Licofelone (ML-3000), a Dual Inhibitor of 5-Lipoxygenase and Cyclooxygenase, Reduces the Level of Cartilage Chondrocyte Death *in Vivo* in Experimental Dog Osteoarthritis: Inhibition of Pro-Apoptotic Factors

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ABSTRACT. ***Objective.*** To evaluate *in vivo* therapeutic efficacy of licofelone, a novel competitive dual inhibitor of 5-lipoxygenase (5-LOX) and cyclooxygenase (COX) in chondrocyte death in the canine ligament transection model of osteoarthritis (OA), and to explore its effect on factors involved in the apoptotic phenomenon, i.e., caspase-3, COX-2, and inducible nitric oxide synthase (iNOS).

Methods. Cartilage specimens were obtained from 3 experimental groups of dogs: Group 1, dogs subjected to sectioning of the anterior cruciate ligament of the right knee and given placebo treatment; Groups 2 and 3, operated dogs that received oral treatment with licofelone (2.5 or 5.0 mg/kg/day, respectively) for 8 weeks starting immediately after surgery. All dogs were killed 8 weeks postsurgery. The cartilage level of chondrocyte death was detected by TUNEL reaction. Cartilage distribution of caspase-3, COX-2, and iNOS was documented by immunohistochemistry using specific antibodies, and other levels were quantified by morphometric analysis.

Results. In cartilage specimens from placebo treated dogs a large number of chondrocytes in the superficial layers stained positive for TUNEL reaction. Treatment with therapeutic concentrations of licofelone (2.5 and 5.0 mg/kg/day) markedly reduced the level of chondrocyte apoptosis to the same extent in both therapeutic groups ($p < 0.0001$, $p < 0.002$, respectively). In these groups, the levels of caspase-3, COX-2, and iNOS in cartilage from both condyles and plateaus were also significantly decreased ($p < 0.0001$, $p < 0.0001$, $p < 0.0002$, respectively) compared to the control (placebo) group.

Conclusion. Licofelone is an effective treatment *in vivo*, capable of reducing the level of OA chondrocyte death. This effect is likely mediated by a decrease in the level of caspase-3 activity, which may be related to the reduced production of 2 major factors involved in chondrocyte apoptosis, NO and prostaglandin E_2. These findings may explain some of the mechanisms by which licofelone reduces the progression of experimental OA. (J Rheumatol 2002;29:1446–53)

Key Indexing Terms:
LICOFELONE CHONDROCYTE DEATH EXPERIMENTAL OSTEOARTHRITIS

Osteoarthritis (OA), the most common arthritic disease, includes changes at all levels of all joint tissues, namely cartilage, synovial membrane, and the subchondral bone[1]. The progressive loss of cartilage during the course of the disease is complex and likely has many origins. Based on recent studies on the pathophysiology of OA, the loss of cartilage matrix, one of the most characteristic morphological changes of OA, is related to several factors leading to an imbalance in synthetic and degradative processes[1,2]. Research has focused on factors responsible for reducing the anabolism of OA chondrocytes. Today, the role of proinflammatory cytokines and nitric oxide (NO) seems preponderant[1-6]. However, an additional factor recently proposed is the death of chondrocytes. Studies have shown that apop-

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tosis seems to be the most common phenomenon by which OA chondrocytes die[7-9]. This phenomenon is accentuated by the loss of matrix, indicating some kind of positive relationship between these 2 phenomena[10].

The chondrocyte death/apoptotic phenomenon in OA is complex and seems related to the excess synthesis of factors having pro-apoptotic activity in cartilage and synovium. Among these, NO and prostaglandin E_2 (PGE_2) seem predominant[1-4,11]. The synthesis of many of these factors is likely due to excess production of cytokines. Mechanical factors also seem to play an important role[12].

Using an experimental dog model of OA created by anterior cruciate ligament sectioning of the knee joint, we found that licofelone, a new antiinflammatory drug with competitive dual inhibitory activity on 5-lipoxygenase (5-LOX) and cyclooxygenase (COX), could reduce the progression of experimental OA[13]. This effect was associated with a decrease in the levels of interleukin 1ß (IL-1ß) and matrix metalloprotease-1 (MMP-1).

We explored whether the effect of licofelone in reducing cartilage damage could also be related to other mechanisms, particularly to chondrocyte death, as well as the regulators of this phenomenon.

MATERIALS AND METHODS

Specimen selection. Specimens of cartilage obtained from dogs from different experimental groups included in our previous study[13] were used: Group 1 — dogs subjected to sectioning of the anterior cruciate ligament of the right knee and given placebo treatment; Group 2 — OA dogs treated with licofelone (2.5 mg/kg/day orally); Group 3 — OA dogs treated with licofelone (5 mg/kg/day orally) beginning immediately after surgery.

