In vitro cytoprotective effects of acetylsalicylic acid, carprofen, meloxicam, or robenacoxib against apoptosis induced by sodium nitroprusside in canine cruciate ligament cells

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Objective—To determine whether incubation of cruciate ligament cells with acetylsalicylic acid, carprofen, meloxicam, or robenacoxib provides protection against apoptosis induced by sodium nitroprusside (SNP).

Sample—Explants of cranial (CCL) and caudal (CaCL) cruciate ligaments from eight 1-day-old Beagles.

Procedures—Primary cultures of CCL and CaCL cells were created via enzymatic dissociation of cruciate explants. Purified cell cultures were incubated for 2 hours without (controls) or with 1 of 3 concentrations of 1 of 4 NSAIDs (10, 100, or 200 µg of acetylsalicylic acid/mL; 0.1, 1, or 10 µg of carprofen/mL; 0.1, 1, or 10 µg of meloxicam/mL; or 0.1, 1, or 10 µg of robenacoxib/mL) and subsequently incubated for 18 hours with 1 of 3 concentrations of SNP in an attempt to induce mild, moderate, or severe cytotoxic effects. Cell viability and apoptosis were analyzed via a cell proliferation assay and flow cytometry, respectively. Prostaglandin E_2 concentrations were measured via an ELISA.

Results—Cytoprotective effects of NSAIDs were dependent on the extent of SNP-induced apoptosis and were greatest in CCL and CaCL cell cultures with moderate SNP-induced cytotoxic effects. Preincubation with an NSAID improved cell viability by 15% to 45% when CCL and CaCL cells were subsequently incubated with SNP. Carprofen (10 µg/mL) had the greatest cytoprotective effects for CCL and CaCL cells. Incubation with NSAIDs resulted in a nonsignificant decrease in PGE_2 production from SNP-damaged cells.

Conclusions and Clinical Relevance—Results indicated that carprofen, meloxicam, and robenacoxib may reduce apoptosis in cells originating from canine cruciate ligaments. (*Am J Vet Res* 2012;73:1752–1758)

Nonsteroidal anti-inflammatory drugs are commonly used to alleviate clinical signs associated with orthopedic diseases; however, the effect of NSAIDs on the progression of osteoarthritis remains controversial. Evidence suggests that NSAIDs favorably modify the metabolism of proteoglycans, collagen, and the extracellular matrix and decrease the release of proteases or toxic oxygen metabolites.[1] Other evidence indicates that some NSAIDs adversely affect clinically normal cartilage by decreasing proteoglycan synthesis (eg, aspirin) and inducing apoptosis (eg, phenylbutazone).[1] Conversely, COX-2 selective NSAIDs appear to protect joints against some degenerative processes.[2–4] Dogs with disorders associated with the CCL are generally considered to have an underlying degenerative process; increased production of inflammatory mediators by synovial cells of the stifle joint may be secondary to or may be an inciting cause of CCL degeneration. Nitric oxide is a major catabolic factor associated with joint disease and is involved in the regulation of apoptosis.[5]

Other investigators[6,7] have proposed that apoptosis alters the internal strength of the CCL, which results in CCL degeneration. Areas in the CCL that have an ab-

Abbreviations

CaCL	Caudal cruciate ligament
CCL	Cranial cruciate ligament
COX	Cyclooxygenase
DMEM	Dulbecco modified Eagle medium
FCS	Fetal calf serum
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
OD	Optical density
PGE_2	Prostaglandin E_2
SNP	Sodium nitroprusside

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normally high number of apoptotic cells are most susceptible to mechanical injury that can lead to partial or total rupture of the CCL. Thus, decreasing the proportion of CCL cells that are undergoing apoptosis at any given time may aid in the prevention of CCL damage. The objective of the study reported here was to determine whether incubation of canine cruciate ligament cells with 1 of 4 NSAIDS (acetylsalicylic acid, carprofen, meloxicam, or robenacoxib) would affect apoptosis of those cells when they were subsequently incubated with SNP, an NO donor.

