Advantageous Use of Glucosamine Combined with S-Adenosylmethionine in Veterinary Medicine: *Preservation of Articular Cartilage in Joint Disorders*

Louis Lippiello, PhD
Alex Prudhomme, BS

Nutramax Laboratories, Inc.
Edgewood, Maryland

KEY WORDS: S-Adenosylmethionine, cartilage, metabolism, glucosamine hydrochloride, arthritis.

ABSTRACT

Glucosamine (GLN) hydrochloride and S-adenosylmethionine (SAMe) are therapeutic agents used to reduce symptoms associated with osteoarthritic disorders. Less is known about their direct effects on articular cartilage. Chondrocytes isolated from mature Holstein cows were cultured in alginate beads, as monolayers or as explants, and examined for evidence of synergistic activity in metabolic responses that would indicate a beneficial effect in preserving the integrity of articular cartilage. SAMe alone stimulated uptake of radiolabeled sulfate into glycosaminoglycan (GAG) at 0.052 μg/mL (+15%), increasing to 50% at 10.0 μg/mL, the highest dose tested. No response was seen with GLN at 5 μg/mL. A combination of SAMe and GLN at doses of 0.052 μg/mL–0.52 μg/mL and 5 μg/mL, respectively, resulted in a 15%–20% greater increase in activity compared with the additive value of each agent tested separately. A similar synergistic effect was noted when testing for anticatabolic activity. GLN and SAMe were also tested under two conditions of stress: a simulation of early osteoarthritis by partial proteoglycan depletion and chondrocytes cultured from an osteoarthritic joint. In each case, the combination of SAMe and GLN had a significantly greater stimulatory effect on matrix neosynthesis compared with each agent used separately. The data suggest that a combination of SAMe and GLN at physiologically obtainable levels may be a more effective chondroprotective agent than either agent used separately, and thereby provide for an enhanced therapeutic potential.

INTRODUCTION

Glucosamine (GLN) is considered to be a "chondroprotective" systemic disease-modifying osteoarthritic drug (SYS/DMOAD) favorably modulating the metabolic activity of chondrocytes. Multiple clinical trials confirm its efficacy for symptomatic relief of osteoarthritis in humans.[1–3] In vitro and animal studies also suggest it has cartilage-preserving effects by virtue of stimulating synthetic activity and inhibiting catabolic enzymes.[4–6] S-adenosylmethionine (SAMe) is a popular agent used in veterinary medicine for liver disorders and has an effect on

multiple cellular processes.[7] The therapeutic application of SAMe in joint disorders is also well known, with extensive clinical trials suggesting it is as effective as nonsteroidal anti-inflammatory drugs (NSAIDs) in reducing pain and improving joint function.[8–11] In this application, the therapeutic efficacy of SAMe is based on potent antioxidant and anti-inflammatory activity.[12] Unlike glucosamine, very little is known about the direct effect of SAMe on articular cartilage. SAMe exhibits anti-inflammatory activity in tumor necrosis factor-stimulated synovial tissue, suggesting a protective action in joint synovitis.[13] Its participation in synthesis of phosphoadenosyl phosphosulfate, a sulfation factor for synthesis of proteoglycans,[14] and as an antioxidant, could be of considerable benefit in preserving joint cartilage under conditions of excessive stress or trauma.

Based on these observations, we examined whether a combination of SAMe and glucosamine would provide a more effective therapeutic modality for the treatment of joint disorders including osteoarthritis. Our concern was to define whether the combination had a direct effect on chondrocyte metabolism in a way that would aid in preserving the integrity of articular cartilage. To this end we conducted in vitro studies examing synthesis and catabolism of cartilage matrix macromolecules. In addition, we examined their effects on an in vitro simulation of joint stress by partial enzyme-induced matrix depletion as well as response of articular cartilage from an osteoarthritic joint.[15] We particularly tested each agent at physiologically obtainable oral doses to explore any synergism in promoting cartilage matrix synthesis and inhibiting those catabolic processes leading to cartilage degradation.