In situ detection of apoptosis. Cartilage sections were fixed in TissuFix #2 (Laboratoires Gilles Chaput, Montreal, Quebec, Canada) for 24 h and embedded in paraffin. Sections (5 µm) were floated onto Superfrost Plus slides (Fisher Scientific, Nepean, ON, Canada). *In situ* detection of cell death was performed as described[14] using the Apoptag® Plus peroxidase *in situ* apoptosis detection kit (Intergen, Purchase, NY, USA). Briefly, sections were digested with chondroitinase ABC (0.25 units/ml; Sigma-Aldrich, Oakville, ON, Canada) in phosphate buffered saline (PBS; Sigma-Aldrich) for 45 min at 37°C, permeabilized with Triton X100 0.3% for 30 min, and further digested with proteinase K (20 µg/ml; Sigma-Aldrich) for 30 min at room temperature. Nucleotides labeled with digoxigenin were enzymatically added to the DNA by TdT (terminal deoxynucleotidyl transferase). This was followed by binding to anti-digoxigenin antibody peroxidase conjugate, which was detected with peroxidase substrate (Intergen). Sections were counterstained with eosin.

Immunohistochemical studies. Cartilage specimens from condyles and plateaus were processed for immunohistochemical analysis as described[15,16]. Briefly, specimens were fixed in Tissufix #2 (Laboratoires Gilles Chaput) for 24 h, then embedded in paraffin. Sections (5 µm) of paraffin embedded specimens were placed on Superfrost Plus slides (Fisher Scientific), deparaffinized in toluene, hydrated in a graded series of ethanol, and preincubated with chondroitinase ABC (0.25 units/ml) in PBS for 90 min at 37°C or after heating at 65°C for 20 min in 10 mM sodium citrate buffer, pH 6.0 (for caspase-3 immunodetection). Then the specimens were washed in PBS, permeabilized in Triton X100 (0.3%) for 30 min, and washed again and then incubated with 0.3% hydrogen peroxide/methanol for 30 min. Slides were further incubated with Universal Blocking Solution (Dako Diagnostics Canada Inc., Mississauga, ON, Canada) for 30 min, blotted, and then overlaid for 18 h at 4°C in a humidified chamber with: (1) a rabbit polyclonal (IgG) anti-caspase-3 antibody (5 µg/ml; R&D Systems, Minneapolis, MN, USA), which recognized only the mature form (p20 subunit) of the enzyme; (2) a rabbit polyclonal antibody (IgG) against inducible NO synthase (iNOS) (1 µg/ml, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); and (3) a rabbit polyclonal antibody (IgG) against rhCOX-2 (dilution 1/250; Oxford Biomedical Research Inc., Oxford, MI, USA).

Each slide was washed 3 times in PBS (pH 7.4), then stained using the avidin-biotin complex method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). This method entails incubation in the presence of the biotin conjugated secondary antibody for 45 min at room temperature followed by the addition of the avidin-biotin-peroxidase complex for 45 min. All incubations were carried out in a humidified chamber, and the color developed with DAB substrate-chromogen (Dako) containing hydrogen peroxide.

To determine the specificity of staining, 3 control procedures were employed according to the same experimental protocol: (1) use of adsorbed immune serum (1 h, 37°C) with 10 to 20-fold molar excess of recombinant or purified antigen; (2) omission of the primary antibody; and (3) substitution of the primary antibody with an autologous preimmune serum. All these antibodies have been shown to be specific for these dog proteins[14,17].

Several sections were made from each block of cartilage, and slides from each specimen were processed for immunohistochemical analysis. Each section was examined under a Leitz Orthplan light microscope and photographed with Kodak Ektachrome 64 ASA film.

Morphometric analysis. Three sections from each femoral condyle and tibial plateau specimen were examined using a Leitz Diaplan microscope (×40), and each section was scored separately. These data were then integrated as a mean for each specimen. The number of chondrocytes staining positive in the superficial zone (superficial and upper intermediate layers) of cartilage for TUNEL reaction, caspase-3, COX-2, or iNOS was estimated as described[15,17]. Briefly, each section was divided into 3 different areas at the superficial layers of cartilage. For each specimen, it was ensured before the evaluation that an intact cartilage surface could be detected for use as a marker to validate the morphometric analysis.

Cell count scores were determined separately for the lateral and medial sides of the condyles and plateaus. The total number of chondrocytes and the number of chondrocytes staining positive were then quantitated separately for the superficial zone. The final results were expressed as the percentage of positive chondrocytes. The maximum score for each cartilage specimen was 100%. Each slide was evaluated by 2 independent observers under blinded conditions (CB and JPP): variation between the observers' findings was < 5%.

