Effects of anti-inflammatory drugs on lipopolysaccharide-challenged and -unchallenged equine synovial explants

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Objective—To evaluate the effects of anti-inflammatory drugs on lipopolysaccharide (LPS)-challenged and -unchallenged equine synovial membrane in terms of production of prostaglandin E2 (PGE2) and hyaluranon, viability, and histomorphologic characteristics.

Sample Population—Synovial membranes were collected from the carpal, tarsocrural, and femoropatellar joints of 6 adult horses.

Procedure—Synovial membranes from each horse were minced and pooled and explants were treated with one of the following: no drug (control), drug, LPS alone, or LPS and drug. Treatment drugs were phenylbutazone (PBZ), flunixin meglumine (FNX), ketoprofen (KET), carprofen (CRP), meloxicam (MEL), low-concentration methylprednisolone (METH), high-concentration METH, dimethyl sulfoxide (DMSO), or an experimental COX-2 inhibitor (dissolved in DMSO). Following 48 hours of culture, medium was assayed for PGE2 and hyaluronic concentration. Synovial explants were assessed for viability and histomorphologic characteristics.

Results—For the LPS-challenged explants, PBZ, FNX, KTP, CRP, MEL, and low-concentration METH suppressed PGE2 production, compared with LPS challenge alone. Only MEL suppressed PGE2 production from LPS-challenged explants, compared with unchallenged explants. Synovial explants maintained > 90% viability and there was no significant difference in viability or hyaluronic production among explants. Histomorphologic scores were significantly decreased for explants treated with low-concentration METH or DMSO.


Inflammatory joint disorders are a common cause of lameness in horses and are the leading cause for early retirement of athletic horses. Chronically inflamed joints are characterized by pain, synovitis, and progressive deterioration of articular cartilage. Inflamed synovial membrane plays a major role in initiating and perpetuating cartilage destruction that ultimately leads to osteoarthritis and irreversible joint damage.

Prostaglandins, specifically prostaglandin E2 (PGE2), are potent mediators of joint inflammation, which occur naturally in osteoarthritic joints and are responsible for clinical signs associated with osteoarthritis. Inhibition of PGE2 production is associated with decreased joint effusion, improved lameness, and decreased inflammatory cells within synovial fluid.

Clinical management of inflammatory joint disorders includes use of pharmacologic agents that inhibit prostaglandin production, most commonly NSAID and corticosteroids. Although these drugs successfully alleviate clinical signs of joint inflammation, their comparative effects on equine synovium are not known.

The NSAID elicit their effects through inhibition of cyclooxygenase (COX) which converts arachidonic acid to prostaglandins. There are 2 isozymes of COX: COX-1 and COX-2. Cyclooxygenase-1 is the constitutive form of the enzyme found in most tissues and is responsible for normal cellular regulation. Cyclooxygenase-2 is the inducible form of the enzyme and is expressed in response to inflammatory stimuli. Most commonly used NSAID are nonselective COX inhibitors that inhibit COX-1 and COX-2, with COX-1 inhibition being responsible for the adverse effects of these drugs.

There are new selective COX-2 inhibitors, such as meloxicam (MEL) and an experimental COX-2 inhibitor (DFU), which are not yet marketed for use in horses. Meloxicam is approved for use as an antiarthritic in dogs in Europe. These drugs have the potential benefit of decreased adverse effects, specifically ulceration of the gastrointestinal tract and renal disease. Currently, MEL is in clinical trials and DFU is being tested. Other anti-inflammatory mechanisms exist for certain NSAID, such as carprofen (CRP), which is clinically effective despite a minor decrease in prostaglandin production. The most commonly used NSAID approved for use in the horse include phenylbutazone (PBZ), flunixin meglumine (FNX), and ketoprofen (KET). Carprofen is approved for use in horses in Europe and for use in dogs in the United States.

Unlike NSAID, corticosteroids inhibit the entire arachidonic acid cascade (cyclooxygenase and lipooxy-
nase) by inhibition of the initiating enzyme phospholipase A₂ and are therefore potent inhibitors of inflammation. Their use is also associated with adverse effects caused in part by inhibition of COX-1. Methylprednisolone (METH) is a steroid commonly used intra-articularly or applied topically. ²⁻⁵ When injected intra-articularly or applied topically, METH has anti-inflammatory and analgesic effects used in our study as a solvent for DFU. Dimethyl sulfoxide (DMSO) is used in vivo as a droprotective.²³,²⁴ It is not known whether a low concentration of METH has sufficient anti-inflammatory effects to be useful clinically, or if a high concentration of METH has detrimental effects on synovium.