Materials and Methods

Cruciate ligament cell collection and culture—All animal procedures were reviewed and approved by the Animal Care and Experimentation Committee of the Canton of Bern, Switzerland. Eight 1-day-old Beagles (5 male and 3 female) were euthanized via IV injection with a pentobarbituate. Within 1 hour after death, the CCLs and CaCLs from each puppy were aseptically harvested and placed in sterile DMEM as described.[8] Briefly, the portion of each ligament at the tibial and femoral ends was trimmed and discarded. The outer synovial layer of each ligament was removed via sharp dissection, and the ligament was cut into sections 0.1 to 0.2 mm in length. To isolate cruciate ligament cells, each section of CCL and CaCL was digested with collagenase type IV[a] and cultured in DMEM supplemented with 15% FCS, 250µM ascorbic acid, penicillin (100 U/mL) and streptomycin (100 U/mL).[9] The cells were incubated in 5% CO_2 at 37°C. When the cell cultures achieved 80% confluence, the cells were treated with 0.025% trypsin and 0.01% EDTA. Then the cells were harvested and placed in 20% FCS and 10% dimethyl sulfoxide and frozen until used.

Purity of the cell cultures from the first through the fifth passages was determined via an immunohistochemistry assay that used antibodies against fibronectin[b] and collagen I.[c] The immunohistochemical staining characteristics of cultured cruciate ligament cells were compared with those of the CCL or CaCL explant from which they had originated as described.[8] Cruciate ligament cell cultures with a purity of > 98% were considered acceptable for use in the experimental treatments described in the present report.

Experimental design—Cruciate ligament cells from the fourth passage were used and incubated with DMEM supplemented with 10% FCS for 24 hours to synchronize cell cycles. The cell cultures were then preincubated without (control) or with a nonselective COX inhibitor (acetylsalicylic acid) or a preferential COX-2 inhibitor (carprofen, meloxicam, or robenacoxib) to assess whether NSAIDs prevented apoptosis when the cells were subsequently incubated with SNP. For all cell cultures except those designated as controls, 1 of 3 concentrations of 1 of the 4 NSAIDs (10, 100, or 200 µg of acetylsalicylic acid/mL; 0.1, 1, or 10 µg of carprofen[d]/mL; 0.1, 1, or 10 µg of meloxicam[e]/mL; or 0.1, 1, or 10 µg of robenacoxib[f]/mL) was added to the culture media of each cell culture, and the cells were incubated for 2 hours. The concentrations evaluated for each NSAID were chosen on the basis of results of preliminary experiments conducted by our laboratory group, in which the extent of apoptosis for untreated control cultures of cruciate ligament cells was compared with that for cultures of cruciate ligament cells that were incubated with various concentrations of each NSAID, and represented what we believed to be experimentally relevant therapeutic ranges for the respective NSAIDs. The highest concentration evaluated for each NSAID in the present study represented the maximum concentration that did not induce cytotoxic effects in the cruciate ligament cell cultures of the preliminary experiments.

Sodium nitroprusside,[g] an NO donor, was used to induce apoptosis in the cruciate ligament cell cultures. Results of preliminary experiments conducted by our laboratory group indicated that viability of CCL and CaCL cells in culture decreased in a dose-dependent manner after incubation with SNP,[10] and CCL cells that were incubated with the NO donors S-nitroso-N-acetyl-D,L-penicillamine or (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1 2-diolate were more susceptible to NO-induced apoptosis than were CaCL cells.[8] Thus, CCL and CaCL cells were incubated with 1 of 3 concentrations of SNP to induce mild (≤ 20% apoptotic cells), moderate (40% to 50% apoptotic cells), or severe (≥ 70% apoptotic cells) cytotoxic effects as identified with flow cytometry. Because the susceptibility of CCL and CaCL cells to SNP-induced apoptosis differs, the concentration of SNP used to induce the 3 levels of cytotoxic effects varied between CCL and CaCL cell cultures and was calculated to allow for an 8% maximum variation in apoptosis between CCL and CaCL cells. Sodium nitroprusside concentrations of 0.1mM and 0.15mM were used to induce mild cytotoxic effects in CCL and CaCL cell cultures, respectively; whereas an SNP concentration of 0.2mM was used to induce moderate cytotoxic effects in both CCL and CaCL cell cultures, and SNP concentrations of 0.35mM and 0.5mM were used to induce severe cytotoxic effects in CCL and CaCL cell cultures, respectively. After 2 hours of preincubation with the assigned NSAID, the assigned concentration of SNP was added to the culture medium of each respective cell culture and the cells were incubated for an additional 18 hours. Each combination of NSAID and SNP concentration was replicated in triplicate.

