In vitro effects of meloxicam on metabolism in articular chondrocytes from dogs with naturally occurring osteoarthritis

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Objective.—To assess effects of in vitro meloxicam exposure on metabolism in articular chondrocytes from dogs with naturally occurring osteoarthritis.

Sample.—Femoral head cartilage from 16 dogs undergoing total hip replacement.

Procedures.—Articular cartilage samples were obtained. Tissue sulfated glycosaminoglycan (SGAG), collagen, and DNA concentrations were measured. Collagen, SGAG, chondroitin sulfate 846, NO, prostaglandin E

Results.—Meloxicam had no effect on chondrocyte SGAG, collagen, or DNA concentrations. Expression of ADAMTS-5 was significantly decreased in all groups on all days, compared with the day 0 value. On day 3, culture medium PGE

Conclusions and Clinical Relevance.—Results suggested that in vitro meloxicam treatment of osteoarthritic canine cartilage for up to 30 days did not induce matrix degradation or stimulate MMP production. Meloxicam lowered PGE

Meloxicam, an NSAID, is frequently used to treat pain and discomfort in dogs with osteoarthritis. However, little is known about the effects of this class of drugs on either healthy or osteoarthritic canine cartilage. Data available on the effects of NSAIDs on human, rat, mouse, and rabbit cartilage suggest a variety of effects (often contradictory) on proteoglycan synthesis when chondrocytes or cartilage explants are incubated with different NSAID compounds in vitro. The most pronounced effects have been detected in chondrocytes from osteoarthritic joints. Aspirin is uniformly reported to cause inhibition of proteoglycan synthesis in joint tissues. Carprofen, diclofenac, ketoprofen, and piroxicam have been shown either to have no effect or to cause increased proteoglycan synthesis in these tissues. In 1 study, meloxicam strongly inhibited PGE production in vitro without causing observable deleterious effects on chondrocytes from osteoarthritic human cartilage. Further, meloxicam has been shown

ABBREVIATIONS

| ADAMTS | ADAM metallopeptidase with thrombospondin type 1 motif |
| COX    | Cyclooxygenase |
| CS-846 | Chondroitin sulfate 846 |
| DMEM   | Dulbecco modified Eagle medium with high glucose |
| IL     | Interleukin |
| INOS   | Inducible nitric oxide synthase |
| MMP    | Matrix metalloproteinase |
| PGE    | Prostaglandin E |
| SGAG   | Sulfated glycosaminoglycan |
| TIMP   | Tissue inhibitor of metalloproteinase |
to have a positive effect in vitro on the metabolism of proteoglycans and hyaluronan in osteoarthritic human cartilage.14 Because chondrocyte responses in cell culture can vary among species, extrapolation of human chondrocyte data to dogs may not be valid.15,16 In 2005, Giannoni et al.15 evaluated the use of cartilage from sheep and dogs as an in vitro model for the study of autologous chondrocyte implantation in humans. Results of that study indicate that species variability exists with respect to chondrocyte metabolism and in vitro chondrogenesis, and the authors cautioned against the use of animal cartilage as a model for human cartilage in studies. In experimental studies17–20 of canine osteoarthritis, in vivo inhibition of COX enzymes slowed the histologic, biochemical, and radiographic progression of osteoarthritis. However, the clinical relevance of these findings is unknown17–20 and many questions remain regarding the effect of NSAIDs on the progression of canine osteoarthritis and metabolism in articular chondrocytes in vivo.21 Additionally, to our knowledge, only 1 report21 has described the residual effects of NSAIDs on cartilage explants after cessation of NSAID treatment, and that study was performed with equine tissues. The results indicate that proteoglycan synthesis remained decreased, compared with baseline values, 14 days after NSAID treatment was discontinued.