MATERIALS AND METHODS

Synthetic Studies in Cell Culture

Articular cartilage was resected from the radial carpal joints of retired aged Holsteins and chondrocytes isolated by overnight digestion with collagenase.[16] Cells were cultured in alginate beads, a more natural in vitro environment that maintains chondrocyte phenotype.[17] Each bead containing 40,000 cells was equilibrated for 10 days in Dulbecco modified Eagle medium (DMEM)/F-12 plus 10% fetal calf serum (FCS) and antibiotics. Serum factors were minimized by reequilibration of the beads for 24 hours in DMEM/F-12 plus 0.5% FCS prior to testing. Our initial study tested for a direct effect of SAMe at levels of 0.005 μg/mL, 0.05 μg/mL, 0.50 μg/mL, 1.0 μg/mL, 3.0 μg/mL, 5.0 μg/mL, and 10.0 μg/mL using the alginate bead system and 35-sulfate uptake as an index of glycosaminoglycan (GAG) synthesis. Based on these data, studies exploring synergistic effects were completed using suboptimal levels of each agent; 5 μg/mL GLN (FCHG49, Nutramax Laboratories, Inc.) and SAMe (a stabilized salt containing not less than 70% of the S,S biologically active isomer) at 0.0052 μg/mL, 0.052 μg/mL, and 0.52 μg/mL or combinations of each. Cells were exposed for 24 hours to agents in the presence of 5 μCi/mL 35-sulfate to label neosynthesis of GAG. The beads in replicates of eight each were rinsed thoroughly with ice-cold DMEM/F-12, dissolved in 1 N sodium hydroxide, and uptake of isotope assayed in a Wallac microplate scintillation counter (Perkin Elmer Life Sciences).

Anticatabolic Studies

To test whether GLN or SAMe alone or in combination prevented cytokine-induced breakdown of proteoglycans, chondrocytes were embedded in alginate beads at a concentration of 60,000 cells/bead. After 10 days equilibration, the beads were exposed to 5 μCi/mL 35-sulfate for 72 hours to label newly synthesized and deposited proteoglycans. The system was exposed to 25 mg/mL GLN, 0.052 μg/mL SAMe, or a combination of both in the presence of 10 ng/mL interleuken-1 (IL-1) plus 0.4 μM plasminogen, agents known to elicit metalloprotease activity in cartilage.[18] A fivefold higher dose of GLN combined with the middle dose of

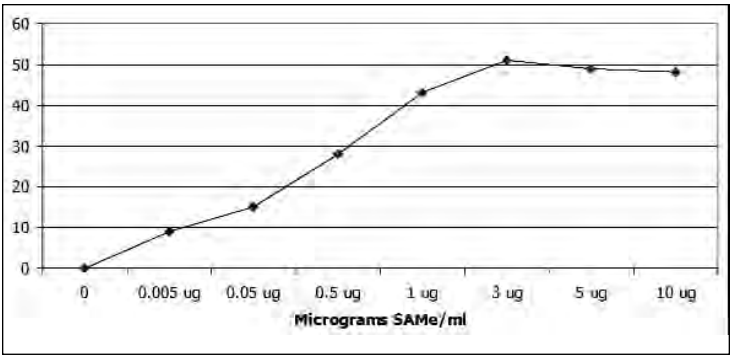

Figure 1. Direct effect of S-adenosylmethionine on glycosaminoglycan synthesis by bovine chondrocytes. Data presented as the mean percent change from control value of 35-sulfate uptake into trichloroacetic acid precipitable macromolecules.

SAMe was chosen for this portion of the study because previous observations indicate that factors causing breakdown of cartilage are less sensitive than those involved in synthetic activity. The cultures were maintained for 72 hours, and daily media changes were monitored for released of isotope. Radioactivity was determined by dissolving beads in 1 N sodium hydroxide and residual radioactivity determined. The data were expressed as percentage release of total incorporated radioactivity per 24-hour period. The data were compared with control cultures including controls with and without cytokine addition.

