Osteoarthritis (OA) is one of the most common joint diseases in many kinds of mammals. Approximately 20% of adult dogs could have osteoarthritic joints [14]. OA has been defined as an inherently noninflammatory disorder of movable joints characterized by degeneration of articular cartilage and by the formation of new bone at joint surfaces and their margins [24]. Recent studies indicated that the inflammatory reaction of the synovium could play an important role in the degenerative process of the osteoarthritic structure [22, 27]. In osteoarthritic joints, the reactive synovium shows hypertrophy and hyperplasia with an increased number of lining cells and also infiltration of inflammatory cells, revealing that inflammation would certainly be participating in the process [21]. Severe synovitis, which is consistently seen in rheumatoid arthritis pannus, is one of the common findings in some pathologies of canine OA [9, 13]. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, play a central role in OA. Activated synoviocytes are one of the main sources of cytokines, and an increase in synoviocytes worsens inflammation of the synovium and other structures of the entire joint [25, 28]. Thus, inducing apoptosis of the activated and increased numbers of synoviocytes is thought to be a part of the anti-inflammatory effects that improve osteoarthritic conditions [3].

In small animal practice, nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used as a symptomatic treatment for OA [24, 26]. It tends to be believed that NSAIDs promote anti-inflammatory and analgesic effects by inhibition of cyclooxygenase (COX), which catalyses the conversion of arachidonic acid to inflammatory mediators such as prostaglandins (PGs) and thromboxanes [24, 29]. Most of the favorable effects of NSAIDs in OA patients, however, seem to be limited to relieving pain and not direct reduction of joint inflammation [26].

In recent years, COX-2 inhibitors, a type of NSAID, such as carprofen, meloxicam, firocoxib and deracoxib have been widely used to treat canine OA as symptomatic osteoarthritis agents, which minimize fatal adverse effects, such as gastrointestinal hemorrhage [18]. Tepoxalin is currently used as one of the other options for canine OA treatment and has been mentioned as an unique NSAID that acts as a dual inhibitor, inhibiting both COX and lipooxygenase (LOX) [12]. The harmful gastrointestinal effects of tepoxalin are thought to be as same as the effects of meloxicam [7]. Anti-inflammatory properties related to LOX inhibition are likely to suppress reactive synovitis in OA affected joints. However, knowledge of the clinical efficacy of the anti-inflammatory properties of tepoxalin is very limited in the literature [1, 11, 20, 23].

The objectives of the present study were to evaluate the magnitude and manner of cytotoxicity of tepoxalin in cultured osteoarthritic synoviocytes in vitro.
MATERIALS AND METHODS

**Cell culture:** Canine fibroblast-like synoviocytes (CFLSs) were freshly isolated from inner layers of joint capsules removed from 3 dogs undergoing surgery for cranial cruciate ligament rupture. All the dogs suffered from secondary OA and had an enlarged synovium, from which a portion was to be removed. In order to use this tissue in this study, informed consent was obtained from the owners of the patients. Synovial tissue was dissected out, minced and incubated for 4 hr at 37°C in 5% CO2 with serum-free Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco-BRL, Paisley, U.K.) supplemented with 4 mg/ml of collagenase type IA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and antibiotics (100 IU/ml of penicillin and 0.1 mg/ml of streptomycin). Cells were suspended in DMEM with 10% fetal bovine serum (FBS; Biomedical Inc., Aurora, OH, U.S.A.), 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Cells were incubated at 37°C in 5% CO2 to obtain monolayer adherent cultures. At confluence, cells were trypsinized and passed. Cells between the second and fifth passage were used for experiments. Cell viability, assessed by trypan blue exclusion test, always exceeded 90%.