The objective of the study reported here was to assess the effects of in vitro meloxicam exposure on metabolism in articular chondrocytes from dogs with naturally occurring osteoarthritis. We hypothesized that meloxicam treatment would not alter collagen synthesis in chondrocytes from osteoarthritic cartilage at 0.3 µg/mL (a concentration found in synovial fluid of dogs given a clinically therapeutic dose)12,22 but would significantly decrease collagen synthesis at higher concentrations (10 and 100 times this value). Additionally, we hypothesized that meloxicam treatment would not alter proteoglycan or collagen degradation in these cells at 0.3 µg/mL, but would significantly increase cartilage degradation at the higher concentrations.

Materials and Methods

Animals—Cartilage was collected from 18 client-owned dogs undergoing total hip replacement surgery. The study was approved by the Clinical Research Committee at the University of Georgia. The study was explained to the owner prior to enrollment of any dog, and informed consent was obtained from each owner prior to total hip replacement surgery and collection of articular cartilage.

Sexually intact or neutered dogs of either sex between 1 and 10 years of age with no history of current, active systemic disease were eligible to enter the study. Each dog included in the study had a complete physical examination performed by one of the investigators and was diagnosed as having lameness and pain attributable to chronic osteoarthritis of either coxofemoral joint with radiographic evidence of secondary osteoarthritic changes in affected joints. Each dog underwent routine hematologic and serum biochemical analysis and was required to have results within respective reference ranges (or if not within ranges, acceptable according to the clinical judgment of the investigator) and to be considered a suitable candidate for total hip replacement surgery.

Causes for exclusion from the study included failure to meet the described inclusion criteria, fractures, behavior, pregnancy or likelihood of pregnancy during the study period, and presence of complicating systemic disease conditions pertaining to the liver, kidney, or gastrointestinal tract or thought to increase the risk of surgical complications. Additionally, dogs were excluded if they were known to receive any of the following treatments prior to hip replacement surgery: intra-articular injection of any material into any joint within 30 days, prior surgery on any joint within 180 days, corticosteroid administration within 30 days, or NSAID administration within 14 days.

Tissue collection—Full-thickness pieces of articular cartilage were aseptically obtained from the affected femoral heads of dogs during total hip replacement surgery. For inclusion in the study, at least 12 pieces of cartilage (size, approx 5 × 5 × mm) were required to be collected from the femoral head of each dog following excision and placed in sterile DMEM. However, enough cartilage was obtained from each animal to create the 4-mm explants required for all the test groups and time points for this study, and therefore all animals were represented at all time points. Articular cartilage was collected by inserting a scalpel blade into the cartilage at the region just dorsal to the fovea capitis and sectioning toward the periphery. Care was taken not to include tissue near enthesophytic regions that had developed on the periphery near the joint capsule attachment.

At the time of surgery, 1 cartilage sample/dog was weighed to determine wet weight and then stored at −20°C to be used for baseline (day 0) extracellular matrix composition analysis. A second sample was stored in an RNA stabilization reagent at −20°C for later baseline (day 0) gene expression analysis. Full-thickness, 4-mm-diameter cartilage explants were created from remaining cartilage with a dermal biopsy punch and cultured for 3, 12, or 30 days.

Cartilage explant culture—Control medium was prepared by supplementation of DMEM with penicillin (1 U/mL), streptomycin (100 µg/mL), amphotericin B (2 µg/mL), i-glutamine (2 mM), ascorbic acid (50 µg/mL), modified Eagle medium nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), and 1X serum replacement solution containing insulin, transferrin, and selenous acid. Test medium was prepared by adding meloxicam at concentrations of 0.3, 3.0, or 30.0 µg/mL. These concentrations were selected on the basis of synovial concentrations of the drug detected in a pharmacokinetics study23 in dogs.

From each dog, two 4-mm-diameter explants for each culture group and time point (n = 24 total explants/dog) were cocultured in 1 mL of medium in a 24-well plate. The plates were incubated at 37°C with 5% CO₂ and 95% humidity. The medium was changed every 3 days, and explants and medium were collected on days 3, 12, and 30 for various analyses (with the first full day of culture considered day 1). One explant from each time point was weighed to determine wet weight and then stored at −20°C for tissue matrix analysis; the
other was stored in RNA stabilization reagent at 20°C for gene expression analysis. Collected medium was stored at −20°C until analyzed.