Sodium nitroprusside generates NO and releases cyanide ions, which can also cause apoptosis. To ascertain that cruciate ligament cell death in the cultures was not caused by cyanide, thiosulfate (10:1) was added to the culture medium to detoxify any cyanide released by the SNP. Therefore, SNP concentrations of 0.1mM to 0.5mM were used and the cytotoxic effects in CCL and CaCL cell cultures were compared with and without additional thiosulfate. Each combination of SNP concentration with and without thiosulfate was replicated in triplicate.

Assessment of apoptosis—Apoptosis of cruciate ligament cells was assessed by means of a colorimetric MTT assay. Briefly, each well of a 96-well plate was seeded with 3×10^4 ligamentous cells, and the colorimetric MTT assay was performed as described.[8] The remaining cell viability within each well was expressed as a percentage of cell viability for cells in untreated

control wells minus cell viability for cells in treated wells relative to that for cells in untreated control wells and calculated as follows: ([OD of control well – OD of blank well] – [OD of sample well – OD of blank well])/(OD of control well – OD of blank well) × 100.

Concurrent with the colorimetric MTT assay, flow cytometry was used to confirm apoptotic cell damage. Briefly, each well of a 6-well plate was seeded with 10^6 ligamentous cells. Cells were then double-stained by use of fluorescein isothiocyanate–conjugated annexin V and propidium iodide and analyzed via a flow cytometer.[h] The double-staining method used allowed discrimination of cells in the early stages of apoptosis (ie, before the loss of cell membrane integrity) from cells in the late stages of apoptosis (ie, after the loss of cell membrane integrity).

PGE_2 production—Results of another study[11] indicate that PGE_2 induces apoptosis in articular chondrocytes; therefore, the effect that 2 hours of preincubation of 2×10^6 cruciate ligament cells in 1 mL of culture medium/well with the respective NSAIDs had on PGE_2 concentration in culture media after cell damage was induced by 18 hours of incubation with SNP was determined. The PGE_2 concentration in the culture medium after incubation with each respective NSAID and SNP at a concentration of 0.2mM, after incubation without NSAIDs and with SNP at a concentration of 0.2mM and 1mM, and after incubation with no NSAIDs and no SNP (control) was determined via a commercially available ELISA[i] in accordance with the manufacturer's instructions.

Statistical analysis—All statistical analyses were performed with statistical software.[j] A 1-way ANOVA with a post hoc Dunnett test was used to make comparisons between the control and each combination of NSAID and SNP. Data were reported as the mean ± SEM or the mean ± SD. For all analyses, values of $P < 0.05$ were considered significant.

Results

Cytoprotective effects of NSAIDs—The percentage reduction of viable cells did not differ between CCL and CaCL cell cultures incubated with and without thiosulfate, which indicated that the SNP concentrations used did not release a sufficient amount of cyanide to affect apoptosis. The cytoprotective effects of the NSAIDs evaluated were dependent on the concentration of SNP; generally, as the concentration of SNP (ie, cytotoxic effects) increased, the ability of an NSAID to protect cells against apoptosis decreased (**Table 1**). For CaCL cell cultures, prophylactic incubation with a selective COX-2 inhibitor (carprofen, meloxicam, or robenacoxib) decreased apoptosis by approximately 5% when the cells were subsequently incubated with 0.5mM SNP to simulate severe cytotoxic effects. Conversely, for the CCL cell cultures, incubation with an NSAID had no effect on the extent of apoptosis when the cells were subsequently incubated

Table 1—Mean ± SEM percentage of cell viability of canine CCL and CaCL cells that were not incubated initially for 2 hours with an NSAID but were incubated for 18 hours with 1 of 3 concentrations of SNP (control) and that for CCL and CaCL cells that were incubated initially for 2 hours with 1 of 3 concentrations of 1 of 4 NSAIDs (10, 100, or 200 µg of acetylsalicylic acid/mL; 0.1, 1, or 10 µg of carprofen/mL; 0.1, 1, or 10 µg of meloxicam/mL; or 0.1, 1, or 10 µg of robenacoxib/mL) and then subsequently incubated for 18 hours with 1 of 3 concentrations of SNP.