Dimethyl sulfoxide (DMSO) is an organic liquid used in our study as a solvent for DFU. Dimethyl sulfoxide has anti-inflammatory and analgesic effects when injected intra-articularly or applied topically.²⁵⁻²⁶ To our knowledge, the effects of DMSO on synovium have not been reported.

Lipopolysaccharide (LPS) is used in vivo as a model for synovitis-induced arthritis and stimulates PGE₂ production when injected intra-articularly.²⁷⁻³⁰ In vitro, LPS stimulates PGE₂ production from equine synovial monolayers and articular cartilage explants.³¹,³²

The purposes of the study reported here were to challenge equine synovial explants with LPS to induce PGE₂ production, and to quantify and compare the ability of various anti-inflammatory drugs to alter this response. Our hypothesis was that the anti-inflammatory drugs would suppress PGE₂ production, with the COX-2 inhibitors more selectively suppressing PGE₂ production in LPS-challenged explants. We also hypothesized that the drugs would not affect synovial viability, function, or histomorphologic characteristics.

**Materials and Methods**

*PGE₂ concentrations in culture medium—* Culture medium samples were thawed to room temperature (approx. 25°C) and PGE₂ concentrations were measured using a commercially available enzyme immunoassay kit. Briefly, 100 µl aliquots of culture medium were incubated overnight at 2 to 8°C in a 96-well culture plate precoated with goat anti-rabbit IgG containing 100 µl of PGE₂ antibody/well. Following incubation overnight, 100 µl of alkaline phosphate conjugate was added to each well and the plate allowed to incubate an additional 3 hours at 2 to 8°C. Wells were washed with 400 µl of wash buffer, and 300 µl of para-Nitrophenyl phosphate substrate was added and the plate was incubated a final 2 hours at 37°C. Stop solution (50 µl) was added to all wells and the absorbance read in a spectrophotometer at 405 nm. Standards ranging from 10 to 5000 pg/ml were similarly incubated and a standard curve was generated. Prostaglandin E₂ concentrations were recorded in pg/ml. Prostaglandin E₂ concentrations were and expressed in pg/mg of synovium calculated on a wet weight basis.

**Hyaluronan concentrations in culture medium—** Culture medium samples were thawed to room temperature (approx. 25°C) and hyaluronan concentrations were measured, using a previously described method. Briefly, 1000 µl aliquots of culture medium were dialyzed (membrane cut-off 12,000 to 14,000 daltons) against buffer (0.1 mM sodium acetate, 0.15 M sodium chloride, 0.15 mM sodium azide [pH 7.4]) for 96 hours at 22°C with daily exchange of buffer. Aliquots of 500 µl were collected for measurements before hyaluronidase digestion. The remaining 500 µl was digested with 10 Units of Streptomyces hyaluronidase and dialyzed for an additional 96 hours at 22°C with daily exchange of buffer. At the end of dialysis, the final volume was recorded and 500 µl aliquots were collected for measurements after hyaluronidase digestion. Hyaluronan was measured by use of a hexuronic acid assay. Briefly, over ice, 200 µl of dialysis aliquots were added to borosilicate glass tubes that contained 1250 µl of sulfuric acid with 0.25 M sodium borate. Samples were incubated in boiling water for 10 minutes. One hundred µl of 0.125% solution was added to the tubes and incubated an additional 15 minutes in boiling water. Sample absorbance was read in a spectrophotometer at 530 nm. Standards ranging from 5 to 50 µg/ml were similarly incubated, and a standard curve was generated. Hyaluronan concent-

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tration in the medium was calculated as the difference between before and after hyaluronidase digestion measurements following volume correction, and recorded as µg/ml.

Synovial cell viability—Synovial explant viability was estimated using a method adapted from previously described techniques. Briefly, synovial explants from 1 of the duplicate wells/horse (n = 6) were washed 3 times with fresh culture medium and placed in digestion vials containing 3 ml of 0.25% trypsin-EDTA and stirred for 30 minutes at 37°C. Explants were rinsed and incubated for an additional 15 minutes in culture medium containing 200 Units of collagenase type IV. Incubation medium was collected and centrifuged for 2 minutes and the supernatant discarded. The remaining cells were stained with 30 µl of 0.1% trypan blue in PBS solution. Cell viability was assessed by counting 100 cells and recording the percentage of unstained cells (trypan blue exclusion).

