Effects of continuous oral administration of phenylbutazone on biomarkers of cartilage and bone metabolism in horses

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Objective—To evaluate the effects of continuous oral administration of phenylbutazone on serum and synovial fluid biomarkers of skeletal matrix metabolism in horses.

Animals—11 adult female horses without clinical or radiographic evidence of joint disease.

Procedures—Horses were randomly assigned to control or treatment groups. Phenylbutazone was administered orally twice daily at a dose of 4.4 mg/kg for 3 days to the treatment group and subsequently at a dose of 2.2 mg/kg for 7 days. Serum and radiocarpal synovial fluid samples were obtained at baseline and thereafter at regular intervals for 4 weeks. Biomarkers of cartilage aggrecan synthesis (chondroitin sulfate 846) and type II collagen synthesis (procollagen type II C-propeptide) and degradation (collagen type II cleavage) were assayed. Biomarkers of bone synthesis (osteocalcin) and resorption (C-terminal telopeptide of type I collagen) were also measured.

Results—No significant differences were found between control and treatment groups or temporally for the biomarkers chondroitin sulfate 846, procollagen type II C-propeptide, collagen type II cleavage, and C-terminal telopeptide of type I collagen in serum or synovial fluid. A significant increase in osteocalcin concentration occurred in synovial fluid during treatment in the treated group. No treatment effect was detected for serum osteocalcin concentration.

Conclusions and Clinical Relevance—Results suggested that continuous phenylbutazone administration at recommended doses altered some biomarkers in healthy equine joints after short periods of administration. Increased osteocalcin concentration may indicate an undetermined anabolic effect of phenylbutazone administration on periartericular bone or transient induction of osteogenesis in articular chondrocytes or a mesenchymal subpopulation of synoviocytes. (Am J Vet Res 2007;68:128–133)

Phenylbutazone is a nonselective NSAID that inhibits the inflammatory response mediated by prostaglandins and thromboxanes by blocking COX.1 Despite the fact that it is the most widely used NSAID in horses, there is still controversy in the literature concerning the effects of its administration on cartilage and bone matrix metabolism because prostaglandins play a role in skeletal extracellular matrix turnover. Both favorable and detrimental effects have been detected experimentally.2,3 Phenylbutazone, when administered orally for 14 days, suppresses proteoglycan synthesis in normal equine articular cartilage explants harvested at the end of a study.4 In contrast, no significant effects of clinically relevant doses of phenylbutazone on proteoglycan synthesis are detected when equine cartilage explants are studied in vitro.5,6 At the other end of the spectrum, more recently, it has also been reported that a selective COX-2 inhibitor has a beneficial effect on proteoglycan turnover in osteoarthritic cartilage explants from humans, suggesting chondroprotective properties of the NSAID family of drugs in the treatment of degenerative joint disorders.7 Effects of phenylbutazone on equine bone have also been studied, and its administration induces a significant decrease in mineral apposition rate in cortical bone.8 Studies9,10 in other animals also suggest that nonselective NSAIDs may delay fracture healing. Although no difference in bone fracture repair is observed when COX-2–specific inhibitors are evaluated in rats,11 others found that, conversely, their administration in the early phase of fracture healing creates a risk of delayed healing.12

Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drug</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<td>CTX-1</td>
<td>C-terminal telopeptide of type I collagen</td>
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<td>OC</td>
<td>Osteocalcin</td>
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<tr>
<td>CP II</td>
<td>Procollagen type II C-propeptide</td>
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<tr>
<td>C2C</td>
<td>Collagen type II cleavage</td>
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<td>CS 846</td>
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Articular cartilage is composed of chondrocytes, which both synthesize and degrade the matrix in which they are embedded. This matrix is composed principally of type II collagen and aggrecan. Bone, however, contains 2 cell types that either deposit (osteoblasts) or degrade (osteoclasts) its mineralized matrix, which is composed mainly of type I collagen. Skeleton-specific biomarkers are now available that can be used to measure the cellular turnover, either synthesis or degradation, of these matrices, both in vivo and in vitro. Such immunoassays measure specific molecular products of tissue turnover released into various body fluids.