		Level of cytotoxic challenge with SNP					
		Severe (n = 4)		Moderate (n = 6)		Mild (n = 3)	
NSAID	NSAID concentration (µg/mL)	CCL	CaCL	CCL	CaCL	CCL	CaCL
Control	0	25.9 ± 3.1	18.7 ± 2.2	50.2 ± 2.6	57.9 ± 1.1	89.3 ± 3.2	83.2 ± 3.8
Acetylsalicylic acid	10	25.8 ± 1.9	15.7 ± 1.3	66.6 ± 3.9*	60.7 ± 1.5	88.6 ± 3.9	80.8 ± 5.9
	100	23.0 ± 1.1	18.0 ± 1.2	59.0 ± 6.0	65.9 ± 3.9	82.9 ± 3.5	69.5 ± 3.7
	200	19.2 ± 1.4	16.2 ± 1.3	60.3 ± 4.9	72.2 ± 2.2*	80.7 ± 3.9	76.7 ± 4.6
Carprofen	0.1	22.7 ± 1.5	18.7 ± 1.4	64.6 ± 2.0*	58.8 ± 1.2	92.7 ± 4.4	83.2 ± 5.0
	1	24.6 ± 1.3	23.9 ± 0.8*	77.5 ± 2.1*	57.2 ± 1.1	102.7 ± 4.4*	84.8 ± 4.6
	10	31.0 ± 2.3	23.5 ± 0.7*	94.9 ± 2.3*	81.9 ± 1.1*	113.8 ± 4.7*	139.9 ± 2.4*
Meloxicam	0.1	22.2 ± 1.5	19.2 ± 0.8	61.4 ± 5.4	59.6 ± 1.2	88.6 ± 3.8	65.9 ± 3.6
	1	25.0 ± 2.1	18.4 ± 1.4	68.1 ± 4.0*	52.0 ± 1.6	87.8 ± 2.8	55.7 ± 1.4
	10	24.0 ± 2.3	24.1 ± 0.9*	65.1 ± 2.3*	73.8 ± 0.5*	78.4 ± 3.3	68.4 ± 5.3
Robenacoxib	0.1	20.8 ± 1.7	17.9 ± 1.5	54.8 ± 2.1	66.9 ± 3.0	76.7 ± 1.8	74.4 ± 2.9
	1	27.7 ± 1.6	18.6 ± 1.2	56.1 ± 3.9	83.8 ± 10.1*	78.9 ± 2.4	81.0 ± 0.9
	10	33.0 ± 1.7	25.0 ± 0.5*	66.2 ± 3.4*	79.9 ± 6.2*	72.1 ± 2.4	77.6 ± 1.2

Eight 1-day-old Beagles were euthanized; within 1 hour of death, both CCLs and CaCLs were aseptically harvested from each puppy and processed to isolate and purify the cruciate ligament cells. Fourth-passage cells obtained from the CCLs and CaCLs were used for all experiments. For all cell cultures except those designated as controls (not incubated with an NSAID), the assigned NSAID was added to the culture medium and the cells were incubated for 2 hours. To induce apoptosis, each cell culture was subsequently incubated with SNP for 18 hours to simulate mild, moderate, or severe cytotoxic effects. Because the susceptibility of CCL and CaCL cells to SNP-induced apoptosis differs, SNP concentrations of 0.1mM and 0.15mM were used to induce mild cytotoxic effects in CCL and CaCL cell cultures, respectively; an SNP concentration of 0.2mM was used to induce moderate cytotoxic effects in both CCL and CaCL cell cultures, and SNP concentrations of 0.35mM and 0.5mM were used to induce severe cytotoxic effects in CCL and CaCL cell cultures, respectively. The number of different cell cultures from different dogs (n) varied dependent on the severity of apoptosis induced by SNP, and each combination of control and SNP concentration and combination of NSAID and SNP concentration was replicated 3 times for each cell culture. Cell cultures that were not incubated with an NSAID or SNP were assumed to have 100% cell viability.