Stressed Cartilage Explant System

Adverse tissue culture conditions simulating the matrix-depleted state of cartilage in osteoarthritic joints[15] were generated by a short-term exposure to the cartilage-degrading enzyme stromelysin (MMP-3, metalloprotease 3), one of the predominant enzymes degrading cartilage in osteoarthritis.[19] Cartilage explants in replicates of 5 were cultured to a metabolic steady state in 6-well culture plates. Half the tissue was exposed to 1.47 mU/mL stromelysin (Calbiochem; specific activity, 1,473 mU/mg protein) for 3 hours. The tissues were thoroughly rinsed and fresh DMEM/F-12 medium-containing testing agent was added for 24 hours. Six hours prior to termination, 5 μCi/mL 35-sulfate were added to monitor synthetic activity. Cultures were terminated by multiple rinsing in phosphate-buffered saline (containing 1M sodium sulfate) to remove unincorporated radioisotope, dehydrated in 100% ethanol, lyophilized, and weighed to the nearest microgram. Tissue was dissolved in 200 μL 1 N sodium hydroxide and uptake of isotope assayed by liquid scintillation counting. Data were expressed as counts per minute (CPM) ± SEM/μg dry tissue weight.

A single study was conducted using chondrocytes isolated from a human osteoarthritic femoral head. Cells plated in a 96-well multiwell culture plate at a density of 100,000 cells/well were cultured to a metabolic steady state for 5 days in plain F-12 medium containing 10% fetal calf serum (FCS), antibiotics, and 50 μg/mL ascorbic acid. The media was changed to plain F-12 without serum containing 10 μg/mL Cosequin Double Strength (CDS) (Nutramax Laboratories Inc.) with or without 0.6 μg/mL SAMe and cultured for 24 hours. Six hours prior to termination, 5 μCi/mL 35-sulfate was added to the medium. The cell layer was analyzed for incorporated 35-sulfate after washing with cold F-12 medium to remove unincorporated isotope. Isotope uptake into macromolecules was assayed by dissolving the cell layer with 1 N sodium hydroxide and counting in a liquid scintillation counter. The data are expressed as the percentage change from the control value.

Statistical Analysis

The mean and standard error of the mean were calculated for each sample group. Means were compared using the Student *t*-test and ANOVA using multiple group comparisons and the Mann-Whitney rank sum test.

RESULTS

Exposure of bovine chondrocytes to SAMe alone demonstrates a dose-dependent stimulation of GAG synthesis that plateaus at 3 μg/mL (Figure 1). Under the conditions of the experiment, a maximum 50% increase in GAG synthesis was observed at 3 μg/mL\L–10 μg/mL\L. Twenty-four hour exposure of chondrocytes in alginate beads to low-dose GLN and varying low doses of SAMe also elicited a dose-dependent stimulation of GAG synthesis (Table 1). Each dose of SAMe significantly increased synthetic activity. A maximum effect of 29% was seen at 0.52 μg/mL SAMe, a fivefold increase over GLN and at one-tenth the dose. Maximum response was noted in the positive insulin-like growth factor-1 (IGF-1) control, the natural growth stimulator for chondrocytes. The combination of GLN and SAMe resulted in a synergistic effect (effect greater than an additive response) with a two-fold increase in synthetic activity (Table 2).

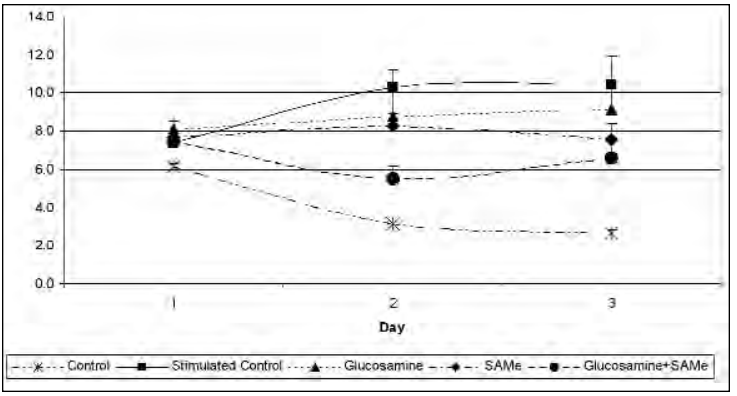

Figure 2. Anticatabolic effect of glucosamine (GLN) and S-adenosylmethionine (SAMe) measured by release of 35-sulfate from prelabeled chondrocytes in alginate beads. Data plotted as mean ± SEM. Agents were tested at 5 mg/mL and 0.05 mg/mL of GLN and SAMe, respectively.