Synovial explant histomorphologic characteristics—Synovial explants from the other duplicate well for each horse (n = 6) were placed in 10% solution of formalin, sectioned, and stained with H&E. Synovial membrane cellularity was estimated by evaluating 3 representative microscopic fields at 20X magnification. Grading was recorded as the percentage of the synovial surface covered by cells as follows: 0) no cells, 1) <25%, 2) >25% but <75%, 3) >75%. Synovial cell nuclear degeneration was estimated by evaluating 3 representative microscopic fields at 40X magnification. Cells were considered degenerate if the nuclei were pyknotic or fragmented, the cytoplasm was vacuolated, and there was loss of differential staining. Grading was recorded as the percentage of cells that appeared nondegenerate (plump and well-rounded nuclei, homogeneous cytoplasm, and differential staining) and followed the same scale as for cellularity. A total histologic score was calculated as the sum of the synovial membrane cellularity and synoviocyte nuclear degeneration scores (0 to 6).

Statistical analysis—Prostaglandin E2 and hyaluronan concentrations were analyzed by means of a 2-way ANOVA with repeated measures for LPS and drug. When a significant difference was detected between treatments, explants were compared by use of a Newman Keuhl post hoc test. Assumptions of normality or homogeneity of variance were not met; therefore, PGE2 concentrations were analyzed following log transformation of data. Histologic scores were analyzed by use of a nonparametric Kruskal-Wallis ANOVA by ranks and a Friedman ANOVA. Significance for all tests was set at P < 0.05.

Results

PGE2 concentrations in culture medium—In LPS-challenged explants, PBZ, FNX, KET, CRP, MEL, and low-concentration METH (4 µg/ml) significantly suppressed PGE2 production when compared with LPS-challenged explants without drugs (Fig 1). In unchallenged explants there were no significant effects of drug treatments compared with control explants. Meloxicam was the only drug for which suppression of PGE2 production from LPS-challenged explants was significantly greater than suppression of PGE2 production from unchallenged explants.

Hyaluronan concentrations in culture medium—Mean hyaluronan concentrations in the culture medium ranged from 30.3 to 45.38 µg/ml for all explants. There were no significant differences among explants.

Synovial cell viability—Mean viability of synovial explant cells (exclusion of trypan blue stain) was 90.9% for all explants. There were no significant differences among explants.

Synovial explant histomorphologic characteristics—Challenge with LPS did not affect histomorphologic scores for synovial explant cellularity. Therefore, a total histologic score (0 to 12) was calculated by combining LPS-challenged (0 to 6) and unchallenged (0 to 6) explants to better assess drug effects. Total histologic scores were significantly decreased for low-concentration METH, DMSO, and DFU (Fig 2).
In our study, PBZ, FNX, KET, CRP, MEL, and low-concentration METH significantly decreased PGE2 production from LPS-challenged explants, indicating inhibition of COX-2 activity by these drugs. Phenylbutazone, FNX, and KET are approved for use in horses and are used commonly for their ability to inhibit COX. These drugs are nonselective COX inhibitors that inhibit COX-1 and COX-2 to varying degrees.

In our study, PBZ significantly suppressed PGE2 production following LPS challenge of explants, which suggests COX-2 inhibition. Results of a recent study indicate that PBZ is selective for canine COX-2, however, clinical toxicosis caused by COX-1 inhibition is found in dogs and horses.35-37

In vitro, FNX is found to decrease glycosaminoglycan synthesis by equine cartilage explants at concentrations of 100 µg/ml, but not at 1 or 10 µg/ml.3 FNX and KET decreased PGE2 production in vitro by LPS-stimulated equine synovial monolayers at similar concentrations as those used in our study.31 Recent study results indicate that CRP is a potent selective inhibitor of COX-2 in canine macrophage-like cell cultures following stimulation with LPS.35 In our study, CRP significantly suppressed PGE2 production following LPS challenge of explants, which is likely related to COX-2 inhibition. Carprofen is also reported to decrease PGE2 production from equine chondrocyte monolayers before and after IL-1β addition, but the type of COX isoenzyme inhibition has not been specified.36 These results indicate that CRP may exert selective COX-2 inhibition, which could explain the low incidence of adverse effects. A recent study on the pharmacokinetics of CRP in equine plasma and synovial fluid found that the concentrations of CRP in synovial fluid following an IV injection of 0.7 mg/kg were <1 µg/ml.39 This is lower than the concentration chosen for use in our study, however, concentrations of NSAID, which are highly protein bound, are expected to be higher in inflamed joints as a result of increased permeability across the synovial membrane. Ketoprofen concentrations are 6.5 times higher in joints with synovitis compared with normal joints.40 Following CRP administration, a 6.5 times increase in CRP concentration in inflamed joints would result in a concentration similar to those used in our study.

