ABSTRACT

Studies were conducted to test the hypothesis that exposure of articular cartilage to a combination of the “chondroprotective” agents glucosamine hydrochloride, low-molecular-weight chondroitin sulfate, and manganese ascorbate (Cosequin® DS [CDS], Nutramax Laboratories, Inc.) prevents the potentially adverse effects of NSAIDs on cartilage. Articular cartilage proteoglycan synthesis and degradation were used to monitor in vitro cartilage activity following timed exposure to levels of NSAIDs routinely applied in veterinary medicine. Etodolac exposure for 144 hours induced significant inhibition of cartilage matrix synthesis and increased the rate of matrix breakdown. Addition of CDS reversed both activities. Cartilage metabolism was not affected by exposure to indomethacin and remained responsive to CDS with an increase in synthetic activity. Aspirin significantly stimulated chondrocyte synthetic activity in the presence and absence of CDS. Carprofen at therapeutic levels had a mild (15%) stimulatory effect on cartilage metabolism and reduced matrix breakdown. Addition of CDS significantly accelerated (30%) matrix synthesis. At higher levels carprofen was toxic, reducing cell activity by 80%. The data suggest that CDS may be a useful adjunct therapy for preservation of articular cartilage by reversing the adverse metabolic effects of etodolac and providing accelerated cartilage synthetic activity in the presence of therapeutic levels of carprofen.

INTRODUCTION

Nonsteroidal antiinflammatory agents have been proven effective in the treatment of inflammation and painful symptoms associated with both rheumatoid arthritis and osteoarthritis. However, there is evidence that chronic use of many NSAIDs may adversely alter cartilage metabolism. Administration of some NSAIDs may result in arthropathy progression in osteoarthritis because of inhibitory effects on synthesis of cartilage proteoglycans. Additionally, there is evidence that NSAIDs affect cytokine production, collagen metabolism, and
superoxide radical production, all processes known to be involved to some extent in joint
degeneration.⁵,⁶ Although there is disagreement
regarding the magnitude of effects of individ-
ual NSAIDs, several categories of antiinflam-
matory agents have been recognized based on
their effect on articular cartilage. These agents
include compounds such as aceclofenac,
tenidap, and tolmetin, which stimulate matrix
synthesis; piroxicam, tiaprofenic acid, and as-
pirin, which have little or no effect; naproxen
and ibuprofen, which significantly inhibit ma-
trix synthesis; and indomethacin, for which ef-
fects are dose dependent.⁷

A new type of treatment for osteoarthritis,
which uses symptomatic slow-acting or chon-
droprotective agents, has been investigated.
Evidence suggests that these agents provide
symptomatic relief with no apparent adverse
physiologic effects. The chondroprotective
compounds chondroitin sulfate and glu-
cosamine hydrochloride (HCl) have been ex-
tensively studied in the laboratory as well as in
clinical trials.⁸⁹ Cosequin® DS (CDS; Nutra-
max Laboratories, Inc., Edgewood, MD) is a
patented chondroprotective compound consist-
ing of 500 mg of FCHG49™ (a highly purified
glucosamine HCl), 400 mg of TRH122™ (a
low-molecular-weight sodium chondroitin sul-
fate), and 38 mg of manganese ascorbate. This
commercial product has been shown to in-
crease proteoglycan synthesis in vitro and to re-
tard the progression of cartilage lesions in an
animal model of osteoarthritis.¹⁰ Veterinary tri-
als using a canine model of inflammatory
arthritis demonstrated substantial efficacy in
reducing scintigraphic evidence of joint in-
flammation.¹¹ In a study of surgical reconstruc-
tion of canine cruciate ligament, oral CDS
stimulated cartilage metabolism as measured
by an increase in chondroitin sulfate epitopes
3B3 and 7D4.¹² Availability of therapeutic lev-
els was assessed in a canine study and con-

firmed by detection of cartilage-stimulatory
agents in the sera of animals receiving oral dos-
es of the product.¹³ Therefore, it was of interest
to examine whether CDS could modulate (re-
verse) any adverse metabolic responses in
bovine articular cartilage exposed to NSAIDs.

