

Original Article

Collagen Synthesis in Tenocytes, Ligament Cells and Chondrocytes Exposed to a Combination of Glucosamine HCl and Chondroitin Sulfate

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Clinical testing of the nutraceuticals glucosamine (glcN) and chondroitin sulfate (CS) has shown efficacy in providing relief from symptoms in osteoarthritic patients. *In vitro* and *in vivo* studies support existence of a synergistic relationship upregulating synthetic activity in chondrocytes. A combination of glcN and CS may also be useful as adjunct therapy in sports-related injuries if similar upregulation of collagen synthesis is elicited in accessory ligament and tendon joint tissue. Collagen and non-collagenous protein (NCP) synthesis in cultures of bovine tenocytes, ligament cells and chondrocytes exposed to glcN + CS were assayed by uptake of radiolabeled proline into collagenase-sensitive material. Assay of radiolabel in hydroxyproline (a specific marker for collagen synthesis) following HPLC isolation confirmed the specificity of the metabolic effect. Synthesis of total collagenase-sensitive material was maximally upregulated at physiologically obtainable doses of glcN + CS. Tissue response followed the sequence ligament cells (+69%) > chondrocytes (+56%) > tenocytes (+22%). Labeled hydroxyproline increased by 132% in ligament cells, 27% in tenocytes and 49% in epitendon cells after a 48 h exposure to 5 $\mu\text{g ml}^{-1}$ glcN + 4 $\mu\text{g ml}^{-1}$ CS. Low dose combinations of glcN and CS effectively stimulate *in vitro* collagen and NCP synthesis by ligament cells, tenocytes and chondrocytes. Hence, therapeutic use following accessory joint tissue trauma may help augment repair processes.

Keywords: arthritis – metabolism alternative therapy

Introduction

The combination of glucosamine (glcN) and chondroitin sulfate (CS) has been extensively tested for clinical efficacy of symptomatic relief in patients with osteoarthritic (OA) joints (1–4). Assessment of joint cartilage degeneration and anti-inflammatory effects has also been examined in diverse animal models of arthritis (5–8). In addition, *in vitro* and *in vivo* studies support existence of a synergistic relationship of these two agents associated with upregulation of matrix proteoglycan synthesis and downregulation of metalloprotease activity (1,9–11) suggesting a ‘chondroprotective’ effect. For the most part, these studies have only examined responses of articular chondrocytes but conceptually OA is considered a

disorder of the entire articulating joint including the ligament and tendon accessory joint structures (12).

Ligaments and tendons are dense fibrous connective tissues providing mechanical stability to joints during movement. The cellular fibroblastic-like cells are surrounded by an organized fibrous extracellular matrix composed primarily of type I collagen, elastin, non-collagenous proteins (NCP), and small amounts of keratan and CS. Aging-related alterations or trauma to tendons and ligaments play a role in altering joint dynamics and predispose the joint to early onset of osteoarthritis (13,14). Tendon/ligament failure by traumatic rupture, overuse and/or inflammatory processes is ranked as the 15th most common musculoskeletal condition and 30–50% of all sports injuries (15). Moreover, the annual incidence of acute rupture of the anterior cruciate ligament has been estimated to be one in 3000 in the American population, with ~95 000 new cases per year (16).

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Current therapies for the treatment of ligament/tendon injuries emphasize non-steroidal anti-inflammatory agents (NSAIDs) to minimize inflammation and subsequent damage to tissue integrity. However, caution has been recommended against excessive use of some NSAIDs since these agents have an inhibitory effect on proteoglycan synthesis and cell proliferation (17) and in animal models do not provide any biochemical benefit (18). A number of growth promoting factors including platelet-derived growth factor, transforming growth factor beta and basic fibroblast growth factor have demonstrated significant stimulation of matrix synthesis *in vitro* but have not proved successful *in vivo* (19). There is currently no efficacious therapy for enhancing the rate and/or ability of these tissues to heal (17). Nutraceutical supplements including creatine, ephedra, etc., have been associated with side effects and lack rigorous quality assurance to warrant their use (20).

Clinical trials using nutraceutical preparations for healing and minimizing inflammatory processes in dense connective tissues have not been performed. The significant advantage of such therapy for sports-related injuries is the possibility of enhancing natural repair processes and/or minimizing NSAIDs use.