RESULTS

Drug administration and circulating drug level. Licofelone was administered twice daily (8:00 AM and 4:00 PM) with food, at a total daily dose of 2.5 mg/kg or 5.0 mg/kg[13]. The drug was given orally in a capsule. Drug treatment was initiated immediately after surgery and continued for 8 weeks; then the animals were killed. The plasma concentration of licofelone was measured at 4 weeks and at the end of the study. The 4 week samples were obtained at 2 h [maximum concentration (C_{max})] and the 8 week samples at 12 h [minimum concentration (C_{min})] after the drug was administered. The mean ± SEM 2 h postadministration serum level (C_{max}) of licofelone was 371 ± 119 and 629 ± 331 µg/ml in the 2.5 and 5.0 mg/kg/day groups, respectively. These levels were comparable with those in humans after the administration of licofelone in single doses of 100 mg or 200 mg

(Merckle GmbH: internal data). The drug levels at the time the dogs were killed (C_{min}) were 110 ± 17 and 200 ± 17 µg/ml in the 2.5 and 5.0 mg/kg/day groups, respectively, which are similar to those found in the phase II and III clinical trials in patients with OA (Merckle GmbH: internal data).

Cartilage chondrocyte death (Table 1, Figure 1). A large percentage of chondrocytes in the superficial layers of OA cartilage from placebo treated dogs stained positive for the TUNEL reaction. We have reported that in this OA model, the level of TUNEL positive cells found in OA cartilage was statistically higher than that in normal cartilage[4]. Treatment with therapeutic levels of licofelone significantly reduced the number of apoptotic chondrocytes in the OA cartilage.

Table 1. Level of chondrocyte death (TUNEL reaction) in canine knee cartilage. Values are the mean ± SEM.

Group	No. of Animals	% Positive Cells	
		Femoral Condyles	Tibial Plateaus
OA control (placebo)	7	30.8 ± 1.9	31.0 ± 2.4
Licofelone 2.5 mg/kg/day	7	9.6 ± 1.9	12.0 ± 1.7
		p < 0.0001	p < 0.0001
Licofelone 5.0 mg/kg/day	7	11.5 ± 1.9	14.0 ± 1.8
		p < 0.0001	p < 0.0002

Statistical analysis by Mann-Whitney U test. P values were compared to OA control group.