**In vitro cell viability assay:** The viability of cells was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [4]. In a 96-well flat-bottomed microplate (Corning, Corning, NY, U.S.A.), 2 × 10^4 cells/well of CFLSs were incubated for 24 hr at 37°C. CFLSs were treated with different concentrations of meloxicam (Wako), carprofen (Wako), tepoxalin (Zubrin, Takeda Schering-Plough Animal Health, Osaka, Japan) and AA-861 (Wako) for 24 hr. To determine the activity of caspase-3, 100 µM of a caspase-3-specific inhibitor, N-Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO, Wako), was added 1 hr prior to exposure to tested agents. To investigate the involvement of the 5-LOX pathway, 10 µM of a 5-LOX metabolite, 5-oxoETE (Cayman, Ann Arbor, MI, U.S.A.), was added with tepoxalin and AA-861. At the end of the treatment, 10 µl of MTT solution (10 mg/ml, Wako) was added. After 4 hr of incubation, the cultures were centrifuged (760 × g, 10 min). The supernatants were removed, and 100 µl of solution buffer (47.5 µl of deionized distilled water with 47.5 µl of N-N-dimethylformamide and 20 mg of sodium dodecyl sulfate, pH 4.7) was added to each well and oscillated for 1 min. The light absorbance of each well was measured with a wavelength of 570 nm. The results were presented as the average of triplicate samples. All test drugs were dissolved in dimethyl sulfoxide (DMSO). Final concentrations of DMSO in all tested solutions were less than 0.1%.

**Morphological evaluation by optical microscopic analysis:** On 8-well chamber slide glasses (Nalge Nunc International, Rochester, NY, U.S.A.), 1 × 10^4 cells/well of CFLSs were seeded and incubated for 24 hr at 37°C in 5% CO2 with serum-free DMEM. Solutions of drugs were added to each well, and the cells were then incubated for another 24 hr. Final concentrations of test drugs were 100 µM for tepoxalin, 100 µM for carprofen, 100 µM for meloxicam and 10 µM for AA-861. Slides were air-dried, fixed with methanol for 1 min and stained with Giemsa solution (Merck, Whitehouse Station, NJ, U.S.A.). The morphological changes in the nucleus and cytoplasm of cells were observed with an optical microscope.

**Evaluation of apoptosis induction by fluorescence microscopic analysis:** Treated cells were washed with cold phosphate-buffered saline twice. Cells were suspended in buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl2, pH 7.4) at a concentration of 1 × 10^6 cells/ml. Ten microliters of annexin V, Alexa Fluor® 488 conjugate (Lonzza, Walkersville, MD, U.S.A.) and 1 µM of Hoechst 33342 solution (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) were added to the cells, and the cells were incubated for 15 min at room temperature. The nuclear morphology of the cells and cell membrane were visualized using a fluorescence microscope (FV500, Olympus, Tokyo, Japan).

**Data analysis and statistics:** Each data set was expressed as the mean ± SD. All data were statistically analyzed by performing the Tukey-Kramer test using a commercial statistical software (StatView 5.0, SAS institute Inc., Cary, NC, U.S.A.) to determine significant differences between groups. P values less than 0.05 were considered significant.

**RESULTS**

**Cell viability:** The viability of CFLSs treated with each drug is shown in Fig. 1A significant decrease (P<0.01) in cell viability was observed with 100 µM of tepoxalin in comparison with cells treated with vehicle only (0.1% DMSO). In contrast, carprofen and meloxicam had no significant effects on cell viability at all examined concentrations. Supplementation with AA-861 reduced cell viability by much more than supplementation with tepoxalin.

**Apoptosis-related morphological changes in CFLSs:** As shown in Fig. 2, morphological changes were observed in...
Giemsa-stained CFLSs treated with tepoxalin or AA-861. Severe shrinkage in the nucleus and strong staining in the cytoplasm were evident after culture with either drug. There were no morphological changes found in CFLSs treated with carprofen or meloxicam compared with the control. Annexin V-FITC/Hoechst 33342 double staining was performed to detect cells in the early phase of apoptosis after treatment (Fig. 3). Control cells were not stained by annexin V and were stained only by Hoechst 33342 (Fig. 3A). Annexin V-positive CFLSs were observed after treatment with 100 μM of tepoxalin (Fig. 3B) and 10 μM of AA-861 (Fig. 3C). Shrinkage in the nucleus was detected in tepoxalin- and AA-861-treated cells by Hoechst 33342 nuclear staining (Fig. 3B and 3C). Fragmentation of CFLSs nucleus was not detected in the control and tepoxalin- and AA-861-treated cells.