The objective of the study reported here was to evaluate the effects of the continuous oral administration of phenylbutazone on biomarkers of cartilage and bone metabolism in horses. We hypothesized that administration of phenylbutazone would impair bone and cartilage metabolism.

**Materials and Methods**

**Horses**—Eleven adult female horses from a research herd were used in the study and had mean ± SD weight of 493 ± 47 kg and age of 12 ± 2 years. Horses were randomly assigned to a treatment group (n = 6 horses) and an untreated control group (5). All horses included in the study were free of radiographic evidence of joint disease in the sampled joints. Serum creatinine and γ-glutamyl transpeptidase concentrations were measured at baseline to assess renal and hepatic function. Phenylbutazone (4.4 mg/kg) was administered orally twice daily for 3 days and then at a lower dose (2.2 mg/kg) for 7 days to the treatment group. Horses were allowed to exercise in a small paddock for approximately 5 h/d during the treatment period and were kept at pasture for the rest of the study. The Institutional Animal Care and Use Committee of the Université de Montréal approved this project.

**Serum samples**—Blood was collected in a dry tube from the jugular vein daily between 7:30 and 8:30 AM to avoid diurnal variation. The blood samples were kept at 4°C for 1 h to ensure good clot formation. Baseline control samples were obtained 2 days before treatment. Blood samples were collected for 28 days following initiation of treatment. A final sample was obtained on day 32 after treatment.

**Synovial fluid samples**—An aseptic method was used to obtain samples of undiluted synovial fluid from the radiocarpal joint. Anesthetic cream (lidocaine [2.5%] and prilocaine [2.5%]) was applied 1 h prior to collection to desensitize the skin and minimize adverse reactions to the arthrocentesis procedure. Furthermore, because repeated arthrocentesis was part of the protocol, use of the anesthetic facilitated collection of the samples for the duration of the study. Samples were collected weekly for 4 weeks; the first sample (right radiocarpal joint) was obtained prior to treatment in all horses. Subsequent weekly samples were harvested by alternating between left and right joints. Horses were observed daily and evaluated every 2 days throughout the study for any signs of heat, pain, effusion, or abnormal gait.

Blood and synovial fluid samples were centrifuged at 1,000 g for 20 minutes at 4°C. The supernatant was divided into aliquots and stored at −80°C in Eppendorf tubes until analysis.

**Biomarker assays**—Competitive ELISAs were used to measure all biomarkers except CTX-1, which was analyzed by use of a sandwich immunoassay. Prior to assay of all synovial samples, except for the OC assay, the synovial fluid was digested at 37°C overnight with Streptomyces hyaluronidase (50 turbidity-reducing U/mL) and proteinase inhibitors (0.2M EDTA, 0.2M phenylmethylsulfonyl fluoride in isopropanol, and 0.2M iodoacetamide and 1.0 mg/L of pepstatin [in 95% ethanol] in 0.2M sodium acetate buffer at pH of 5). The digestion reduces the viscosity of the synovial fluid, which improves contact of the fluid with the epitope and pipetting accuracy. The dilution caused by the digestion was estimated to be 10%.

A plate reader was used to measure the optical density, which was inversely proportional to the amount of epitope in the sample. Software was used to calculate the concentration of the epitope in a sample, compared with a standard curve.

**CP II assay**—To evaluate synthesis of cartilage type II collagen, an ELISAI that used the same rabbit polyclonal antibody that was validated for use in samples obtained from horses was used. The assay detected CP II, which is cleaved from type II procollagen after release of newly synthesized procollagen into the cartilage.

**C2C assay**—To evaluate degradation of cartilage type II collagen, C2C (COL2-3/4C long antibody) was assayed by use of an ELISAI to detect a neoepitope created by the specific cleavage of type II collagen by collagenases when degradation occurs. The assay used a mouse IgG antibody.

**CS 846 assay**—To evaluate synthesis of aggrecan, the CS 846 epitope, which is a measure of aggrecan synthesis, was measured by use of a commercial ELISAI kit. The epitope is present on newly synthesized chondroitin sulfate chains of the cartilage aggrecan. The same mouse antibody against CS 846 has been used in radioimmunoassays and validated for use in equine specimens.