**Analysis of tissue matrix**—After all tissues were collected, 1 cartilage tissue sample obtained at the time of surgery and cartilage explants (1/treatment group/time point) from each dog were lyophilized and re-weighed to determine dry weight and water content. After lyophilization, tissue samples were digested overnight at 65°C in 500 µL of papain digestion buffer (20 mM sodium phosphate buffer, 1 mM EDTA, 300 µg of papain/mL [14 U of papain/mg], and 2 mM dithiothreitol) as previously described. Results were standardized to the dry weight of the cartilage explants from the same time point and reported as units per nanogram of DNA.

**Gene expression in cartilage**—One cartilage tissue sample collected at the time of surgery and cartilage explants (1/treatment group/time point) from each dog were evaluated for gene expression of matrix molecules (aggrecan and collagen II), proteinases (MMP-2, MMP-3, MMP-9, MMP-13, ADAMTS-4 [also called aggrecanase-1], and ADAMTS-5 [also called aggrecanase-2]), proteinase inhibitors (TIMP-1, TIMP-2, and TIMP-3), inflammatory indicators (IL-1β, tumor necrosis factor-α, COX-1, COX-2, and iNOS), and a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase) by means of real-time reverse transcriptase PCR assay with previously described species-specific primers. Total RNA was extracted from each cartilage explant with the TRIspin method, and RNA was converted to cDNA with the superscript III enzyme as previously described.

The real-time PCR assay was performed with a commercially available reagent mix and a thermal cycler. Each reaction mixture consisted of 4 µL of diluted cDNA, 0.3 µM forward and reverse primers, 0.3 µM of nucleic acid staining kit for double-stranded DNA, and a fluorescence-based microplate reader with a 96-well protocol as previously described.

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment group</th>
<th>Meloxicam (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaseLine*</td>
<td>Control 0.3 3.0 30.0</td>
<td></td>
</tr>
<tr>
<td>SGAG concentration (µg/mg)</td>
<td>168.35 ± 36.88</td>
<td>150.13 ± 52.76</td>
</tr>
<tr>
<td>Day 0 3 12 30</td>
<td>160.35 ± 48.49</td>
<td>142.79 ± 46.97</td>
</tr>
<tr>
<td>Hydroxyproline concentration (µg/mg)</td>
<td>4.89 ± 0.70</td>
<td>4.26 ± 1.54</td>
</tr>
<tr>
<td>Day 0 3 12 30</td>
<td>4.98 ± 2.80</td>
<td>4.00 ± 2.06</td>
</tr>
<tr>
<td>DNA concentration (µg/mg)</td>
<td>59.56 ± 23.04</td>
<td>62.55 ± 34.35</td>
</tr>
<tr>
<td>Day 0 3 12 30</td>
<td>63.39 ± 31.16</td>
<td>54.85 ± 35.50</td>
</tr>
</tbody>
</table>

**Variable** | **Baseline*** | **Control** | **0.3** | **3.0** | **30.0**
--- | --- | --- | --- | --- | ---
SGAG concentration (µg/mg) | 168.35 ± 36.88 | 150.13 ± 52.76 | 140.12 ± 46.16 | 160.24 ± 47.51 | 160.35 ± 48.49 | 142.79 ± 46.97 | 137.27 ± 38.24 | 138.17 ± 32.11 |
Hydroxyproline concentration (µg/mg) | 4.89 ± 0.70 | 4.26 ± 1.54 | 4.98 ± 2.80 | 4.87 ± 2.50 | 4.98 ± 2.80 | 4.00 ± 2.06 | 4.87 ± 2.50 | 5.05 ± 1.87 |
DNA concentration (µg/mg) | 59.56 ± 23.04 | 62.55 ± 34.35 | 68.26 ± 30.94 | 63.52 ± 24.40 | 63.39 ± 31.16 | 54.85 ± 35.50 | 67.22 ± 32.21 | 73.52 ± 35.21 |

Tissue samples were collected at the time of surgery (day 0). For each dog, a baseline sample was stored for later analysis, and remaining samples were cultured as explants (2 samples/well) in triplicate in a modified DMEM solution without (control treatment) or with meloxicam added at each of 3 concentrations. Assays for SGAG, hydroxyproline concentration, and total DNA content were performed as described elsewhere. Values obtained were standardized to the dry weight of the cartilage explant from the same time point. Values obtained were standardized to the cellular DNA content of the cartilage explant from the same time point and reported as units per nanogram of DNA.