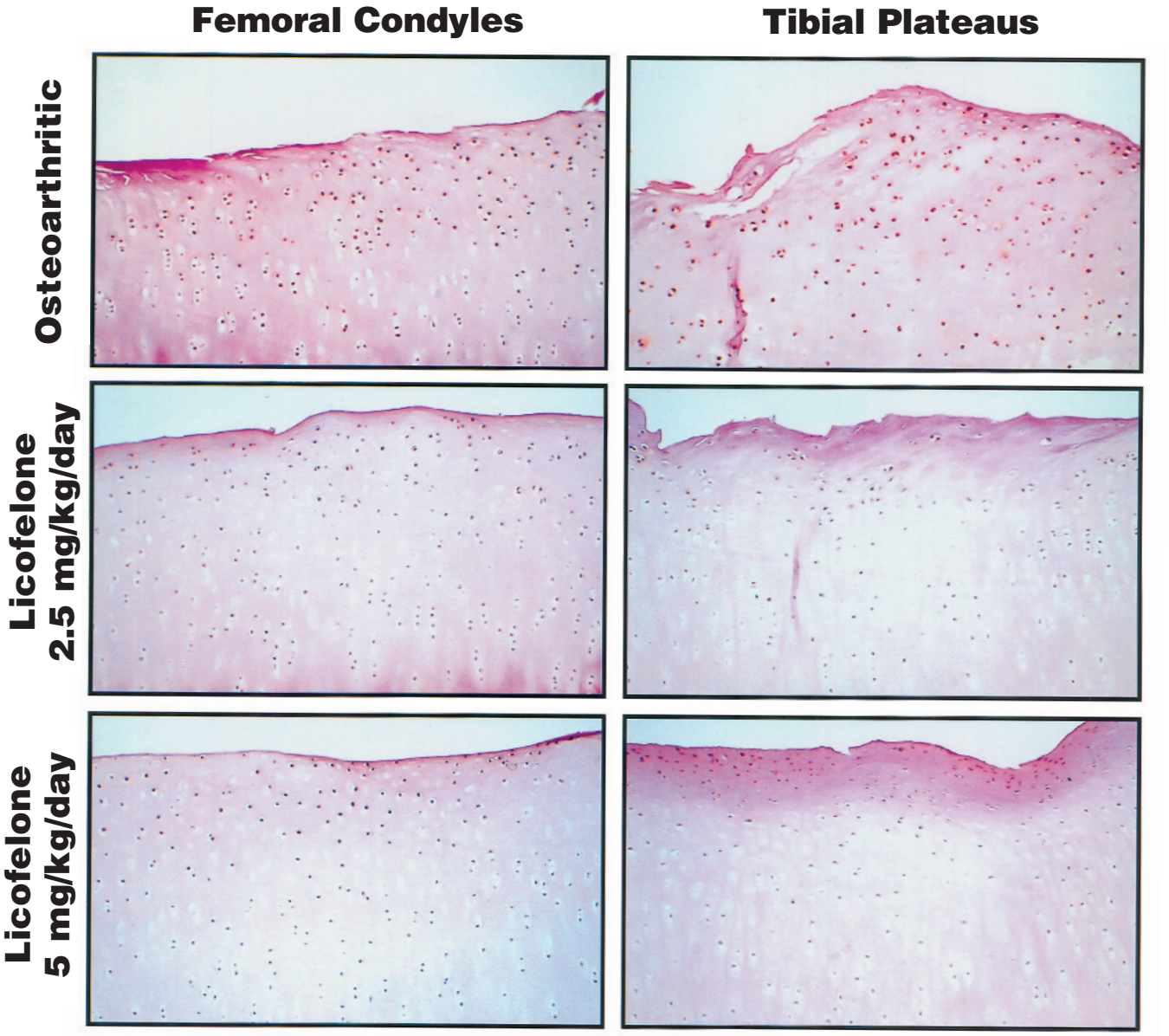

Figure 1. Representative sections of cartilage showing *in situ* detection of chondrocyte death by TUNEL reaction staining in femoral condyles (left) and tibial plateaus (right). A and B. Placebo treated dog OA. C and D. Dog OA treated with licofelone 2.5 mg/kg/day. E and F. Dog OA treated with licofelone 5.0 mg/kg/day (original magnification ×100).

The extent of reduction was similar for both dosages of the drug that were tested in this study. The control done by omission of terminal deoxynucleotidyl transferase showed only background staining (data not shown).

Immunohistochemistry

Caspase-3. About 30% of the chondrocytes in the superficial layers of OA cartilage from placebo treated dogs stained positive for active caspase-3 (Table 2, Figure 2). In dogs treated with licofelone, a marked and significant decrease in the number of chondrocytes staining positive for this enzyme was observed. The extent of this reduction in the level of caspase-3 was more marked with the 5 mg than the

Table 2. Caspase-3 level in canine knee cartilage. Values are the mean ± SEM.

Group	No. of Animals	% Positive Cells	
		Femoral Condyles	Tibial Plateaus
OA control (placebo)	7	30.5 ± 2.7	29.6 ± 3.4
Licofelone 2.5 mg/kg/day	7	6.6 ± 1.5 $p < 0.0001$	5.4 ± 1.1 $p < 0.0001$
Licofelone 5.0 mg/kg/day	7	3.7 ± 1.1 $p < 0.0001$ ($p < 0.05^{\dagger}$)	2.5 ± 0.6 $p < 0.0001$ ($p < 0.04^{\dagger}$)

P value versus the OA group, Mann-Whitney U test. † P value versus licofelone 2.5 mg/kg/day, Mann-Whitney U test.