An in vitro examination of the anticatabolic effect of GLN and SAMe is presented in Figure 2. Compared with unstimulated control cultures, addition of IL-1 plus plas-

Table 1. Uptake of 35-Sulfate by Chondrocytes Exposed to Glucosamine HCl and Varying Doses of S-Adenosylmethionine*

Treatment (n = 4)	CPM ± (SEM)†	% Change	Significance
Control	1185 (27)	–	–
GLN-5 μg/mL†	1306 (122)	+ 6%	NS†
SAMe 0.0052 μg/mL†	1295 (66)	+ 9%	NS
SAMe 0.052 μg/mL	1361 (55)	+ 15%	$P < 0.014$
SAMe 0.520 μg/mL	1530 (40)	+ 29%	$P < 0.001$
GLN-5 μg/mL + SAMe 0.0052 μg/mL	1490 (67)	+ 25%	$P < 0.002$
GLN-5 μg/mL + SAMe 0.0520 μg/mL	1705 (15)	+ 44%	$P < 0.002$
GLN-5 μg/mL + SAMe 0.520 μg/mL	1760 (36)	+ 50%	$P < 0.001$
IGF-1†	2100 (80)	+ 77 %	$P < 0.001$

*Significance determined by Student *t*-test and ANOVA.
†Indicates counts per minute ± SEM; GLN, glucosamine; SAMe, S-adenosylmethionine; IGF-1, insulin-like growth factor-1; NS, not significant.

Table 2. Synergistic Activity of Glucosamine and S-Adenosylmethionine on Uptake of 35-Sulfate by Bovine Chondrocytes

Treatment	Additive Response	Synergistic Response	Difference
GLN-5* μg/mL + SAMe 0.0052μg/mL	15%	25%	+ 10%
GLN-5 μg/mL + SAMe 0.052 μg/mL	21%	44%	+ 23%
GLN-5 μg/mL + SAMe 0.52 μg/mL	35%	50%	+ 15%

*Indicates glucosamine; SAMe, S-adenosylmethionine.

Table 3. Effect of Glucosamine and S-adenosylmethionine on Stromelysin-Stressed Cartilage Explants

Treatment (n = 5)	CPM (SEM)*	% Change	Significance
Control	3680 (300)	–	
GLN-5 μg/mL[†]	3550 (400)	- 4%	NS
SAMe 0.052 μg/mL[†]	3520 (220)	- 4%	NS
GLN + SAMe	4810 (450)	+ 31%	$P < 0.05$

Data given as mean counts per minute (CPM) ± (SEM).
[†]Indicates glucosamine; SAMe, S-adenosylmethionine; NS, not significant.

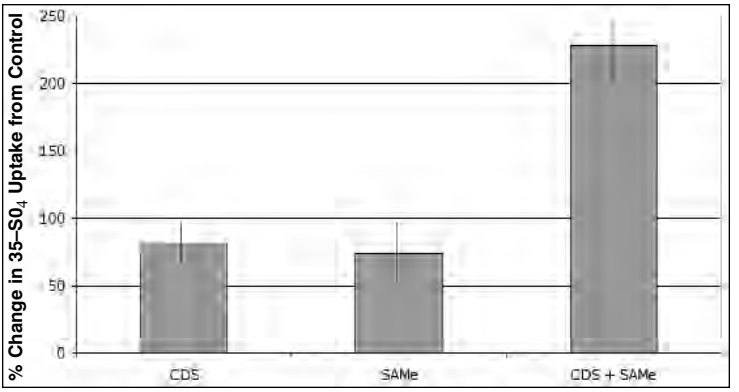

Figure 3. Cartilage synthetic activity in human osteoarthritic chondrocytes exposed to Cosequin DS (CDS) in the presence and absence of S-adenosylmethionine (SAMe). Data represent mean (SEM) of the percent change in counts per minute/well of 8 replicates compared with controls. A 10-mg/mL CDS dose contained 5 mg/mL glucosamine + 4 mg/mL chondroitin sulfate + 8 ng/mL manganese ascorbate. SAMe dose = 0.6 mg/mL.