*Within level of SNP cytotoxicity (mild, moderate, or severe) and cell line (CCL or CaCL), value differs significantly ($P < 0.05$) from that of the control.

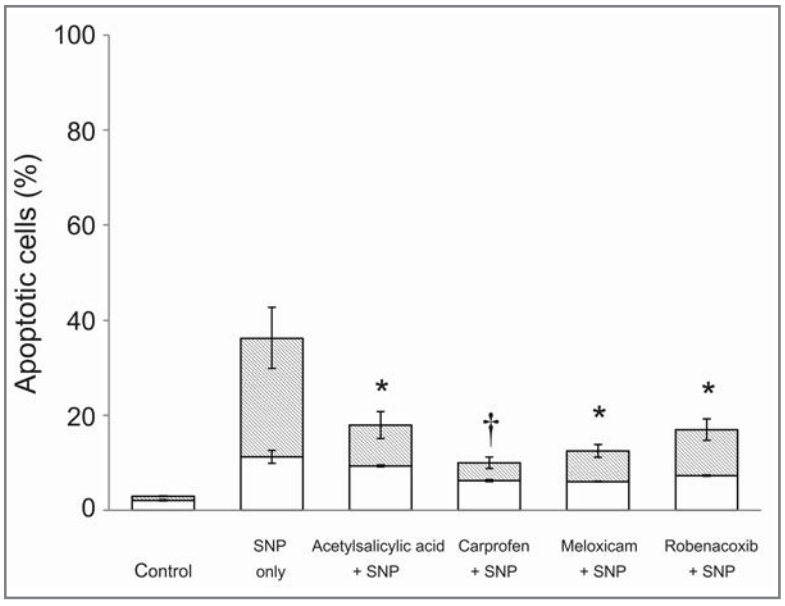

Figure 1—Mean ± SEM percentage of apoptotic cells in cultures of canine CCL cells that were incubated initially without an NSAID and subsequently without SNP (control), incubated initially without an NSAID and subsequently for 18 hours with 0.2mM SNP (SNP only), or incubated initially for 2 hours with acetylsalicylic acid (100 µg/mL), carprofen (10 µg/mL), meloxicam (10 µg/mL), or robenacoxib (10 µg/mL) and subsequently for 18 hours with 0.2mM SNP. Cranial cruciate ligament cells were obtained from eight 1-day-old Beagles within 1 hour after euthanasia. Fourth-passage CCL cells were used for all experiments. Following incubation with the assigned treatments, cells were double-stained with fluorescein isothiocyanate–conjugated annexin V and propidium iodide and analyzed via a flow cytometer, which distinguished cells that were in the early stage of apoptosis (ie, before the loss of cell membrane integrity; white portion of bar) from dead cells (ie, after the loss of cell membrane integrity; shaded portion of bar). Within each treatment, the results represent data obtained from CCL cell cultures obtained from at least 3 dogs, each of which was replicated in triplicate. *Value differs significantly ($P < 0.05$) from that for SNP-only treatment. †Value differs significantly ($P < 0.01$) from that for the SNP-only treatment.

Table 2—Mean ± SD PGE_2 concentration in samples of cell culture medium (determined by an ELISA) obtained from canine CCL and CaCL cells after incubation with various concentrations of different NSAIDs for 2 hours followed by incubation with SNP (0.2mM) for 18 hours, after incubation with SNP only, or after incubation with no NSAID and no SNP (control).