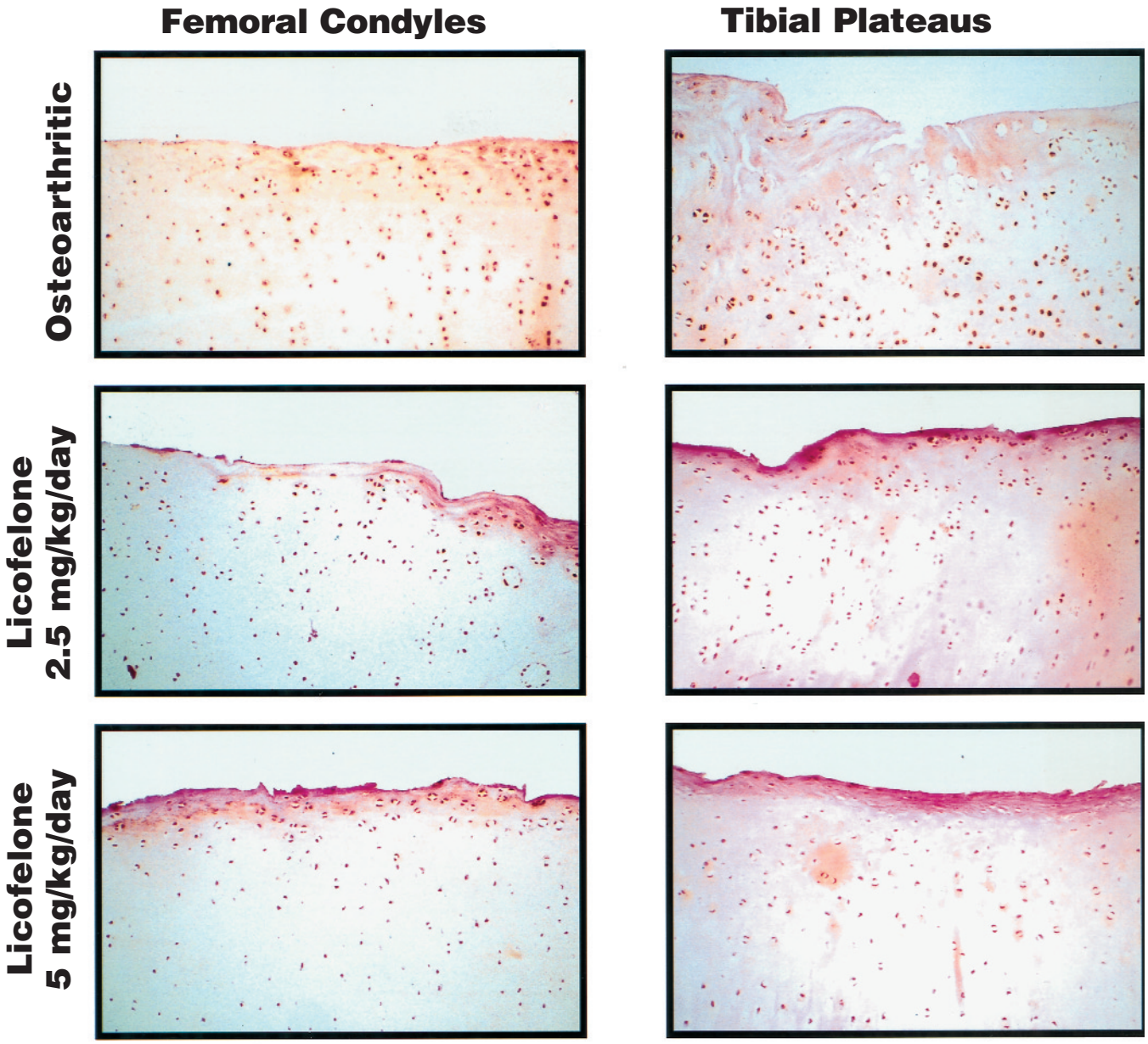

Figure 2. Representative sections of cartilage, showing immunostaining for caspase-3 in femoral condyles (left) and tibial plateaus (right). A and B. Placebo treated dog OA. C and D. Dog OA treated with licofelone 2.5 mg/kg/day. E and F. Dog OA treated with licofelone 5.0 mg/kg/day (original magnification ×100).

2.5 mg/kg/day dose regimen. Controls showed only background staining (data not shown).

Inducible NO synthase. A large number of chondrocytes in the superficial layers of OA cartilage from condyles and plateaus revealed positive staining for iNOS (Table 3, Figure 3). Specimens from the 2 groups of dogs treated with licofelone showed a significant reduction in the percentage of positive chondrocytes. There were, however, no differences between the 2 therapeutic groups, either in femoral condyles or tibial plateaus, concerning the extent of reduction. Controls showed only background staining.

Table 3. INOS level in canine knee cartilage. Values are the mean ± SEM.

Group	No. of Animals	% Positive Cells	
		Femoral Condyles	Tibial Plateaus
OA control (placebo)	7	24.8 ± 2.9	29.4 ± 2.3
Licofelone 2.5 mg/kg/day	7	12.1 ± 0.9	11.1 ± 1.4
		$p < 0.002$	$p < 0.001$
Licofelone 5.0 mg/kg/day	7	10.7 ± 1.7	11.1 ± 1.0
		$p < 0.002$	$p < 0.001$

P value versus the OA group, by Mann-Whitney U test.