The rationale for exploring whether the combination of glcN + CS has a beneficial effect on collagen synthesis in ligaments and tendons is based on previous studies suggesting that they act as biological response modifiers upregulating metabolic activity of chondrocytes (10). Since the cells of ligament/tendon tissue have a similar origin as articular chondrocytes, exhibit similar aging-related changes in metabolism and mechanical properties (21), are less responsive to repair stimuli (22) and are capable of maintaining normal remodeling processes (23), it was of interest to examine whether they respond in similar fashion as articular chondrocytes. Moreover, the majority of published studies with these agents have been on articular cartilage examining proteoglycan synthesis and degradation and anti-inflammatory activity. Little is known of their effect on collagen synthesis, a major component of cartilage as well as dense connective tissue. To this end, we took advantage of the availability of a commercial preparation, Cosamin®DS (CDS; Nutramax Laboratories Inc., Edgewood, MD), a mixture of glucosamine HCl (FCHG49®, 99% purity), CS (TRH122®, 98% purity) and manganese ascorbate in the ratio 5:4:1 for which numerous clinical and *in vitro* data are available. The material was used as a combination rather than testing of individual components since previous data indicates that both agents exert an upregulation of synthetic activity of chondrocytes, but the combination of agents has greater efficacy clinically (1) as well as acting synergistically on articular cartilage *in vitro* (8).

Materials and Methods

Articular cartilage was obtained from the articulating surfaces of metacarpal joints of 3- to 5-year-old Holstein cows. Ligament tissue was resected from between the 3rd and 4th metacarpal bones and a large segment of the extensor tendon

was excised from an area adjacent to the metacarpal joint. All three minced tissues were digested with Type I (tendon and ligament) or Type II (cartilage) bacterial collagenase (Sigma/Aldrich Chemical Co., St Louis, MO) at 220 units ml^{-1} . The cell population was expanded by culturing in 75 cm^2 flasks containing DMEM/F-12 + 10% fetal calf serum, 50 $\mu\text{g ml}^{-1}$ ascorbic acid 2-sulfate and antibiotics. In some studies, the epitenon was dissected from the tendon and cultured separately. After a single passage, sufficient cells were obtained for seeding into multiplate wells. All cultures were brought to a metabolic steady-state by culturing for an additional 5 days in DMEM/F-12 + 10% FCS. Twenty-four hours prior to testing, cell cultures were acclimated to DMEM/F-12 with 1% fetal calf serum, 50 $\mu\text{g ml}^{-1}$ ascorbic acid and 5 mM glucose. All subsequent studies were done with media containing physiological levels of glucose (5 mM) and varying doses of CDS. Two methods were utilized to monitor neosynthesis of collagen and NCP.

Method 1: Incorporation of Tritiated Proline (pro) into Collagenase-Sensitive Material

Cells cultured in 24-well plates at a high density of 200 000 cells per well were exposed at 37°C for 24 h to CDS at doses of 1, 10, 50 and 100 $\mu\text{g ml}^{-1}$ and 5 $\mu\text{Ci ml}^{-1}$ ^3H -proline (specific activity 97 Ci mM^{-1}). There were eight replicates/treatments in a total media volume of 0.5 ml. IGF-1 at 50 ng ml^{-1} was used as a positive control. Cultures were terminated by freeze-thawing and sonication to rupture cells. Soluble collagen and NCP synthesis were assayed following the addition of 50% trichloroacetic acid (TCA) to precipitate all proteins contained in the combined cell layer and media (final TCA concentration 5%). The plates were centrifuged at 3000 r.p.m. in microplate carriers for 15 min and the supernatant removed. TCA (5% containing 1 mM proline) precipitation was repeated until the supernate was free of unincorporated radiolabel. Residual TCA was removed by a final rinse of ethanol:ethyl ether (1:1) and the culture plates air dried.

Assay of collagenase-sensitive material was done according to the method of Diegelmann *et al.* (24). Briefly, collagen in the TCA precipitated air-dried plates was digested by adding an incubation cocktail containing 25 μg purified collagenase (Worthington Biochemical Corp., Lakewood, NJ) in 200 μl of 0.05 M Tris (pH 7.6) containing 0.005 M CaCl_2 . The plates were incubated for 3 h at 37°C. The supernatant was removed after centrifugation and the collagenase digestion repeated a second time. Radioactivity in the pooled supernates (total collagen fraction) was counted in multiplate wells after addition of 300 μl of scintillant (Hewlett Packard) to 100 μl of sample. The residue (NCP) was dissolved by heating at 50°C in 1 N NaOH for 15 min and similar aliquots were counted as described above. The data are expressed as CPM \pm SEM associated with collagenase-sensitive material and NCP. Unless otherwise stated, all tendon cultures consisted of a mixture of three cell types: sheath fibroblasts, epitenon and endotendon tenocytes.