	PGE_2 concentration (pg/mL)	
Treatment	**CCL cells**	**CaCL cells**
Control	41.0 ± 8.4	32.0 ± 4.6
SNP (0.2mM)	95.7 ± 10.3*	87.3 ± 10.9*
SNP (1mM)	239.4 ± 12.6*	179.8 ± 8.2*
Acetylsalicylic acid (100 µg/mL) + SNP (0.2mM)	72.3 ± 8.2	78.0 ± 7.3
Carprofen (10 µg/mL) + SNP (0.2mM)	77.4 ± 6.9	67.2 ± 10.2
Meloxicam (10 µg/mL) + SNP (0.2mM)	74.8 ± 6.2	77.7 ± 6.3
Robenacoxib (10 µg/mL) + SNP (0.2mM)	78.6 ± 4.9	66.2 ± 1.6

*Within a cell line, value differs significantly ($P < 0.01$) from that of the control as determined via a Dunnett test for multiple comparisons.
See Table 1 for remainder of key.

with 0.35mM of SNP to simulate severe cytotoxic effects. For both CCL and CaCL cell cultures, incubation with NSAIDs had the most substantial cytoprotective effects when the cells were subsequently incubated with the SNP concentration (0.2mM) chosen to simulate moderate cytotoxic effects. Specifically, for cell cultures incubated with 0.2mM SNP, incubation of CCL cells with 10 µg of acetylsalicylic acid/mL, 1 µg of meloxicam/mL, 10 µg of meloxicam/mL, or 10 µg of robenacoxib/mL decreased apoptosis by 16 to 18%, compared with the extent of apoptosis for the control CCL cells; preincubation of CCL cells with 10 µg of carprofen/mL decreased apoptosis by almost 45%, compared with that in the control cells. For cell cultures in which moderate cytotoxic effects were induced by SNP (0.2mM), the cytoprotective effects of each NSAID were generally comparable for CCL and CaCL cells. When cell cultures were incubated with the SNP concentration (0.1mM and 0.15mM for CCL and CaCL cell cultures, respectively) chosen to induce mild cytotoxic effects, carprofen was the only NSAID evaluated that provided significant cytoprotective effects in both CCL and CaCL cells. Overall, preincubation of CCL and CaCL cell cultures with 10 µg of carprofen/mL provided the most substantial cytoprotective effects across the spectrum of SNP-induced cytotoxic effects evaluated, whereas prophylactic treatment with acetylsalicylic acid, a nonselective COX inhibitor, provided only minor cytoprotective effects. Results of the flow cytometry analyses indicated that the cytoprotective effects observed for all the NSAIDs were attributable primarily to a reduction of cells in the late stages of apoptosis (ie, cells that had lost cell membrane integrity; **Figure 1**).

Effect of NSAIDs on PGE_2 concentration in culture media—Prostaglandin E_2 concentration was determined in the culture media obtained from cell cultures in which cell damage was induced with 0.2mM SNP because that was the SNP concentration at which preincubation with an NSAID resulted in the most substantial reduction in apoptosis. Incubation of CCL or CaCL cells with 0.2mM and 1mM SNP significantly increased the PGE_2 concentration in the culture media, compared with the PGE_2 concentration in the culture media of CCL or CaCL cells that were not incubated with SNP (ie, controls). Incubation of CCL or CaCL cells with acetylsalicylic acid (100 µg/mL), carprofen (10 µg/mL), meloxicam (10 µg/mL), or robenacoxib (10 µg/mL) and subsequent incubation with 0.2mM SNP resulted in a nonsignificant increase in PGE_2 concentration, compared with that of the respective CCL or CaCL controls (**Table 2**). The magnitude of decrease in PGE_2 concentration in cell culture media did not differ among cell cultures incubated with the various NSAIDs evaluated.