MATERIALS AND METHODS

Tissue Cultures

Articular cartilage segments measuring 5
mm² × 0.5 mm thick were resected from the
metacarpophalangeal joints of adult Holstein
cows, approximately 7 to 10 years of age.
Bovine cartilage was used for several reasons: It
is the most easily accessible cartilage from aged
animals and its metabolic behavior in vitro has
been studied extensively. More importantly,
because cartilage is avascular and generally iso-
lated from the body, there are very few species-
dependent variations in responsiveness to ex-
ogenous stimulants. To attain a metabolic
steady state, tissues were cultured for 5 days as
explants in Dulbecco’s modified eagle medium
(DMEM)/F-12 containing antibiotics, 50
µg/ml ascorbic acid sulfate, and 10% fetal calf
serum.¹⁴ For initial proteoglycan synthesis
studies using CDS alone, six to eight replicate
cartilage segments were distributed into indi-
vidual wells of 24-well culture plates containing
1 ml of DMEM/F-12, 0.5% fetal calf serum,
and varying dosages of CDS (25, 100, and 400
µg/ml). Five µCi/ml 35-sulfate was added to
pulse-label proteoglycans during the final 5 to 8
hours of culture. Cultures were maintained at
37°C in a 5% CO₂ atmosphere for 24 and 144
hours; media were changed daily.

Exposure to NSAIDs in the presence and ab-
sence of CDS was accomplished by similar dis-
tribution of explants into six-well culture
plates. Media were changed daily in the ex-
tended-time (144 hours) study and 5 µCi/ml
35-sulfate added in the final 5 to 8 hours of in-
cubation. Proteoglycan synthesis was moni-
tored by termination of the cultures with multiple cold washes with Hanks balanced salt solution (HBSS) followed by fixation in 100% ethanol at 4°C for 24 hours. Tissues were lyophilized and weighed to the nearest microgram. After dissolving the tissue with 100 µl of 1N NaOH, incorporation of radiolabeled material was monitored by addition of 200 µl scintillant and counting in a microplate scintillation counter. The data were expressed as mean counts per minute (CPM)/mg dry tissue weight, and the percent change from untreated (control) cells was calculated.

Degradative studies were conducted in a similar manner using cartilage explants with tissue proteoglycans prelabeled with 35-sulfate for 24 hours in DMEM/F-12 plus 10% fetal calf serum. In these studies, degradation was monitored by assay of released radioactivity after 24 hours of exposure to NSAID in DMEM/F12 plus 0.5% fetal calf serum. Data were expressed as a percent of total CPM released into the medium.

Preparation of Antiinflammatory Agents

A sampling of commercially available NSAIDs, including etodolac (EtoGesic®, Fort Dodge Animal Health, Kansas City, MO), a partially cyclooxygenase-2 (COX-2)–selective agent; carprofen, (Rimadyl®, Pfizer Inc., Groton, CT), a general COX inhibitor; indomethacin (Sigma Chemical Co.), primarily an indole COX-1 inhibitor; and acetylsalicylic acid (Sigma Chemical Co.), a salicylate prostaglandin inhibitor, were obtained for the study. Organic solvent–soluble NSAIDs were prepared as ethanolic extracts of powdered material at concentrations that allowed for the addition of less than 0.02% ethanol to the cultures. A similar amount of ethanol was added to all controls. The levels of individual NSAIDs tested were derived from published literature and mimic ranges obtainable in serum or synovial fluid. Etodolac was tested at 11 µg/ml, indomethacin at 10 µg/ml, aspirin at 200 µg/ml, and carprofen at 10 and 50 µg/ml.

Statistical Analysis

Results of treatments were compared using Student’s t-test and/or the Kruskal-Wallis non-parametric one-way analysis of variance on ranked values. The number of replicates required to detect a significant difference at \( P < .05 \) with a power of 0.80 and \( \alpha \) of 0.05 was determined using commercial statistical software.

**RESULTS**

The earliest response of normal bovine cartilage explants to CDS alone was observed at 24 hours (Table 1). Significant increases in 35-sulfate incorporation (proteoglycan synthesis) were noted, with the greatest effect observed at the lower

<table>
<thead>
<tr>
<th>Exposure Time (hr)</th>
<th>Percent 35-Sulfate Incorporation at Dosages of CDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>5</td>
<td>2%</td>
</tr>
<tr>
<td>24</td>
<td>57%</td>
</tr>
<tr>
<td>144</td>
<td>65%</td>
</tr>
</tbody>
</table>

**TABLE 1. Effect of Dosages of a Chondroprotective Agent (CDS; Glucosamine Hydrochloride/Chondroitin Sulfate/Manganese Ascorbate) on 35-Sulfate Incorporation by Normal Aged Bovine Cartilage Explants**
dosages and of CDS tested (25 and 100 µg/ml). This pattern of metabolic stimulation was consistent up to 144 hours of exposure (note that the 400-µg/ml level was not tested at the longer time period).