For CP II, C2C, and CS 846 analyses, optical density was measured at 450 nm. For CP II and C2C, the dilution used for serum was 1:2, and no dilution was necessary for the synovial fluid assays. For CS 846, a dilution of 1:3 was used for serum and synovial fluid analyses.

**OC assay**—To evaluate bone formation, a commercial ELISAI validated for use in equine samples was employed to measure OC (bone Gla protein). The assay used a mouse anti-OC antibody. Osteocalcin represents 25% of the noncollagenous proteins of bone and plays a role in bone matrix formation.

Certain serum samples required a 1:2 dilution because of high concentrations of the epitope, but most were analyzed without dilution. Digestion was not performed before analyzing the synovial fluid.
because it is believed to interfere with the epitope, according to the manufacturer. A reverse pipetting method was used, as suggested by the manufacturer, to maximize the precision of manipulation with a viscous liquid. Samples were evaluated at a wavelength of 405 nm.

**CTX-1 assay**—To evaluate bone resorption, during which type I collagen is cleaved, the neoepitope CTX-1 (C-terminal telopeptide of type 1 collagen) was measured by use of a commercial ELISA validated by the manufacturer and used in horses. Optical density was measured at 450 nm. All samples were analyzed without dilution.

**Statistical analysis**—Statistical analyses were performed with software. For each biomarker, a repeated-measures linear model with time as a repeated factor and treatment as a between-subject factor was used. Furthermore, mean concentrations of each biomarker (control and treated values taken together) in the serum were compared with mean concentrations in the synovial fluid to determine whether a significant difference between the 2 compartments was present. A repeated-measures linear model with compartment and time as within-subject factors was used. Analyses were performed at 3 time points when concurrent blood and synovial fluid collection was performed (days –2, 12, and 19). For all analyses, \( P < 0.05 \) was considered significant.

**Results**

**Creatinine and γ-glutamyl transpeptidase analysis**—Mean \( \pm S D \) serum creatinine and γ-glutamyl transpeptidase concentrations (101 \( \pm \) 17 μmol/L and 18 \( \pm \) 5 U/L, respectively) measured at baseline were within the reference range of our laboratory for all horses. These values indicated normal renal and hepatic function at the outset of the study.

**Cartilage biomarkers**—No significant differences were detected in concentrations of CS 846, CP II, or C2C between control and treatment groups in either the serum or synovial fluid at baseline, with treatment or temporally (Figure 1). The difference between control and treated groups for synovial fluid concentrations of CP II were not significant at all time points (\( P = 0.058 \)).

When mean concentrations of the cartilage biomarkers were compared in serum and synovial fluid compartments for CP II and C2C, concentrations in the synovial fluid were significantly lower (2.4 and 3.0 times, respectively). In contrast, mean concentrations of synovial fluid CS 846 epitope were significantly higher (4.1 times) than in serum.

**Bone biomarkers**—Baseline concentrations of biomarkers OC and CTX-1 were not significantly different between groups. Similarly, no significant differences were found between groups or among time points for the serum values. However, a significant (\( P = 0.01 \)) effect of treatment at day 5 was found in the synovial fluid for the OC epitope (Figure 1). Values were higher in the treated group, compared with the control group, which suggested a stimulatory effect of phenylbutazone on bone formation in vivo at the joint level. On day 12, 3 days after cessation of treatment, no difference was detected between groups.

No significant effect of treatment was found in the synovial fluid for CTX-1 concentration. Similarly, no temporal effects were detected in the concentrations of OC and CTX-1 in the synovial fluid.

Mean concentration of OC in the serum was significantly higher (1.4 times) than in the synovial fluid. In contrast, mean concentrations of CTX-1 in the synovial fluid and serum were similar.

**Discussion**

Biomarker technology is now providing interesting new insights on the effects of medication on cartilage and bone metabolism in vivo in humans and other animals. Prior to the advent of biomarker assays of body fluids, it was not possible to address longitudinally in vivo the adverse effects of NSAIDs on skeletal metabolism. The study reported here was novel in that systemic and local joint effects were assessed in vivo simultaneously and temporally throughout the administration period.

