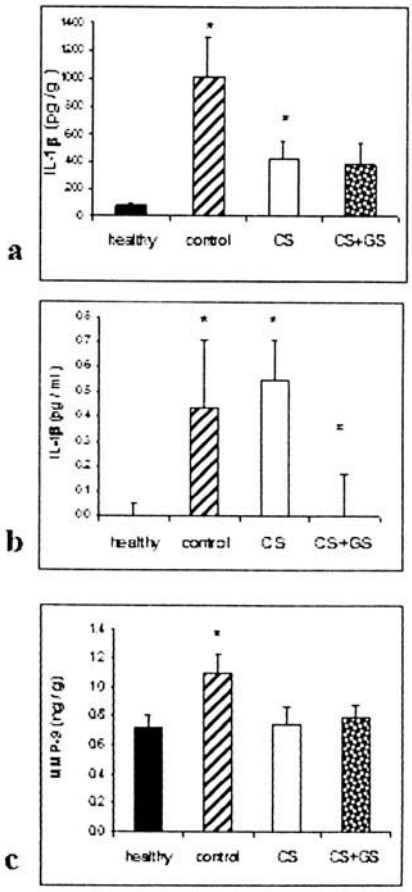

Figure 5. (a) Joint and (b) serum levels of IL-1β, and (c) joint MMP-9 concentrations in rats 17 days after injection. Rats either were placebo-treated healthy rats or were given bars containing CS, CS plus GS, or no active compound (control) before injection. $n = 12$ per group. Values are mean plus or minus SEM. * $P < 0.05$ versus healthy, # $P < 0.05$ versus CS bar.

each group are shown in Figure 6. Cartilage lining the joint surfaces of healthy rats was smooth and continuous (Fig. 6a). In contrast, the joint cartilage in Freund's complete adjuvant–injected control rats was pitted and rough (Fig. 6b). The joint cartilage in rats injected with Freund's complete adjuvant and treated with either CS or CS plus GS was smooth, similar to that seen in healthy rats (Fig. 6c, d). Arthritic control rats had significantly ($P < 0.05$) lower cartilage thickness than healthy rats. Rats treated with CS alone and rats treated with CS plus GS had cartilage thickness that was not different from that of healthy rats. Cartilage thickness in rats treated with CS plus GS was significantly ($P < 0.05$) higher than that in the arthritic control group (Fig. 6).

Discussion

The present experiments assessed the effects of CS alone or a CS plus GS combination delivered in dietary bars on parameters of arthritic disease. Overall, the data from this study showed that ingestion of this dietary formulation containing either CS alone or CS plus GS ameliorated disease in an experimental model of rheumatoid arthritis and

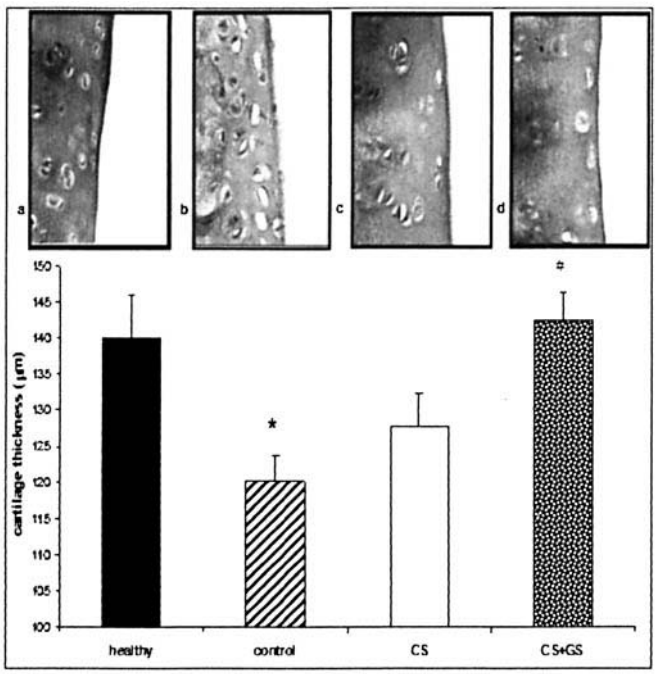

Figure 6. Representative histology of left rat ankle joints 17 days after injection showing a portion of the cartilage, with embedded chondrocytes in the tibiotarsal joint, and corresponding cartilage thickness measurements of each group. Micrographs illustrate cartilage from (a) sham-injected, placebo-treated (healthy) rats, (b) Freund's complete adjuvant–injected, placebo-treated (control) rats, (c) Freund's complete adjuvant–injected, CS bar–treated rats, and (d) Freund's complete adjuvant–injected, CS plus GS bar–treated rats. (b) Freund's complete adjuvant–induced arthritis is associated with degradation and roughing/pitting of the cartilage surface in the joints. Ingestion of (c) CS or (d) CS plus GS in bars maintained joint cartilage integrity. $n = 12$ per group. Values are mean plus or minus SEM. * $P < 0.05$ versus healthy, # $P < 0.05$ versus control. Sections are stained with hematoxylin, saffranin-O, and fast green, and are shown at 400× original magnification.