Change of cell viability by caspase-3 inhibition: Tepoxalin and AA-861 proved to be able to induce apoptosis in CFLSs at certain doses after inhibition of caspase-3. Some of this proapoptotic effect induced by tepoxalin or AA-861 was partially inhibited by Ac-DEVD-CHO (Fig. 4). The survival rate of CFLSs treated with 20 μM of tepoxalin was 7.8 ± 5.7%. The rate was then significantly improved to 44.1 ± 16.2% by supplementation with 100 μM of Ac-DEVD-CHO.
The survival rate of CFLSs in 30 μM of tepoxalin (1.9 ± 1.7%) was likewise raised to 18.1 ± 3.4% by Ac-DEVD-CHO (P < 0.01). Treatment with 10 μM of AA-861 reduced the cell viability of CFLSs to 15 ± 4.6%. The rate was then significantly improved to 71.4 ± 13.6% by 100 μM of Ac-DEVD-CHO (P < 0.01). The survival rate of CFLSs in 20 μM of AA-861 (10.9 ± 3.5%) was raised to 21.1 ± 6.1% by Ac-DEVD-CHO (P < 0.05).

**DISCUSSION**

Osteoarthritis occurs frequently in certain populations of dog, and could cause severe synovitis [22]. An inflamed synovium is one of the main sources of cytokines [15] and needs to be controlled for prevention of catastrophic sequelae. Thus, inducing apoptosis of activated and increased numbers of synoviocytes is thought to be part of the anti-inflammatory effects that modify OA conditions [17]. In this study, tepoxalin showed definite cytotoxic effects on CFLSs. Carprofen and meloxicam had no significant effects on cell viabilities at any examined doses. Inhibition of 5-LOX with a specific 5-LOX inhibitor, AA-861, showed clear cytotoxic effects on CFLSs. Morphological findings and a caspase-3 inhibition assay then showed that these cytotoxic effects of tepoxalin and AA-861 were induced as the results of inducing cell apoptosis. The results indicated that LOX inhibition may suppress synovium growth and that COX-2-selective inhibitors, widely used as anti-inflammatory drugs, would not affect the viabilities of inflammatory synoviocytes. This hypothesis was also supported by our results showing the inhibitory effects of 5-LOX metabolite on the cytotoxicity of tepoxalin and AA-861 and that 5-LOX activity could regulate survival of CFLSs. Tepoxalin, a COX/LOX dual inhibitor, would have unique inflammatory properties as an NSAIDs and directly or indirectly suppress viability of inflammatory synoviocytes by inhibition of LOX. Many reports have shown that 5-LOX plays an important role in various diseases associated with cell proliferation and that inhibition of 5-LOX resulted in cytotoxicity [10, 16, 31]. Overexpression of 5-LOX in tissue samples of primary tumor, as well as in established cancer cell lines, has been reported [5]. Important roles of 5-LOX and its
products in the survival and growth of human tumor cells have also been reported [19, 30]. Tepoxalin might somehow support suppression of LOX-dependent cell growth.

In this study, a higher tepoxalin concentration was necessary to induce definite apoptosis than the physiological concentration at oral administration of the recommended dose. These findings could explain why the LOX inhibitory effects of tepoxalin may suppress LOX-dependent cell growth in vitro but are not strong enough in vivo. A recent report showed that the 5-LOX metabolite, leukotriene B\(_4\) (LTB\(_4\)) promotes migratory and invasive behavior and induces inflammation to fibroblast-like-synoviocytes of mice [6]. It is known that tepoxalin inhibits LTB\(_4 \) in vivo at a low concentration [2]. These reports suggest the advantageous effects of 5-LOX inhibition in addition to COX inhibition. Lipoxin is an eicosanoid produced by LOX catalysis of arachidonic acid. These LOX products play roles as physiological mediators to optimize articular cartilage metabolism [8]. Thus, complete inhibition of LOX could lead to severe adverse effects. It is known that tepoxalin inhibits less than half of the lipoxigenase activity, and production of 5-LOX metabolites is not completely blocked at recommended concentrations in vivo [2]. Mild inhibition of LOX by tepoxalin might be more favorable to use than specific LOX inhibitors.

In the present study, all data were obtained by in vitro study. This experimental condition did not always represent actual synovium conditions in OA. The number of cell sources was also not satisfactory enough to prove the effects of tepoxalin on the OA synovium in vivo. However, regarding its anti-inflammatory properties, it appears that tepoxalin had a significant proapoptotic effect on CFLSs at relatively high concentrations [7]. Further studies are required to determine more accurate clinical efficacy for practical use of tepoxalin.

In conclusion, tepoxalin appeared to reduce the number of proliferating synovial cells in vitro, possibly by inhibiting LOX and direct effects. These findings suggest that the clinical effects of tepoxalin could result not only from alleviation of pain but also from direct antiproliferative effects on the inflamed synovium.

REFERENCES