No significant adverse effects of phenylbutazone on systemic cartilage or bone metabolism were detected at the doses and route of administration investigated. These results suggested that phenylbutazone administration should not markedly affect systemic skeletal tissue metabolism when used clinically to target localized disease processes. The doses used and route of administration reflected what is presently recommended and were representative of treatment protocols in our hospital. Higher doses and more prolonged oral administration of phenylbutazone have, however, been reported by other investigators to have effects on cartilage or bone, or both.

Osteocalcin concentrations were significantly higher in the synovial fluid of the phenylbutazone-treated horses during the treatment period. These findings suggested a stimulatory effect of phenylbutazone administration on bone formation in vivo, at the joint level, because no differences in serum concentrations were detected. However, it has also recently been proposed that OC should be considered a marker of bone turnover and not bone formation alone because release of intact OC molecules and fragments occurs with bone resorption in vitro. No differences, however, were detected during our study in the concentrations of the marker of bone degradation, CTX-1; therefore, the higher concentrations of OC may not necessarily have been attributable to increased bone matrix catabolism.

The source of OC in a healthy joint is unclear. Although diffusion of molecules from the subchondral bone through the mineralized cartilage and nonmineralized cartilage is not fully understood, it is unlikely that this is the source of the molecules. The increased OC concentrations in the synovial fluid that we observed may have therefore reflected increased periarticular bone metabolism with newly formed OC molecules released into the joint fluid. In an osteoarthritic joint with exposed subchondral bone, the presence of OC in the synovial fluid would be more easily explained. In the study reported here, radiographic examination was per-
formed to rule out osteoarthritis in the sampled joints. Because of financial constraints, more sensitive methods to detect early cartilage damage such as arthroscopy or MRI were not performed. It is therefore possible that the subchondral bone could have been exposed in some of these joints, but only a significant rise in OC concentrations in the joints of horses treated with phenylbutazone was detected.

Figure 1—Mean ± SEM concentrations of biomarkers CS 846, CP II, C2C, OC, and CTX-1 in serum and synovial fluid of control horses (gray lines; n = 5) and horses that were treated with phenylbutazone (black lines; 6). X-axis indicates day of the study. Significant (P = 0.01) difference between groups.
Another potential explanation is that phenylbutazone treatment transiently induced osteogenesis in articular chondrocytes or in a mesenchymal subpopulation of synoviocytes. Evidence of osteogenic capacity in both of these tissues is available. It is, however, difficult to explain why these events would not also be reflected in systemic OC concentrations.

Because concentrations of OC were higher in the serum than in the synovial fluid, the molecules could have diffused into the joint from the serum. Synovial fluid OC concentration was also significantly lower than in serum in healthy humans, humans with osteoarthritis, and humans with rheumatoid arthritis, suggesting that OC in the synovial fluid may be derived from the blood. However, because serum OC concentrations did not change with treatment in the present study, it is difficult to attribute the increase in synovial fluid concentrations of OC to diffusion from serum.

Our findings provided evidence that phenylbutazone administration, by decreasing concentrations of prostaglandins or other arachidonic acid cascade metabolites, may somehow promote bone formation in healthy joints. An alternative hypothesis is that phenylbutazone may facilitate release of accumulated OC molecules from the matrix, without an increase in bone formation.

Phenylbutazone administration in horses decreased mineral apposition rate of unicortical healing bone defects in another study. The difference between studies is that the present study evaluated effects on metabolism in normal joints alone. It is also reported that NSAID administration could impair fracture healing in rats. Such results are in conflict with results of other studies because COX-2-specific inhibitors are reported to have either a significant effect on bone repair or no effect in rodent models. The exact mechanism of the effect remains to be determined. In the present study, a significant difference was detected between concentrations of OC in serum (higher) and synovial fluid. In contrast, mean concentrations of CTX-1 were similar in the serum and synovial fluid, which suggested a balance between the 2 compartments.