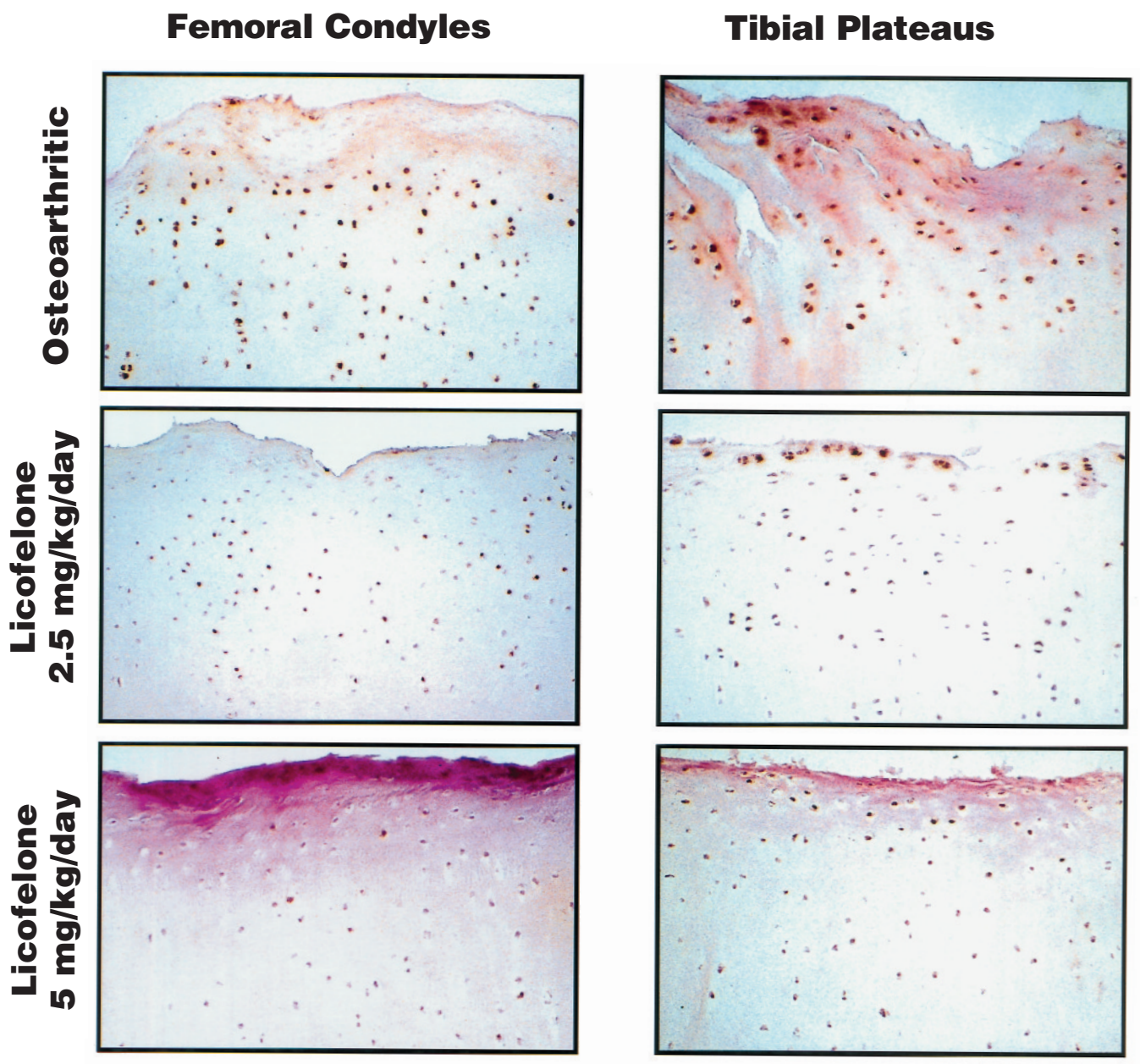

Figure 3. Representative sections of cartilage, showing immunostaining for iNOS in femoral condyles (left) and tibial plateaus (right). A and B. Placebo treated dog OA. C and D. Dog OA treated with licofelone 2.5 mg/kg/day. E and F. Dog OA treated with licofelone 5.0 mg/kg/day (original magnification ×100).

Cyclooxygenase 2. Over 30% of the chondrocytes in the superficial layers of OA cartilage, both in condyles and plateaus, expressed COX-2 (Table 4, Figure 4). There was a very significant decrease in the number of cells expressing COX-2 in specimens from condyles and plateaus in the 2 experimental groups treated with licofelone. There was a slight tendency for the effect to be more marked in the dogs treated with the highest dosage of the drug. Controls showed only background staining (data not shown).

DISCUSSION

This study demonstrates that under experimental conditions, licofelone is a potent inhibitor of OA chondrocyte death *in*

Table 4. COX-2 level in canine knee cartilage. Values are the mean ± SEM.

Group	No. of Animals	% Positive Cells	
		Femoral Condyles	Tibial Plateaus
OA control (placebo)	7	31.1 ± 1.4	30.1 ± 2.0
Licofelone 2.5 mg/kg/day	7	15.9 ± 1.0	16.6 ± 1.2
		p < 0.001	p < 0.001
Licofelone 5.0 mg/kg/day	7	10.8 ± 1.1	10.4 ± 1.3
		p < 0.001	p < 0.001
		(p < 0.004†)	(p < 0.01†)

P value versus the OA group, Mann-Whitney U test. † P value versus licofelone 2.5 mg/kg/day group, Mann-Whitney U test.