The level of CDS used in subsequent testing was adjusted to 100 µg/ml based on data presented in Figure 1. This level of CDS appeared to be equally or more effective than 400 µg/ml in stimulating proteoglycan synthesis. Moreover, other studies have indicated that upregulation of cartilage matrix synthesis was higher at lower doses in vitro. Cartilage explants exposed to CDS at 100 µg/ml had 42% and 38% accelerated synthetic activity after 24 and 144 hours’ exposure, respectively (Table 2). Indomethacin alone was without effect, but the addition of CDS to indomethacin cultures resulted in elevation of synthetic activity to 38% at 24 hours and 24% at 144 hours. At 24 hours, both etodolac and aspirin had little effect. At this interval, addition of CDS elevated activity in the etodolac cultures but not in the aspirin cultures. After 144 hours’ exposure, aspirin stimulated synthetic activity by 68%, an effect not significantly altered by the addition of CDS. However, 22% inhibition (P < .05) was observed with 6 days of exposure to etodolac, and this reversed to 51% stimulation by addition of CDS. Carprofen at therapeutic doses generally increased cartilage synthetic activity by 15% from 24 hours to 144 hours of exposure, but this was not significant. The level of stimulation varied slightly with different bovine specimens. Addition of CDS to carprofen resulted in a significant 30% increase (P < .009) in the level of synthesis. Carprofen at 50 µg/ml almost completely shut down synthetic activity (−78%) after 24 hours’ exposure, and this did not change appreciably by adding CDS (−71%). The toxic effect of the high dose of carprofen precluded further testing of this material.

Degradative Activity

Analysis of cartilage degradative activity after a 24-hour exposure to NSAIDs and CDS is presented in Table 3. Indomethacin did not induce any degradative activity, whereas etodolac

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Figure 1. Metabolic activity of varying dosages of a commercial preparation of glucosamine hydrochloride/chondroitin sulfate/manganese ascorbate (CDS) over three time intervals.
significantly accelerated matrix breakdown (36%). The chondroprotective agent significantly reversed the etodolac-induced increase in degradation to a level 25% less than control tissue. Carprofen at 10 and 50 µg/ml significantly inhibited matrix breakdown; however, live cells are required to proteolyze matrix proteoglycans and the experimental system did not allow for characterizing whether the observed inhibition was a result of true enzyme inhibition or carprofen-induced cell death.

**DISCUSSION**

Articular cartilage explant cultures have been used extensively to assess the metabolic response of exogenous agents. The primary advantage of the system is that chondrocytes em-bedded in a matrix retain their phenotypic mode of expression and maintain a steady-state metabolism for proteoglycans and collagen for 4 to 5 weeks when maintained in serum containing growth medium. The system allows for investigation of metabolic responses in cells under simulated disease-related situations. The culture system used here is considered an “un-stimulated” system compared with a “stimulated” system in which a cytokine such as interleukin-1 (IL-1) is added.

Unlike cartilage in vivo, culture systems are static systems without physiologic parameters such as mechanical pressure that may affect exposure and responses to nutrients. Therefore, a higher level of test article may be required to elicit a cartilage response than that required in

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**TABLE 2. Synthetic Activity Assayed by 35-Sulfate Incorporation in Bovine Cartilage Explants Exposed to NSAIDs and a Chondroprotective Agent (CDS; Glucosamine Hydrochloride/Chondroitin Sulfate/Manganese Ascorbate)**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Time (hr)</th>
<th>Before CDS</th>
<th>After CDS</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etodolac</td>
<td>24</td>
<td>4%</td>
<td>21%</td>
<td>&lt;.04</td>
</tr>
<tr>
<td></td>
<td>144</td>
<td>–22%†</td>
<td>51%</td>
<td>&lt;.004</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>24</td>
<td>3%</td>
<td>38%</td>
<td>&lt;.005</td>
</tr>
<tr>
<td></td>
<td>144</td>
<td>9%</td>
<td>24%</td>
<td>NS</td>
</tr>
<tr>
<td>Aspirin</td>
<td>24</td>
<td>8%</td>
<td>16%</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>144</td>
<td>68%†</td>
<td>42%</td>
<td>NS</td>
</tr>
<tr>
<td>Carprofen</td>
<td>24</td>
<td>11%</td>
<td>4%</td>
<td>NS</td>
</tr>
<tr>
<td>(10 µg/ml)</td>
<td>144</td>
<td>15%</td>
<td>30%</td>
<td>&lt;.009</td>
</tr>
<tr>
<td>Carprofen</td>
<td>24</td>
<td>–78%†</td>
<td>–71%</td>
<td>NS</td>
</tr>
<tr>
<td>(50 µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDS</td>
<td>24</td>
<td>ND</td>
<td>42%</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>144</td>
<td>ND</td>
<td>38%</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