Because prostaglandins play an important role in the physiologic and pathologic responses of skeletal tissue, understanding the effects of blockade of prostaglandins on normal skeletal metabolism and under pathologic circumstances is of value. Reduction of production of prostaglandin E₂ and other arachidonic acid metabolites may affect target tissues and other tissues and therefore affect overall skeletal health. Although effects of prostaglandin E₂, on bone in vivo remain to be fully elucidated, prostaglandin E₂ inhibits human osteoclast formation in vitro. Blocking prostaglandin E₂ production with an NSAID could alter osteoclastic activity. The effect of NSAID administration on serum OC concentrations was studied in patients with rheumatoid arthritis and, like the study reported here, no differences in serum concentrations occurred.

The specific biomarkers of cartilage metabolism studied here were not measurably affected by treatment or time. This finding suggested either a lack of effect of the medication or a lack of sensitivity of the markers to detect changes. The difference between groups approached significance for synovial fluid concentrations of CP II, a type II collagen synthesis marker, with values typically greater in the treated group at all time points. These results could suggest an increase in articular cartilage type II collagen synthesis after systemic administration of phenylbutazone. This is intriguing and could point to a role for prostaglandin metabolites in normal cartilage turnover, with lower prostaglandin E₂ concentrations favoring synthesis. Procollagen type II C-propeptide and CS 846 have been used to measure effects of intra-articular injections of corticosteroids on articular cartilage metabolism. These investigations provide evidence that repeated intra-articular use of corticosteroids alters articular cartilage collagen metabolism by inhibition of procollagen II synthesis; increased release of degradation products of the proteoglycan aggrecan from articular cartilage; and significant increases in CS 846, keratosulfate, C1, 2C, and CP II epitope concentrations. No effects of phenylbutazone on degradation of type II collagen were detected in our study. These findings suggested that phenylbutazone administration does not tip the balance of cartilage metabolism toward catabolic pathways when administered for short durations.

Mean serum biomarker concentrations of type II collagen synthesis and degradation were significantly higher, compared with local joint concentrations, suggesting rapid diffusion of the epitopes from the joint into the serum and perhaps a longer half-life of the epitope in the serum. However, half-lives would be anticipated to be shorter in serum because the epitope should be cleared more effectively from serum, compared with other compartments. The serum half-life of the CP II epitope is estimated to be 18 hours. It is likely that the serum concentrations of these markers reflect total skeletal type II collagen turnover. Further research is required to determine the kinetics and half-lives of the biomarkers to enhance interpretation of their fluctuations.

In contrast, mean concentrations of CS 846, a marker of aggrecan synthesis, were significantly higher in the synovial fluid, compared with serum. This has also been reported in human patients with osteoarthritis and rheumatoid arthritis. Our results indicated that synovial concentrations of CS 846 clearly reflected local production of the epitope. These results could also provide evidence that the epitope had a short serum half-life. Further work is required to determine the distribution of biomarkers in the different fluid compartments.

We recognize that the power in our statistical analyses was rather low given the small number of horses studied, and therefore, only large effects could be detected. Further studies in vivo using larger numbers of horses and different treatment schedules, doses, and routes of NSAID administration, as well as repair or disease models, would help determine whether the biomarker patterns observed in the present study would persist under these circumstances.

a. Emla cream, AstraZeneca, Mississauga, ON, Canada.
   b. CS-6R centrifuge, Beckman Instruments Inc, Brea, Calif.
   c. Streptomyces hyaluronidase, Seikagaku Inc, Tokyo, Japan.
   d. EDTA, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada.
   e. PMSF (in isopropanol), Sigma-Aldrich Canada Ltd, Oakville, ON, Canada.
f. Iodoacetamine, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada.
g. Pepstatin (in 95% ethanol), Sigma-Aldrich Canada Ltd, Oakville, ON, Canada.
h. Sodium acetate buffer, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada.
j. CP II assay, Ibex Diagnostics, Montreal, QC, Canada.
k. C2C assay, Ibex Diagnostics, Montreal, QC, Canada.
l. CS 840 assay, Ibex Diagnostics, Montreal, QC, Canada.
m. Novocaline, Meta Biosystems, Mountain View, Calif.
n. Serum Crosslamps, Nordic BioScience Diagnostics, Herlev, Denmark.
o. SAS, version 8.2, SAS Institute Inc, Cary, NC.

References