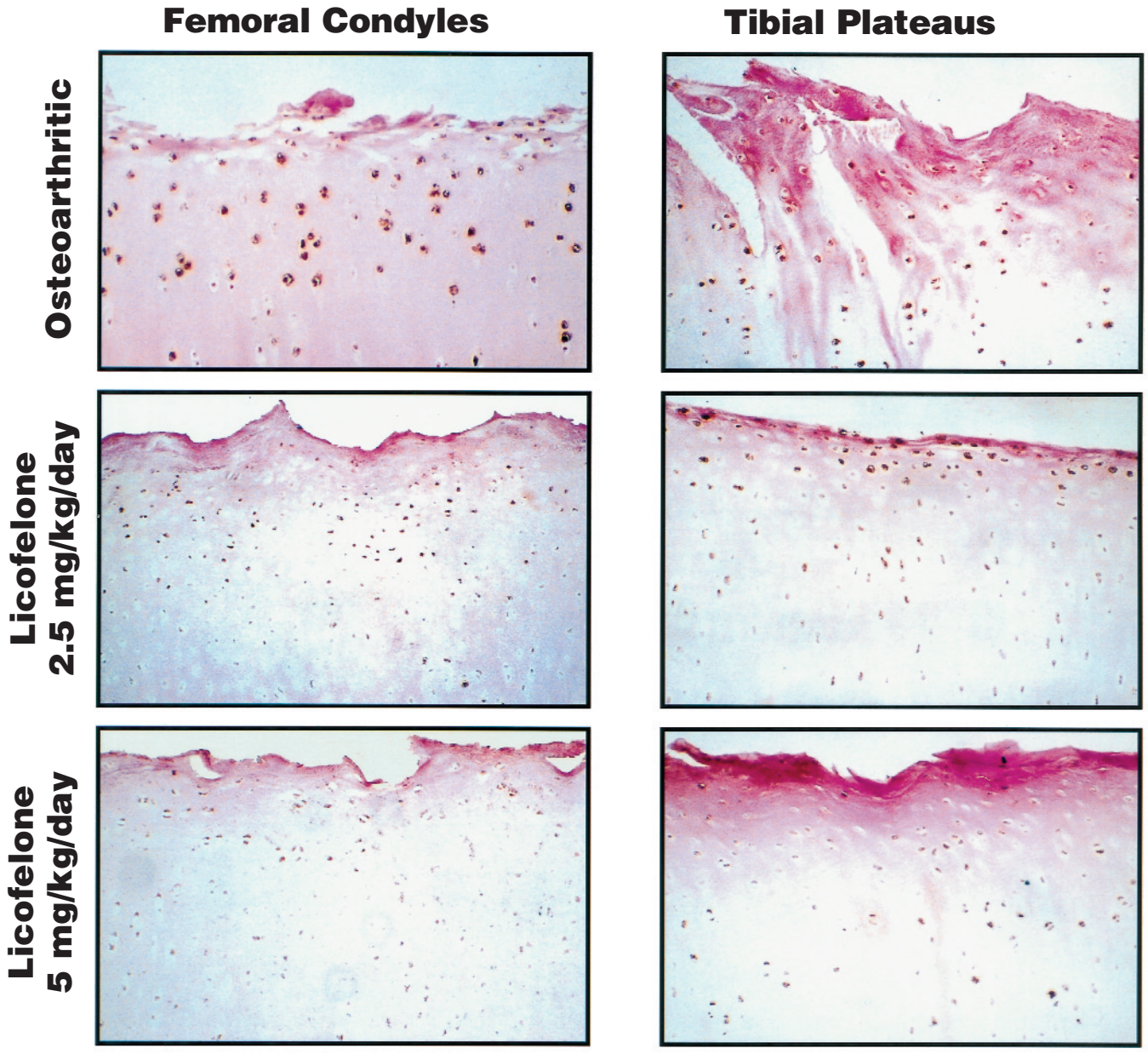

Figure 4. Representative sections of cartilage, showing immunostaining for COX-2 in femoral condyles (left) and tibial plateaus (right). A and B. Placebo treated dog OA. C and D. Dog OA treated with licofelone 2.5 mg/kg/day. E and F. Dog OA treated with licofelone 5.0 mg/kg/day (original magnification ×100).

vivo. This new antiinflammatory compound was reported to be a potent *in vivo* inhibitor of 5-LOX and COX in experimental dog OA, as well as a drug capable of reducing the development of cartilage degradation[13]. This study gives new information on the potential mechanisms by which this drug exerts its cartilage-sparing effects.