Discussion

Results of the present study indicated that incubation of canine cruciate ligament cells with a preferential COX-2 inhibitor (carprofen, meloxicam, or robenacoxib) reduced apoptosis when those cells were subsequently incubated with SNP, which resulted in exposure of the cells to NO, and was dependent on the

extent of SNP-induced cytotoxic effects (ie, exposure to NO). It has been hypothesized that NO plays an important role in the chronic degeneration of the stifle joint in dogs with cruciate ligament disorders on the basis of results from multiple studies,[12–15] which indicate that NO is associated with the regulation of extracellular matrix synthesis and cytokine and matrix metalloproteinase production. The addition of exogenous NO in the form of S-nitroso-N-acetyl-D,L-penicillamine to articular chondrocytes and cartilage explants results in a dose-dependent increase in metalloprotease activity.[16] In vitro exposure of chondrocytes to exogenous NO via the addition of S-nitrosoglutathione to the culture media inhibits the attachment of chondrocytes to fibronectin and disrupts assembly of actin filaments.[17] Also, the endogenous production of NO by articular cartilage inhibits the synthesis of matrix proteoglycans such as aggrecans.[18] Nitric oxide is produced within inflamed joints affected by rheumatoid arthritis or osteoarthritis.[19–21] Results of various in vitro and ex vivo studies suggest that chondrocytes originating from human,[21,22] bovine,[23] and canine joints[24,25] can generate NO in the presence of an appropriate proinflammatory cytokine stimulus. Exposure of explant cultures obtained from ruptured CCLs of dogs to a stimulation cocktail of IL-1, TNF, and lipopolysaccaride results in the production of large amounts of NO from inducible NO synthases,[24,26] which suggests that NO is associated with CCL rupture in dogs. Therefore, SNP, an NO donor, was used in the present study to induce apoptosis in the canine cruciate ligament cells. The susceptibility of cells to chemically induced apoptosis varies by cell type. Murakami et al[27] reported that fibroblasts obtained from the CCL of rabbits were more sensitive to NO-induced apoptosis than were fibroblasts obtained from the medial collateral ligament. Although the CCL and CaCL of dogs have the same nutritional source, blood supply, and extrasynovial environment, the CCL ruptures more frequently than does the CaCL. Results of another study[8] involving canine cruciate ligament cells conducted by our laboratory group as well as preliminary data obtained for the present study indicate that CCL cells are more susceptible to NO-induced apoptosis than are CaCL cells; therefore, we chose to evaluate the cytoprotective effects of NSAIDs on both CCL and CaCL cells in the present study.

The standard of care for the treatment of CCL disorders in dogs includes the use of analgesics and anti-inflammatory drugs and surgery. Treatment of osteoarthritis with anti-inflammatory agents, specifically NSAIDs, versus with an analgesic agent remains controversial. Results of some studies[28–30] indicate that NSAIDs induce apoptosis in various cell lines, including colon cancer cells. Conversely, results of other studies[3,31–33] indicate that NSAIDs have antiapoptotic effects in other cell lines. The mechanism by which NSAIDs regulate apoptosis is complicated and has not yet been completely defined. In the present study, a general COX inhibitor (acetylsalicylic acid) and preferential COX-2 inhibitors (carprofen, meloxicam, and robenacoxib) prevented apoptosis of CCL and CaCL cells when those cells were subsequently exposed to SNP under certain conditions.

The NSAID concentrations evaluated in the present study were chosen to represent the range of concentrations that are expected to be achieved in the synovial fluid of dogs after oral administration of the currently recommended dose of each respective NSAID. In dogs, oral administration of 2.3 to 2.7 mg of robenacoxib/kg results in a maximum robenacoxib concentration of 0.5 µg/mL in the synovial fluid[34] and administration of 0.31 mg of meloxicam/kg results in a maximum meloxicam concentration of 0.6 µg/mL in the synovial fluid.[35] In 1 study,[36] acetylsalicylic acid (23 to 86 mg/kg, PO) administered twice daily to dogs for the treatment of lameness resulted in plasma concentrations ranging from 71 to 281 µg/mL (toxic effects develop when the circulating acetylsalicylic acid concentration exceeds 300 µg/mL); the corresponding concentration of acetylsalicylic acid in the synovial fluid was not determined. Likewise, the synovial fluid concentration of carprofen following oral administration to dogs has not been determined; however, in horses, administration of racemic carprofen (0.7 mg/kg, IV) achieved a concentration of 0.5 µg/mL of carprofen in the synovial fluid.[37]