Meloxicam was the only drug that significantly suppressed PGE2 production in LPS-challenged explants, compared with unchallenged explants. This suggests that MEL more selectively suppressed COX-2 activity, compared with other NSAID. This is supported by previous findings of selective COX-2 inhibition using a human whole blood assay and guinea pig peritoneal macrophages following LPS stimulation.45 In contrast, one study found weak selectivity for COX-2 by MEL from canine macrophage-like cells.35 This may represent differences in COX isoenzymes between species; however, canine and equine COX isoenzymes have not reportedly been sequenced.

In our study, the soluble form of METH (sodium succinate) was used to eliminate possible detrimental effects of the suspension (acetate) on the synovial membrane. Synovial concentration of MPA in the tarsochugal joint following a routinely used dose of 100 mg administered intra-articularly is approximately 40 µg/ml.23 To assess its effects on synovium, 40 µg/ml was selected as the high-concentration METH in our study. A lower concentration of 4 µg of METH/ml is chondroprotective in vitro, but its anti-inflammatory effects are not established.24 In our study, the effects of low-concentration METH were assessed by use of 4 µg/ml.

Discussion

In our study, PBZ, FNX, KET, CRP, MEL, and low-concentration METH significantly decreased PGE2 production from LPS-challenged explants, indicating inhibition of COX-2 activity by these drugs. Phenylbutazone, FNX, and KET are approved for use in horses and are used commonly for their ability to inhibit COX. These drugs are nonselective COX inhibitors that inhibit COX-1 and COX-2 to varying degrees.

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Results of our study were unexpected. High-concentration METH, which corresponds to synovial concentra-
tions following intra-articular injection, should have significantly decreased PGE$_2$ production on the basis of the results of a previous study. However, low-concentration METH resulted in lower mean values for PGE$_2$, suggesting that a low dose may be clinically effective. Both explants had decreased histomorphologic scores when compared with controls, indicating a negative effect on synovial histomorphologic characteristics. This is similar to what is described in vivo following intra-articular administration of MPA using an osteochondral model. In contrast, another similar study found no detrimental effects of MPA on synoviocytes; however, those horses received PBZ after surgery.

As a potent and selective COX-2 inhibitor, DFU inhibits LPS-induced PGE$_2$ production in a human whole blood assay and carrageenan-induced rat paw edema. It also reverses LPS-induced pyrexia in a dose-dependent manner. In our study, DFU did not significantly decrease PGE$_2$ production from either unchallenged or LPS-challenged explants. Because it is relatively insoluble, DFU was dissolved in DMSO. Dimethyl sulfoxide alone did not significantly suppress synovial membrane PGE$_2$ production and had detrimental effects on synovial membrane histomorphologic characteristics similar to DFU. Detrimental effects of the DMSO solvent may explain the lack of activity of DFU when compared with the other COX-2 inhibitor, MEL. Another explanation may be that the final concentration of DFU in DMSO was not verified and may have been less than expected.

In our study, DMSO did not significantly alter synovial explant PGE$_2$ production, but did have a negative effect on synovial membrane as indicated by decreased histomorphologic scores (decreased cellularity and increased nuclear degeneration). Similar effects were seen with DFU and may be related to the DMSO solution. When administered intra-articularly as a 10 to 30% solution, DMSO does not induce inflammatory changes in the joint greater than lactated Ringer’s solution alone. However, DMSO (10%) alters articular chondrocyte metabolism in vitro.

In our study, PGE$_2$ production from unchallenged explants was not significantly different from control explants for any drug; however, mean PGE$_2$ values were lower for all drugs in a pattern similar to the LPS-challenged explants. This may represent a NSAID-induced reduction in baseline PGE$_2$ production from explants in vitro, which may be related to COX-1 inhibition.

In our study, synovial explants were challenged with LPS to induce additional PGE$_2$ production. When given intra-articularly to horses at concentrations similar to what was used in our study, LPS increases cytokine concentrations within the joint fluid and induces synovitis. In vitro, LPS increases PGE$_2$ production by equine synoviocytes. Studies using equine cartilage explants found dose-dependent increases in PGE$_2$ concentrations following varying doses of LPS similar to what was used in our study.