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*One sample obtained on day 0 was used to determine the baseline value of the variable of interest for all sample treatment groups.  
--- = Not applicable.
(0.5 µL each), and 5 µL of 2X master mix resulting in a total volume of 10 µL. The PCR profile consisted of incubation for 5 minutes at 50°C and 2 minutes at 94°C, followed by 50 cycles of 5 seconds at 94°C (melting), 5 seconds at 60°C (annealing), and 10 seconds at 72°C (extension), with a melt curve analysis from 69°C to 95°C. Fluorescence was detected during the extension step of each cycle and during the melt curve analysis at 470 nm (excitation) and 510 nm (emission). Melt curve analysis was performed to ensure specific amplification. Take-off point (cycle threshold) determination and melt curve analysis were performed with manufacturer-provided thermal cycler software. Gene expression levels were determined by comparison with the housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis—For comparisons among the 4 treatment groups, each group included cartilage samples from the same dogs. Thus, a repeated-measures ANOVA model was used to test for differences in gene expression in tissue; SGAG, collagen, and DNA concentrations in tissue; and matrix molecules, proteinases, and inflammatory mediator concentrations in culture medium among different groups and days. The full model included factors for treatment, day, and a treat-
ment-by-day interaction term. A Tukey test was used to adjust for multiple comparisons. An unstructured covariance structure was used in all repeated-measures models. All hypothesis tests were 2 sided, and the significance level was set at an \( \alpha \) of 0.05. The repeated-measures analysis was performed with commercially available statistical software.\(^{1, 2}\)

**Results**

The mean age of the dogs was 3.2 years (range, 1 to 8.5 years), and mean body weight was 35.1 kg (range, 22 to 40 kg). There were 10 males (1 sexually intact and 9 neutered) and 6 females (all neutered). Two of 18 dogs initially enrolled in the study were subsequently removed because an insufficient amount of cartilage was obtained from the femoral head of each.

Cartilage tissue analysis indicated that SGAG, collagen (as determined with hydroxyproline measurement), and DNA concentrations were not significantly different among sample treatment groups (ie, those cultured in control medium without meloxicam, or with meloxicam added at 0.3, 3.0, or 30.0 \( \mu \)g/mL) at any time point (Table 1). Analysis of culture medium revealed significantly \( (P < 0.001 \) for all) lower PGE\(_2\) concentrations on day 3 in all 3 meloxicam-treated groups, compared with the control group (Figure 1). On days 12 and 30, PGE\(_2\) concentrations in the control group were significantly \( (P < 0.001 \) for both) lower than on day 0. However, there were no significant differences in the concentration of NO released into the medium at any time point tested. The SGAG concentrations in the medium were not significantly different at any time point tested. Concentrations of CS-846 in the medium were significantly lower on days 12 \( (P < 0.05) \) and 30 \( (P = 0.05) \) in the control group, compared with the concentration on day 3, but not in any of the meloxicam-treated groups. Further, on day 3, the concentration of CS-846 was significantly \( (P \leq 0.05 \) for all) higher in the control group than in all 3 meloxicam-treated groups.

There were no significant differences in medium concentrations of MMP-2 or MMP-9 (data not shown) or of MMP-13 among days within treatment groups or among groups at any time point (Figure 1). Concentrations of MMP-3 in the medium were significantly \( (P \leq 0.04 \) for all) lower on day 30 than on day 3 for all 3 meloxicam-treated groups.