In the present study, incubation of CCL cells with concentrations of carprofen and meloxicam ranging from 0.1 to 1 µg/mL resulted in a significant reduction in apoptosis, and incubation of CaCL cells with 1 µg of robenacoxib/mL significantly decreased apoptosis. Generally, SNP-induced apoptosis of CCL and CaCL cells decreased as the concentration of the NSAID with which they were preincubated increased. The extent of apoptosis within the cruciate ligament cell cultures was positively associated with the SNP concentration, and the most substantial cytoprotective effects of the NSAIDs were detected at the SNP concentration (0.2mM) used to induce moderate cytotoxic effects. For cell cultures in which severe cytotoxic effects were induced with SNP, the NSAIDs evaluated provided no cytoprotective effects for CCL cells and only reduced apoptosis by approximately 5% for CaCL cells. For cell cultures in which mild cytotoxic effects were induced with SNP, carprofen (10 µg/mL) was the only NSAID that provided significant cytoprotective effects for both CCL and CaCL cells and improved cell viability by > 100%, compared with that for the respective CCL and CaCL control cultures. Although results of the present study suggested that administration of NSAIDs may protect cruciate ligament cells from NO-induced apoptosis, the concentrations of NSAIDs with which the cell cultures were incubated may exceed the in vivo concentrations of NSAIDs that can be achieved in the synovial fluid following administration of therapeutically relevant doses of those NSAIDs. Further research is necessary to determine whether the cytoprotective effects of NSAIDs on cruciate ligament cells in vitro can be replicated in vivo.

Results of other studies[38,39] indicate an association between NO-induced apoptosis and the production of PGE_2. The results of the present study indicated that incubation of CCL and CaCl cells with SNP stimulated PGE_2 production, compared with that of the control cultures that were not incubated with SNP. The incubation of CCL and CaCL cell cultures with the NSAIDs evaluated did not significantly affect PGE_2 concentration,

which suggests that the cytoprotective effects of the NSAIDs are not mediated by the inhibition of PGE_2 production. Additionally, in other experiments performed by our laboratory group, we determined that COX-2 expression was not upregulated in CCL and CaCL cells after incubation with SNP, compared with that in the control cells that were not incubated with SNP as determined by western blot analysis.[10] This finding suggests that the minor increase in PGE_2 production detected in the cell cultures of the present study may have been caused by expression of COX-1 instead of COX-2. Cyclooxygenase-1 is constitutively expressed by many cells in various tissues and generates various prostaglandins for physiologic functions, whereas COX-2 is generally undetectable in clinically normal tissues and is induced by proinflammatory agents.[40] On the basis of the results of the present study, we speculate that cells of canine cruciate ligaments degenerate and undergo apoptosis without producing PGE_2, which is in contrast to other synovial tissue cells. Consequently, we hypothesize that inflammation does not have a prominent role in degeneration of the cruciate ligaments.

The results of the present study further elucidated the molecular mechanisms involved in SNP-induced apoptosis in canine cruciate ligaments and suggested that administration of NSAIDs may be beneficial in alleviating ligament degeneration in dogs with cranial cruciate disorders in addition to providing analgesia for affected dogs. Additional research is necessary to completely define the molecular pathways involved in the apoptosis of ligamentous cells and degradation of the extracellular matrix so effective strategies can be developed to preserve stifle joint function in dogs affected with cruciate ligament disorders.

a. Collagen type IV, Sigma-Aldrich, Buchs, Switzerland.
b. Antifibronectin antibody, Sigma-Aldrich, Buchs, Switzerland.
c. Anticollagen I antibody, Abcam, Cambridge, Mass.
d. Rimadyl, Pfizer Animal Health, Zurich, Switzerland.
e. Metacam, Boehringer Ingelheim, Basel, Switzerland.
f. Onsior, Novartis, Basel, Switzerland.
g. Sodium nitroprusside dihydrate, Enzo Life Sciences, Lausen, Switzerland.
h. FACS LRII, BD Biosciences, New York, NY.
i. Prostaglandin E_2 EIA kit, Cayman Chemical Co, Ann Arbor, Mich.
j. NCSS version 2007, NCSS LLC, Kaysville, Utah.

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