Method 2: Specific Activity of Hydroxyproline (Hyp)

In repeat experiments, cells seeded at a density of 500 000 per well in 12-well multiplates were treated for 48 h with 10 $\mu\text{g ml}^{-1}$ CDS. The supernate from the collagenase digestion (total collagen fraction) was made up to 6 N HCl with concentrated acid and hydrolyzed at 120°C under vacuum for 24 h. After evaporation of the HCl, the amino acid residue was dansylated by adding 100 μl of 500 mM NaHCO_3 and 100 μl of 20 mM Dns-Cl in acetone to 100 μl of hydrolyzed sample. Samples were reacted in the dark for 40 min at 65°C. High-pressure liquid chromatography (HPLC) separation of the dansylated imino acids was accomplished on a Ultrasphere ODS C-18 (250 mm \times 4.6 mm) column using a stepwise gradient of 25 mM NaH_2PO_4 + 25 mM acetic acid/acetonitrile (86:14) (Solvent A) and 100% acetonitrile (Solvent B) (25). Peaks corresponding to authentic hyp and pro were collected and assayed for incorporated radioactivity and quantitated by comparison with known standards. The data were expressed as specific activity (cpm hyp or pro μg^{-1} hyp or pro).

Statistical Analysis

The cell culture data were expressed as the mean CPM \pm SEM. Experiments were done with cells from different animals to insure the validity of the results. The percent change from control cultures was also calculated and the means compared using ANOVA and Student's *t*-test for multiple group comparisons. An unpaired two-tailed Student's *t*-test was used to test the percentage differences for statistical significance. Significance was accepted at $P < 0.05$.

Results

Characterization of Cell Cultures

Phase microscopy of tendon cell cultures revealed a mixture of cell types derived from tendon tissue. Morphologically, epitendon (sheath) cells appear as large oval fibroblasts while

tenocytes are small spindle-shaped fibroblasts. Ligament and chondrocyte cultures were homogeneous in cell type.

Upregulation of Collagen Synthesis

An inverse dose-response in uptake of tritiated proline into collagenase-sensitive material was observed in all three cell types exposed to varying dosages of CDS (Table 1). In each cell type, maximum uptake into collagen occurred at 1–10 $\mu\text{g ml}^{-1}$, the lowest doses tested. At doses higher than 50 $\mu\text{g ml}^{-1}$, no effect or slight inhibition of collagen synthesis was noted (Table 1). The sensitivity of response of each cell type with regard to collagen synthesis was ligament cells (+ 69%) \geq chondrocytes (+ 56%) $>$ tenocytes (+ 22%).

Confirmation of CDS-induced stimulation of collagen synthesis was observed by assessment of the specific activity of hyp and pro. Expressed as a percentage change from control cultures, the specific activity of hyp in ligament cells was significantly greater than that in tenocytes or epitendon cells (132% versus 27% and 49%) (chondrocytes not analyzed) (Table 2).

Calculation of Collagen to NCP Ratio

In Method 2, calculation of NCP synthesis was based on the assumption that the ratio of pro to hyp in type I collagen was similar to that in type II collagen and is equal to 1.42. It also assumes that the specific activity of the two imino acids is identical since hyp derives from pro in post-translational reactions. Hence, the synthesis of NCP was calculated as:

$$\text{Labeled hyp} \times 1.42 = \text{labeled pro in collagen}$$

$$\text{Total labeled pro} - \text{labeled pro in collagen} = \text{labeled pro in NCP}$$

Based on this information, the ratio of collagen to NCP synthesis in the three cell types was calculated by measurement of the amount of labeled proline in collagen and NCP. The data presented in Table 3 indicate that