that the combination of CS and GS in bars is more efficacious than CS alone in improving several of the disease markers examined. This observation is consistent with a previous report suggesting that CS and GS in combination reduce the progression of cartilage degradation in a model of osteoarthrosis in rabbits, and that CS and glucosamine HCl may act synergistically to stimulate glycosaminoglycan synthesis (31). Previous reports indicate that IL-1β and active MMP-9 are increased during joint diseases, including in human rheumatoid arthritis (3–11, 48, 49). The pathogenic significance of MMP-9 is further supported by a recent study that showed that this protease is elevated both in latent and in active human rheumatoid arthritis (50). Observations from the present report now suggest that combined CS and GS in dietary bars ameliorates clinical scores, disease incidence, joint temperature and swelling, and cartilage damage, as well as IL-1β and MMP-9 synthesis associated with experimental rheumatoid arthritis. Another major finding is that reduction of IL-1β levels occurred not only in the joints, but also in the serum of arthritic rats fed bars with CS plus GS. Consistent with the present data, previous reports support direct chondroprotective and systemic effects for CS and GS. In human articular chondrocytes cultured from femoral heads, CS stimulated proteoglycan synthesis and decreased total prostaglandin E_2 (PGE_2) synthesis (24). In addition, glucosamine HCl downregulated neutrophil chemotaxis, inhibited the release of lysozyme from neutrophils (51), and decreased inducible nitric oxide synthase protein expression by macrophages in a dose-dependent manner (52). Moreover, in a rabbit model of chymopapain-induced joint injury in rabbits, uninjected contralateral joints of rabbits given oral glucosamine HCl had higher levels of sulfated GAG content than the contralateral joints of rabbits treated with a diet containing no glucosamine (23). Future studies are needed to identify the mechanisms responsible for the alteration of IL-1β and MMP-9 production in the joints, as well as the cause of the systemic effects on IL-1β discovered here. In this study, as well as in previous reports using rat models, the amount of CS and GS administered to rats in the feed exceeds the doses recommended for humans. This discrepancy reflects the significantly higher metabolic rate of rats. Future research investigating different dosages in larger experimental pools is now warranted to determine whether the apparent beneficial effects of CS plus GS delivered in dietary bars from this study may be further enhanced.

Results from the present study show that joint inflammation coincided with cartilage damage in the ankle joints. Treatment with dietary CS alone and treatment with CS plus GS attenuated cartilage damage in arthritic rats. Past studies have demonstrated that CS and/or glucosamine may reverse arthritis-induced proteoglycan loss *in vitro* and *in vivo* (23, 53). IL-1β promotes adhesion molecule production, prostaglandin synthesis, synthesis of MMPs (including MMP-9) that catalyze collagen, proteoglycan degradation, and bone resorption in rheumatoid arthritis and osteoarthritis (4–7, 54). Studies examining the effects of CS and glucosamine on IL-1β–conditioned articular cartilage have shown that these agents can alter components of the inflammatory cascade, including decreasing the production of PGE_2 (4, 24). Moreover, PGE_2 has the demonstrated ability to inhibit the production of proteoglycans by bovine articular chondrocytes (55). Therefore, by reducing PGE_2 production, CS and glucosamine indirectly inhibit cartilage injury. CS and glucosamine also reversed direct harmful effects of IL-1β on cartilage *in vitro* by reversing the increase in MMP expression and activity, the increase in glucuronosyltransferase I mRNA expression, and the decrease in proteoglycan synthesis induced by IL-1β (4, 24, 56). Results from recent studies *in vitro* also suggest that in combination, CS and GS reduce the proteolytic activity of MMP-9 (26). Findings from the present report demonstrate that *in vivo*, these agents may reduce IL-1β and MMP-9 levels associated with arthritis, which in turn may provide direct, as well as indirect, chondroprotective effects on the joint. These observations will be extended into future studies investigating the prophylactic versus therapeutic benefits of a dietary bar containing CS and GS.

In summary, results from the present study indicate that treatment with combined CS plus GS in a dietary bar, more so than CS alone, ameliorates parameters of rheumatoid arthritis. The mechanisms of action of both CS and GS in this novel bar formulation involve the modulation of IL-1β and MMP-9 synthesis. Other mechanisms of action, perhaps involving other MMPs and/or other cytokines implicated in the pathogenesis of arthritic disease (i.e., MMP 1, 2, 3, 8, 12, 13, IL-6, IL-8) (7, 12, 54, 57), remain important topics for future investigations. Comparative experiments of various dosages of the CS-GS combination, as well as experiments on the benefits of glucosamine alone in this bar, also constitute logical extensions from this report. Together, these studies will help develop innovative treatment strategies to control arthritic disease in humans and in companion animals.

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