*Significance of difference between before and after CDS.
†NSAID alone differed significantly from control (P < .05).
NS = not significant.
It is also possible that serum levels of chondroitin sulfate and glucosamine will not reflect actual bioactivity because they have a tropism for such glycosaminoglycan-rich tissues as cartilage.20,21 However, doses used in vitro can be compared with actual concentrations obtained in vivo. Assuming 10% bioavailability of active ingredients, the recommended dose of CDS (1-g capsule) for a 10-kg dog (with an estimated blood volume of 750 ml)22 would give a maximum fluid level of 53 µg chondroitin sulfate/ml and 67 µg of glucosamine/ml.23 In the present study, CDS powder contained 500 mg glucosamine and 400 mg chondroitin sulfate/g; therefore, administration of 100 µg CDS/ml provided 50 µg glucosamine/ml and 40 µg chondroitin sulfate/ml. These doses are in the range of levels that may be obtained in synovial fluid with long-term oral administration of CDS.

The stimulatory effect of CDS on cartilage explants suggests that the highly anionic cartilage matrix may only slightly impede diffusional transport of glucosamine and chondroitin sulfate. The recent work by O’Grady and coworkers,19 also with aged bovine explant cultures, showed significant upregulation (stimulation) of proteoglycan and collagen synthesis. A second observation is that explants showed activity at the therapeutic levels tested (25 µg/ml).

Although indomethacin is generally described as having inhibitory effects on cartilage metabolism, stimulatory results have been obtained depending on culture system, time of exposure, and dose.24 Therefore, the slight stimulatory effect of indomethacin is not an unusual finding. The inhibitory effect of etodolac on cartilage synthetic activity has been reported by others using rabbit chondrocytes.15 A lack of effect was observed with high doses on human chondrocytes.25 Cell cultural differences and/or species specificity may be responsible for this disparity of findings. Aspirin has been shown to both stimulate and inhibit synthetic activity in cartilage depending on dose, culture system, and the state of the tissue.26 In the present study, aspirin administration elicited a mild positive response after 24 hours of exposure, which increased to 68% by

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TABLE 3. Effect of the Chondroprotective Agent (CDS; Glucosamine Hydrochloride/Chondroitin Sulfate/Manganese Ascorbate) on NSAID-Induced Degradative Activity of Cartilage Explants

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mean Percent 35-Sulfate Released During 24-Hour Incubation Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before CDS</td>
</tr>
<tr>
<td>Control</td>
<td>6.07% (0.35)</td>
</tr>
<tr>
<td>Etodolac</td>
<td>8.27% (0.22)*</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>6.40% (0.37)</td>
</tr>
<tr>
<td>Carprofen (10 µg/ml)</td>
<td>4.18% (0.45)</td>
</tr>
<tr>
<td>Carprofen (50 µg/ml)</td>
<td>3.80% (0.01)</td>
</tr>
</tbody>
</table>

*Significantly different from control (untreated) tissue (P < .05).
†Significantly different from NSAID without CDS (P < .05).

Data are expressed as mean ± SEM.
144 hours. Administration of the chondroprotective material had no effect on this response. In spite of the positive effect of aspirin on cartilage, its gastrointestinal side effects (e.g.: ulcers, bleeding) preclude its chronic use.

Carprofen is a popular veterinary product proven effective as an antiinflammatory agent. At doses of 1 to 10 µg/ml, Benton and coworkers observed 23% to 35% stimulation of synthetic activity after 24 hours' exposure using "normal-appearing" cartilage resected from canine osteoarthritic joints. Toxic effects were also seen with carprofen at dosages greater than 20 µg/ml. A second study by Armstrong and Lees found significant stimulation (approximately 53%) with carprofen at low doses and marked inhibition in explants at 50 µg/ml using normal equine cartilage from aged animals. The greater degree of synthetic activity in those studies compared with the present study may reflect their tissues not precultured to a metabolic steady state or the inappropriate use of "normal" tissues from an osteoarthritic joint for biochemical studies. A more accurate picture of tissue response to exogenous agents requires a steady-state metabolism, suggesting that the stimulatory effect of low-dose carprofen is generally mild.

In summary, CDS was shown to be effective in counteracting the potentially adverse metabolic effects of the NSAID etodolac and to enhance the mild stimulatory effect of carprofen. Further studies using animal models should be completed to confirm these in vitro results. Nevertheless, the data suggest that there is a firm rationale for incorporating CDS as adjunct therapy with some NSAIDs, in particular etodolac and perhaps carprofen.

REFERENCES