minogen stimulated release of prelabeled proteoglycans by 21%, 220%, and 304% after 24, 48, and 72 hours, respectively. Neither SAMe, GLN, or a combination of the two prevented release after 24 hours. At 48 hours only the combination of agents significantly reduced release by 47%. However, by 72 hours, all three treatments significantly reduced catabolic activity in the following order: SAMe + GLN > SAMe > GLN.

Previous studies indicate that the synthetic processes of articular chondrocytes is markedly inhibited during recovery from a transient in vitro stress elicited by partial matrix digestion with stromelysin.[15] In the presence of 5 μg/mL GLN or 0.052 μg/mL SAMe, no change from this response was noted. However, a combination of these agents at the stated doses resulted in a stimulation (+31%) of synthetic activity to levels approaching the controls that were not exposed to enzyme (Table 3). In the second "stress" test using chondrocytes from an osteoarthritic joint that are considered to be in a stressed state, the synthetic activity of chondrocytes were markedly stimulated when exposed to a very low dose (10 μg/mL) of Cosequin DS (+70%) or 0.6 μg/mL SAMe (+50%). However, the combination of CDS and SAMe produced a substantially greater increase (+228%) in 35-sulfate uptake than the additive value of each agent alone (Figure 3).

DISCUSSION

Low doses of GLN and SAMe were tested for "chondroprotective" activity monitored as an increase in GAG and protein synthesis and inhibition of IL-1-induced matrix breakdown. A synergistic phenomenon was observed in GAG synthesis. A synergistic phenomenon was also observed in inhibition of IL-1-induced matrix breakdown and in the synthetic activity of chondrocytes under stress. A surprising finding was the potency of SAMe. A significant upregulation of GAG synthesis at 52 ng/mL was seen, becoming more prominent with increasing doses up to 10 μg/mL (the highest dose tested). Based on pharmacokinetic studies in canines, a single 280-mg dose of SAMe (20 mg/kg) gives peak serum levels of 12

μg/mL, and in felines a dose of 53 mg/kg resulted in peak levels of 2.5 μg/mL.[20,21] It would be reasonable to state that synovial fluid levels of both components is probably lower that that observed circulating in plasma but still may be in the range that effectively exerts a "chondroprotective" effect on cartilage integrity. Taken together, this information is suggestive that the combination of agents may indeed be considered as a SYS/DMOAD.

There are very few studies examining the direct effect of SAMe on articular chondrocytes. In one study, assays of fibronectin and levels of keratin sulfate following treatment with SAMe, diclofenac sodium, and GAG polysulfate suggests that SAMe decreases in a dose-dependent manner the synthesis of these matrix molecules, and at high concentrations cell viability is impaired.[22] In contrast, Harmand et al[23] looked at the effect of SAMe on human chondrocytes and found effective stimulation of protein and glycosaminoglycan synthesis at a dose of 10 μg/mL. The authors also suggest that SAMe has a direct effect on chondrocytes.

MMP-3 is one of the major proteoglycan-degrading enzymes in cartilage.[24] In a previously published study, we showed that transient exposure of cartilage explants to MMP-3 has a marked inhibitory effect on proteoglycan synthesis, which is very slowly reversible when the enzyme is removed.[15] In the current study, this enzyme inhibition is reversed when the tissues are treated with a combination of GLN and SAMe but not with low doses of either agent used alone. The net effect of such activity is the possible prevention of cartilage breakdown when the joint is exposed to proteolytic enzymes.

CONCLUSION

These observations suggest that an effective SYS/DMOAD veterinary therapy for joint disorders, including osteoarthritis, should retard progression of joint degeneration. The in vitro demonstration that a combination of SAMe and GLN has significantly more biological activity in stimulating GAG synthesis and in inhibiting enzyme-induced matrix breakdown, supports its role as a more efficacious chondroprotective therapy than either agent used alone.

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