Chondrocyte aggrecan gene expression in the control treatment group was significantly higher on days 12 \( (P = 0.03) \) and 30 \( (P = 0.006) \), compared with the value on day 0 (ie, that for the sample collected on the day of surgery; Figure 2). Although apparent increases in aggrecan gene expression were observed for meloxicam-treated explants over time, differences were not significant among time points. Gene expression of

![Figure 2](image-url)
ADAMTS-5 was significantly lower in all groups on days 3 (P = 0.05, 0.007, and 0.001 for the 0.3, 3.0, and 30.0 µg of meloxicam/mL treatment groups, respectively; P < 0.001 for the control group), 12 (P = 0.02 for the 0.3 µg/mL treatment group and P < 0.001 for all other groups), and 30 (P = 0.002 for the 0.3 µg/mL treatment group and P < 0.001 for all other groups), compared with the day 0 value. Gene expression of TIMP-2 was significantly (P = 0.02) higher on day 30 than on day 0 in the control group. None of the meloxicam-treated groups had significant changes in TIMP-2 gene expression over time, although samples cultured with 0.3 µg of meloxicam/mL had an apparent trend toward increasing expression over time. Gene expression of INOS was significantly lower in all groups on days 3 (P = 0.05 and 0.002 for the 0.3 and 30.0 µg/mL treatment groups, respectively; P < 0.001 for the 3.0 µg/mL and control groups), 12 (P = 0.03 for the 30.0 µg/mL treatment group and P < 0.001 for all other groups), and 30 (P < 0.001 for all) than on day 0. No significant changes or differences were found within or between groups at any time points for gene expression of collagen II, ADAMTS-4, MMP-2, MMP-9, TIMP-1, TIMP-3, IL-1β, tumor necrosis factor-α, COX-1, or COX-2.

**Discussion**

It can be a challenge to determine the concentrations of an agent that should be used for in vitro tissue culture when the goal is to evaluate the effects of exposure. Data from a pharmacokinetic study indicate that maximum concentrations of meloxicam in serum and synovial tissue following oral administration of the drug were 0.78 µg/mL and 0.65 µg/mL, respectively, in healthy Beagles used for research. The oral dose in that study (0.31 mg/kg) was well above the current recommended dose of 0.1 mg/kg in dogs; however, these data provided a starting point and a justification for the 3 meloxicam concentrations used in the test medium for culture of cartilage explants from the affected hip joints of dogs with osteoarthritis in the present study. We chose to use 0.3 µg/mL as the low concentration (which we considered a likely synovial fluid concentration in dogs administered a dose of 0.1 to 0.2 mg of meloxicam/kg), and then we used concentrations 10 and 100 times this value to evaluate potential toxic effects at higher concentrations.

On the basis of results from the present study, we accepted the hypothesis that detrimental effects to chondrocytes in cultured cartilage explants would not be detected at a 0.3 µg/mL concentration of meloxicam. Furthermore, we rejected the second part of the hypothesis that cartilage degradation would be significantly increased at higher concentrations, because no detectable detrimental effects on cartilage were found when tissues were cultured with meloxicam at 10 and 100 times the expected synovial concentrations after oral drug administration.

Compared with results for samples cultured in medium without meloxicam (ie, the control group), meloxicam treatment at 0.3, 3.0, or 30.0 µg/mL did not affect cartilage tissue DNA, SGAG, or collagen concentrations for up to 30 days in culture. Additionally, similar release of SGAG into the culture medium throughout the study indicated that meloxicam treatment did not stimulate the degradation of cartilage tissue matrix at any of the concentrations used. The control group did have a significantly higher concentration of CS-846 in the medium on day 3, compared with all 3 meloxicam-treated groups, potentially indicating a greater degree of aggrecan synthesis at that time point. These data were somewhat surprising because no corresponding increase in aggrecan gene expression was detected in the control group on day 3. However, it is possible that the greater release of CS-846 from these tissues was part of a carryover inflammatory response to osteoarthritic conditions in the joint in vivo, which was decreased by meloxicam treatment in the other 3 groups. This supposition is supported by the higher production of PGE2 by the control group on day 3, compared with all meloxicam-treated groups, combined with the observation that CS-846 concentrations in control group culture medium decreased to concentrations similar to those for the meloxicam-treated groups after day 3. Thus, these data suggest that in cartilage from affected hip joints of dogs with naturally occurring osteoarthritis, meloxicam was neutral in its effects on tissue chondrocyte content and matrix composition in vitro.