The participation of chondrocyte death by apoptosis in cartilage destruction is very likely during OA[3,6,8-10,18]. The reduction in the number of living cells, particularly in the superficial layers of OA cartilage where a great deal of extracellular matrix degradation has taken place, is certainly an important factor limiting the capacity of this tissue for self-repair[5]. Moreover, the death of chondrocytes leads to the release of a number of catabolic factors such as proteases, which could also contribute to cartilage matrix degradation. A study in an experimental OA model revealed that an agent that can reduce the level of chondrocyte apoptosis was also able to protect against the progression of cartilage lesions[19]. The relevance of these specific antiarthritic agents for the treatment of clinical OA is questionable, since one may argue that the administration of agents able to specifically and selectively inhibit apoptosis may also increase the risk of inducing malignant disease when given systemically. Indeed, drugs that can induce apoptosis in intestinal cells have been clearly established as reducing the incidence of certain colon cancers[20,21]. In regard to OA, there is a need for agents that will inhibit apoptosis selectively in the joints or at the site of inflammation.

The mechanisms leading to OA chondrocyte death/apoptosis have been extensively studied. Recent publications have shown that a number of pathways, linked together, are involved in this phenomenon. For instance, the implication of the caspase cascade has recently been documented[22] and is to some extent related to the excessive production of NO within the OA joint and more particularly by chondrocytes[23,24]. The exact mechanisms(s) by which NO induces chondrocyte apoptosis remains to be elucidated. Recent reports indicate several possibilities, including direct DNA damage, generation of peroxynitrite, and inactivation of antioxidant enzymes[25-28]. Studies using human leukemic cell lines indicate that NO might induce apoptosis via mitochondria damage and by cytochrome c release, leading to activation of the caspase enzymes[29].

The cytosolic aspartate-specific proteases (caspases) involve a family of enzymes responsible for the "ordered" disassembly of cells[22]. Caspase-8, caspase-9, and caspase-3 are the primary enzymes involved in cell apoptosis. These enzymes induce cell death by a number of mechanisms, including DNA fragmentation and inactivation of the proteins that protect cells against apoptosis[22,30]. They are present in the cytoplasm as proenzymes and can undergo self-activation or be activated in a cascade-like manner by enzymes with similar specificity. Caspase-9 is activated in response to agents or insults that induce the release of cytochrome c from the inner mitochondrial membrane[31]. Caspase-8 is activated by the cytoplasmic death domains of the receptors. Caspase-8 and caspase-9 can activate caspase-3, which could amplify the caspase-8 and caspase-9 signals. Upon activation, caspase-3 cleaves vital intracellular proteins and additional caspases.

Recent studies have revealed that the induction of apoptosis by NO in OA chondrocytes is mediated via the induction of COX-2 gene expression and subsequent increase in the production of endogenous PGE_2 [11]. The exact mechanism by which PGE_2 is involved in chondrocyte apoptosis is under exploration in our laboratory. This finding is notable because, in cancer cells, prostaglandins are believed to have an inverse effect by having anti-apoptotic properties[20,21,32].

We found that licofelone was capable of dramatically reducing OA chondrocyte death/apoptosis, particularly in the lesional area of the cartilage. This effect was present in concentrations of licofelone within the therapeutic range[13]. This effect was also closely associated with a reduction in the level of the mature form of caspase-3, explaining the mechanism by which the drug reduced chondrocyte death/apoptosis. Moreover, licofelone was found to significantly inhibit the expression of both iNOS and COX-2. Together, these results show that this drug reduces chondrocyte death/apoptosis by inhibiting the production of 2 major factors involved in the activation of the caspase cascade, namely NO and PGE_2. There are several hypotheses to explain the effects of licofelone on these pathways. First, this drug was found to reduce the synthesis of IL-1ß by the synovial cells in experimental dog OA[13]. This action could possibly be related to the capacity of licofelone to inhibit the activity of 5-LOX and the synthesis of leukotriene B_4 (LTB_4)[33]. Recent studies have also identified that mutual cross-talking exists between the iNOS and COX pathways, which may provide an additional explanation for the action of licofelone. These findings can be summarized as follows: the level of COX-2 is autoregulated by PGE_2 and upregulated by NO, and the level of iNOS is autoregulated by NO and upregulated by PGE_2 [34]. Therefore, licofelone being a potent inhibitor of COX-2, the inhibition of PGE_2 production could explain the reduction in the level of COX-2 and iNOS. Moreover, recent findings from our laboratory indicate that LTB_4 could also upregulate COX-2 expression[35], and therefore the inhibition of 5-LOX by licofelone may provide an additional explanation for the reduction in the level of COX-2 induced by this drug in OA chondrocytes.

This study reveals that licofelone, a new antiinflammatory drug with dual balanced inhibitory activity against COX and 5-LOX, reduces chondrocyte death/apoptosis *in vivo* in OA cartilage. This effect is regulated by the inhibition of 2 potent pro-apoptotic pathways in chondrocytes, namely COX-2 and iNOS, and the subsequent decrease in the activation of the caspase cascade. These findings

provide additional information about the possible mechanisms by which licofelone could reduce the progression of the structural changes of experimental dog OA.

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