Table 1. Collagen synthesis by chondrocytes, ligament cells and tenocytes exposed to varying doses of CDS

| | Control | IGF (50 ng ml^{-1}) | CDS (1 $\mu\text{g ml}^{-1}$) | CDS (10 $\mu\text{g ml}^{-1}$) | CDS (50 $\mu\text{g ml}^{-1}$) | CDS (100 $\mu\text{g ml}^{-1}$) |
|----------------|----------|-------------------------------|--------------------------------|---------------------------------|---------------------------------|----------------------------------|
| Chondrocytes | 380 (60) | 600 (96) [†] | 592 (112) [†] | 560 (60) [†] | 580 (112) | 352 (58) |
| Ligament Cells | 520 (32) | 944 (132) [†] | 880 (120) [†] | 920 (148) [†] | 656 (120) | 576 (56) |
| Tenocytes | 316 (43) | 460 (35) [†] | 385 (35) [†] | 419 (30) [†] | 364 (45) | 345 (43) |

Data presented as Mean (\pm SEM) ($n = 8$) CPM tritiated proline uptake into collagenase-sensitive material (collagen).

[†]Denotes statistical significance at $P < 0.05$ to 0.001.

Table 2. Comparative analysis of hydroxyproline and proline-specific activity in collagen of connective tissue cells exposed to CDS and IGF-1

| Assay | Ligament | | | Tendon | | | Epitendon | | |
|--------|----------|-----------------------|-----------------------|----------|-----------------------|------------------------|-----------|------------------------|------------------------|
| | Control | CDS | IGF | Control | CDS | IGF | Control | CDS | IGF |
| SA Hyp | 68 (13) | 158 [†] (35) | 195 [†] (38) | 250 (23) | 318 [†] (25) | 626 [†] (120) | 378 (45) | 563 [†] (120) | 740 [†] (160) |
| SA Pro | 120 (26) | 196 [†] (25) | 348 [†] (69) | 230 (35) | 326 [†] (45) | 555 [†] (110) | 180 (24) | 167 (28) | 300 [†] (55) |

Data given as Mean (\pm SEM) specific activity (cpm hyp μg^{-1} hyp and cpm pro μg^{-1} pro) in cultures exposed to agents for 48 h.

[†]Indicates values statistically significant from control value at $P < 0.05$.

Table 3. Ratio of labeled proline in collagen to labeled proline in NCP

| | Chondrocytes | Tenocytes | Ligament cells |
|---------|--------------------------|--------------------------|----------------|
| Control | 1.09 (0.12) | 0.72 (0.11) | 0.90 (0.08) |
| CDS | 0.80 (0.04) | 1.06 (0.10) | 0.97 (0.25) |
| IGF-1 | 3.15 (0.66) [†] | 2.15 (0.55) [†] | 0.83 (0.20) |

Data expressed as ratio of CPM (\pm SEM) of labeled proline in collagen versus labeled proline in NCP.

[†]Indicates statistically significant at $P < 0.05$.

IGF-1 has a greater stimulatory effect on collagen synthesis versus NCP synthesis in both chondrocytes and tenocytes but not on ligament cells. In contrast, exposure to CDS did not significantly alter the collagen to NCP ratio from control values in any cell type.

Discussion

The combination of glcN and CS effectively stimulates neosynthesis of collagen in cell cultures of ligament, tendon and cartilage tissue. Based on the ratio 5:4:1 of the commercial product Cosamin[®]DS, a 10 $\mu\text{g ml}^{-1}$ dose exposed cells to 5 $\mu\text{g ml}^{-1}$ glucosamine ($\sim 23 \mu\text{M}$), 4 $\mu\text{g ml}^{-1}$ CS ($\sim 0.25 \mu\text{M}$) and 1 $\mu\text{g ml}^{-1}$ Mn ascorbate. In comparison to *in vivo* levels, CS at 0.25 μM is probably at the low range of that obtainable with repeated dosing (26). The doses of glcN are within the range of serum levels according to the latest published data [23 μM versus 10–60 μM (27,28)]. The effects noted *in vitro* can be attributed to glucosamine and CS since our media contained 50 $\mu\text{g ml}^{-1}$ ascorbate, negating any effect by the small amount of ascorbate present in the formula.

We observed these results under *in vitro* conditions in media containing glucose at levels existing in serum and at cell densities known to maintain cell phenotype (29). Standard DMEM/F-12 culture media, optimized for cell growth, contains 17 mM of glucose. Such high levels of sugar may be found in serum of diabetics while normal serum levels of glucose are closer to 5 mM. Previous studies using fibroblastic-like cells indicate that high glucose levels ($>5 \text{ mM}$) reduce collagen type I synthesis (30,31). In a preliminary study using chondrocytes, we observed up to a 28% reduction in radiolabeled proline uptake into collagenase-sensitive material at 17 mM glucose compared to 5 mM glucose (data not presented). The addition of 1% fetal calf serum was considered to be the minimal amount necessary for maintenance and adherence of the cells in culture.