Previous studies have shown that large amounts of PGE2 and NO are produced in osteoarthritic joints, contributing to impaired chondrocyte and synoviocyte proliferation and enhanced activity of MMPs. As expected, the osteoarthritic cartilage explants in our study produced both PGE2 and NO under the culture conditions used. Further, all meloxicam-treated groups had decreased production of PGE2, but not NO, compared with the control group on day 3. Although NO production was steady throughout the culture period in all groups, there was a significant decrease in PGE2 production by the control group after day 3. It has been shown that cartilage PGE2 production is highly mechanosensitive, and therefore the significant decrease in PGE2 production by the control group after day 3 may be a result of the transition of the tissue from a mechanically unstable in vivo environment to the culture environment where swelling was unrestricted. The production of NO during culture, although steady, was also somewhat low in all groups. In a previous study, cartilage NO production was shown to be responsive to cytokine stimulation, but not mechanical stimulation. Therefore, it is possible that the production of NO decreased rapidly after removal from the inflammatory joint environment during transport from the surgical location to the laboratory for culture, and the NO concentrations detected reflected only basal in vitro production and not the concentrations produced in the environment from which tissues were obtained. The significant decrease in INOS gene expression (indicating loss of induction) in the control group as well as in meloxicam-treated groups on all days after day 0 supports the idea that the explants downregulated this response after being removed from the in vivo environment. Taken together, these data suggest that meloxicam can regulate PGE2 production by cartilage tissue and either does not affect NO production or does not affect the pathways involved in NO production in this in vitro model. However, a second explanation for the lack
of changes in NO production may be that the number of chondrocytes in the explants was too small, and thus a difference in NO release was below the detection limit for the assays used.

The concentration of MMP-13 released into the culture medium was not significantly different between the control and meloxicam-treated groups. This was surprising because a previous study found that chondrocytes exposed to a variety of NSAIDs had increased MMP-1 and MMP-13 production. Those data were used to speculate on the reason that NSAIDs were ineffective in retarding cartilage destruction despite favorable effects on proinflammatory mediators. However, that study was performed with chondrocytes in monolayer, not cartilage explants, and IL-1β stimulation was used to induce the response. Without IL-1β stimulation, NSAIDS did not increase chondrocyte MMP production, which is in agreement with the results of the present study. Our data indicate that meloxicam treatment did not stimulate increased MMP production by osteoarthritic cartilage explant tissue in vitro, even at supra-physiologic doses.

It could be argued that the release of smaller amounts of CS-846 from control group samples on days 12 and 30, compared with day 3, may indicate slowing cartilage metabolism in general, limiting the ability to assess changes mediated by different meloxicam concentrations with the study protocol used. However, it is not surprising that measures of synthesis did not increase because growth-simulating factors were not included in this study. Persistent aggrecan, collagen II, and TIMP-1 and TIMP-2 gene expression combined with the steady production of MMP-2 in all groups through 30 days in culture indicated a metabolically active cartilage explant in culture.

Interestingly, results of the present study suggest that in vitro meloxicam treatment of cartilage from affected joints of dogs with naturally occurring osteoarthritis does not induce matrix degradation or stimulate MMP production over 30 days of treatment, even at doses up to 100 times the expected synovial concentrations. Although meloxicam did lower PGE2, concentrations in this tissue, its effects on tissue chondrocyte content and matrix composition were neutral.

References

7. Rainsford KD, Ying C, Smith FC. Effects of meloxicam, compared with other NSAIDs, on cartilage proteoglycan metabolism, synovial prostaglandin E2, and production of interleukins 1, 6 and 8, in human and porcine explants in organ culture. J Pharm Pharmacol 1997;49:991–998.
22. Beluche LA, Bertone AL, Anderson DE, et al. Effects of oral ad-