Other studies reported decreased proteoglycan synthesis from cartilage explants after exposure to LPS, but only at concentrations > 0.001 µg/ml. In our study, PGE$_2$ concentrations were higher for LPS-challenged explants but were not significantly different from unchallenged explants, despite preliminary dose-response data that indicated 0.001 µg of LPS/ml is necessary to effectively increase PGE$_2$ concentrations. However, the effects of the NSAID on PGE$_2$ production were greater in the LPS-challenged explants. Culture conditions alone, prior to incubation with LPS, were associated with PGE$_2$ production. It is possible that baseline PGE$_2$ production was already increased as a result of explants being in culture, thereby reducing the effect of LPS. Adverse culture conditions cause release of PGE$_2$ from equine chondrocytes in culture.

Another explanation for the variable response to LPS may be that the LPS dose-response study was performed on synovial membrane from only 1 horse, which does not account for variability between horses (n = 6). A synovial explant culture system was used in our study because it has the advantages of maintaining the 3-dimensional architecture of the tissue and does not preferentially select for 1 cell type compared with monolayer culture systems. The disadvantage is, despite using only villous synovial membrane for explants, synoviocyte numbers may vary between explants. This may also affect PGE$_2$ production in response to LPS between explants.

Time of exposure to LPS may also affect PGE$_2$ production. On the basis of the results of an in vivo studies, which had peak synovial fluid PGE$_2$ concentrations as early as 2 hours following intra-articular injection of LPS, a culture period of 24 hours was considered sufficient to allow expression of the effect of LPS in our study. Other in vitro studies with positive results have also used a 24-hour culture period for LPS exposure to evaluate the effects of anti-inflammatory drugs on synovial monolayer PGE$_2$ production. Findings in in vitro studies also suggest that synovial explant viability may be questionable after 72 hours of culture. In our study, differences in PGE$_2$ concentrations may have been more significant with a higher concentration of LPS.

Cell viability and hyaluronan concentrations were not significantly different among explant treatments. Cell viability was estimated following digestion of synovial explants and counting of individual cells. It is possible that dead cells were sloughed during culture and were not counted as nonviable using this method, explaining the differences between viability and histomorphologic observations in the METH, DMSO, and DFU treated explants. Therefore, assumptions of viability on the basis of trypan blue exclusion should be interpreted with caution.

Hyaluronan is a large, nonsulfated glycosaminoglycan produced by synoviocytes and functions as a boundary lubricant within the joint giving joint fluid its characteristic viscosity. Production of hyaluronan is altered with synovitis or arthritis and manifests clinically as a decrease in the synovial fluid viscosity. Hyaluronan was measured in our study as a marker for synoviocyte function. Concentrations of hyaluronan reported in our study are similar to other reports using equine synovial explant cultures and suggest that incubation with LPS or drugs did not alter synoviocyte function in vitro. Hyaluronan is produced by synoviocytes located deep within the synovial membrane.
which may have decreased exposure to drugs. Alternatively, these cells may be less susceptible to the toxicologic effects of drug.

In conclusion, findings in our study suggest that selective COX-2 inhibition would be beneficial in long-term treatment of pain associated with chronic inflammatory joint disorders, because it would eliminate the adverse effects resulting from COX-1 inhibition often encountered with long-term use of these drugs. In our study, all of the NSAID (ie, PBZ, FNX, KET, CRP, MEL) suppressed PGE2 production in LPS-challenged explants, with MEL appearing to have a more selective inhibition of COX-2. Methylprednisolone also suppressed synovial explant PGE2 production following LPS challenge, but only at the low concentration. High-concentration METH, DMSO, and DFU did not suppress PGE2 production, but did induce negative effects on synovial histomorphologic characteristics.


Escherichia coli LPS, Sigma Chemical Co, St Louis, Mo.

Bacterial, Schering-Plough Animal Health Corp, Kenilworth, NJ.

DMEM, Gibco BRL, Grand Island, NY.

Phenylbutazone, Animals Products Co, Arcadia, Calif.

Pamamine, Schering-Plough Animal Health Corp, Kenilworth, NJ.

Ketofox, Fort Dodge Laboratories Inc, Fort Dodge, Iowa.


Meloxicam, Boehringer Ingelheim Animal Health, St Joseph, Mo.


Domosso, Fort Dodge Laboratories Inc, Fort Dodge, Iowa.

DFU, Merck Sharp and Dohme Research Laboratories, Rahway, NJ.

Tierzyme PGE2, ETA Kit, Perceptive Diagnostics, Cambridge, Mass.

Streptomycyes hyaluroniadase, Sigma Chemical Co, St Louis, Mo.

Collagenase Type IV, Sigma Chemical Co, St Louis, Mo.


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