Epitenon cells are considered to be the collagen-producing cells involved in healing of tendons (21), but *in vivo* ligaments are considered to be functionally and metabolically the more active tissue compared to tendons or chondrocytes (32). Our data confirms these findings insofar as ligament cells incorporated almost twice the radioactivity into collagenase-sensitive material than tenocytes or chondrocytes (Table 1). This was also seen in calculation of the specific activity of hyp (+132% in ligament versus +27% in tenocytes). Interestingly,

when comparing the ratio of radiolabeled proline in collagen versus radiolabeled proline in NCP, a 3-fold increase was seen with IGF-1 in chondrocytes and tenocytes but not ligament cells. Moreover, the ratio in cells exposure to CDS did not significantly differ from controls. This suggests that the response of chondrocytes and tenocytes to CDS may differ mechanistically from that of IGF-1.

Few publications have been devoted to the effect of glcN or CS on collagen synthesis. Bassleer *et al.* (33) found that CS had no effect on human cartilage collagen synthesis and Anderson *et al.* (34) failed to detect an effect of glucosamine on canine chondrocyte collagen synthesis. However, the data of Jimenez *et al.* (35) and O'Grady *et al.* (36) indicate increased gene expression for collagen synthesis with both agents as well as with a combination of agents. The disparity in results may be a reflection of the doses used, the culture system, i.e. cells clusters versus explants or may indicate that the increase in mRNA levels is not reflected in the actual assayable final product.

The inhibitory effect of high levels of CS on glycosaminoglycan synthesis by chondrocytes has been observed by others (9,37). Although our preparation consisted of a mixture of glcN and CS, the inhibition of collagen synthesis observed at doses higher than 50 $\mu\text{g ml}^{-1}$ (20 $\mu\text{g ml}^{-1}$ CS) suggest that the effect is due to the CS component. An explanation for the inverse dose–response of connective tissue cells to CS was not investigated in this study nor has any explanation been forthcoming by other authors (9,37). It may be a reasonable conjecture that the response resembles a typical non-monotonic dose–response (i.e. a non-linear curve where the slope of the dose–response reverses sign somewhere along the curve).

There are several complementary and alternative therapies related to connective tissue metabolism and repair. For example, a recent review by Ahmed *et al.* (38) describes the use of botanicals in osteoarthritis. Bromelain (39) and bee venom acupuncture (40) have also been proposed for a similar application. The addition of glucosamine and CS to the list of alternative therapies provides an additional tool to alternative non-invasive treatment.

The clinical significance of these data relates to documentation that patients with meniscus and ligament injuries of the knee have a high incidence of developing degenerative radiological changes within a 10–20 year period (14). Since collagen is the major component of these tissues, our hypothesis is that upregulation of its synthesis by a combination of glcN and CS may accelerate tissue repair and diminish the probability of OA development. However, it is too premature to extrapolate our *in vitro* data to *in vivo* circumstances by stating that these agents accelerate ligament and tendon healing. However, under conditions of trauma/stress where collagen degradation is occurring the combination of glcN and CS may circumvent and/or accelerate repair processes (Fig. 1). It should also be noted that these results pertain to *in vitro* direct effects of the agents on connective tissue cell metabolism. Clinical efficacy *in vivo* relates to symptomatic

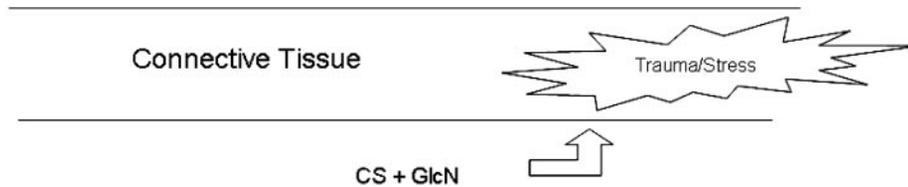


Figure 1. CS + GlcN represents increased collagen synthesis and Trauma/Stress represents collagen synthesis.

relief by virtue of their anti-inflammatory action and therefore does not necessarily equate to the observed metabolic responses.

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