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Limited inhibitory effects of non-steroidal anti-inflammatory drugs on *in vitro* osteogenic differentiation in canine cells

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Abstract

Cyclooxygenase (COX)-2 participates essentially in bone healing, demonstrated by COX-2 knockout mice that showed delayed fracture repair. Considerable controversy still exists on inhibitory effects of COX-2 inhibitors on bone healing in clinical cases. To assess stage-dependent effects of short-term treatment of COX-2 inhibitors on osteogenic differentiation, a canine POS osteosarcoma cell line which spontaneously differentiates into osteoblastic cell was exposed to COX-2 inhibitors such as carprofen and meloxicam for 72 hours during three different stages of osteoblast differentiation, including day 0 to 3 (pre-osteoblastic stage), day 4 to 7 (transitional stage) and day 8 to 11 (mature osteoblastic stage). As osteogenic markers, expression of alkaline phosphatase (ALP) was estimated by analysis of mRNA expression, enzymatic activity and ALP staining, and expression of osteocalcin was estimated by analysis of mRNA expression after the drug treatments. Calcified matrix formation was finally observed by von Kossa staining on day 14. Expressions of ALP showed no significant suppression by carprofen and meloxicam during all three stages. However, expressions of osteocalcin mRNA and non-calcified nodule formations were delayed by carprofen and meloxicam during transitional stage. Nevertheless, fully calcified nodule formation was observed in all experimental groups during post-medication period. These results indicate that short-term treatment of carprofen and meloxicam would reversibly suppress the differentiation of osteoblasts.

Key Words: bone, canine, prostaglandin E₂, non-steroidal anti-inflammatory drug,

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) have been used as an analgesic agent

for wide range of specific orthopedic diseases, including osteoarthritis³³⁾ and fracture repair¹⁴⁾ in animals and human beings. Deterrent effects of NSAIDs on inflammation and pain are obtained

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by suppressing the action of cyclooxygenase (COX), which catalyzes the conversion of arachidonic acids into prostaglandins (PGs)³⁵⁾. Over the last decades, with development of new generation of NSAIDs which specifically inhibit inducible COX-2, incidence of major adverse effects of conventional NSAIDs, including gastrointestinal tract bleeding, are dramatically reduced by preserving the function of COX-1 on gastric mucosa and platelet aggregation⁴¹⁾. While COX-1, a housekeeping enzyme, is constitutively expressed in almost all types of cells to maintain homeostatic levels of PGs⁴⁰⁾, COX-2 is rapidly induced to produce inflammatory PGs²⁵⁾ by various stress factors related to structural damage, including physical trauma, mechanical stimulation, and by numerous inflammatory mediators^{8,26,39)}. High selectivity of the NSAIDs for COX-2 inhibition can control pain with benefits from COX-1 sparing effects. However, COX-2 selective NSAIDs also seem to have risks of some inhibitory effects on essential physiological phenomenon.

The most abundant PG is PGE₂ that plays a critical role in bone repair^{10,38)}. The concentration of PGE₂ reached at the highest level around the fracture site within the day after fracture, and after then the level of PGE₂ diminished steadily as bone was healing in experimental fractures in rats¹²⁾. In the same study, administration of selective COX-2 inhibitors resulted in lower levels of PGE₂ production at fracture sites and delayed fracture union, compared with non-selective NSAIDs treated rats¹²⁾. In COX-2 knockout (KO) mice, far less fracture callus formation was identified than that in wild type mice, and the osteoblastogenesis of mesenchymal stem cells that cultured from COX-2 KO mice was rescued by PGE₂ supplementation⁴³⁾. The results of these studies suggest that COX-2 participates in bone healing through mediation of PGE₂ production.

However, controversy regarding clinical use of COX-2 inhibitors in fracture patients still exists in the literature^{5,12,37)}. Although animal

studies showed high dose and long-term treatment of COX-2 inhibitor delayed fracture repair, inhibitory dose and duration in those experiments could not be converted to clinical use directly. On the contrary to the COX-2 KO animals, wild-type animals could express COX-2 gene⁹⁾, which may also partially act to restore deficient PGE₂, leading to keep osteogenic cells to proceeding bone forming process. Thereby, it is assumed that COX-2 inhibitor would temporarily affect bone healing within the duration of its use.

The present study was aimed to elucidate the cellular behavior of canine osteogenic cells after exposure to the COX-2 inhibitors, such as carprofen³¹⁾ and meloxicam¹¹⁾, which are among the most available NSAIDs in veterinary medicine. The time course of genetic, biochemical and morphological changes during osteoblast differentiation were determined by using canine POS osteosarcoma cell line^{16,29)} that shows the same characteristics over a series of osteogenic differentiation as other osteosarcoma cell lines^{32,34)} and osteoblasts from mesenchymal stem cells²¹⁾ or calvarial cells²⁸⁾.

Materials and methods

Culture of canine osteoblast-like cells: Canine POS cells were cultured in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 25 mM NaHCO₃, 100 U/ml penicillin and 100 µg/ml streptomycin. To induce osteogenic differentiation, 10 nM 1,25-dihydroxyvitamin D3 (calcitriol; Sigma-Aldrich, St. Louis, MO, USA) was also added into the medium^{2,20,22)}. POS cells were seeded at a density of 4.5×10^4 cells/well in 12-well, flat-bottomed culture plates (Costar 3513, Corning Inc., Corning, NY, USA). Media were changed every other day. All cultures were maintained in an incubator at 37°C with humidified 5% CO₂ atmosphere.

Determination of cell viability under various doses of carprofen and meloxicam: Concentrations of carprofen (LKT Laboratories, Inc., St. Paul, MN, USA) and meloxicam (Wako, Pure Chemical Ind., Osaka, Japan) that have no cytotoxicity on the bioavailability of POS cells were determined by following colorimetric assay. The cells were seeded at a density of 5×10^3 cells in 96-well plate (Costar 3595, Corning Inc.) with 100 μ l of RPMI-1640 culture medium. The cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. After 24 hours of incubation for cell adaptation to the culture condition, cells were treated with various concentrations (0.01, 0.1, 1, 10 and 100 μ M) of drugs for 48 hours of exponential growth phase. The cell viability was measured by the colorimetric assay based on the conversion of 3-(4,5-dimethyl-2-thiazol) 2,5-diphenyl-2H-tetrazolium bromide (MTT) (Wako) to a formazan pigment by mitochondrial enzymes in viable cells during four hours of incubation²³⁾. Colorimetric measurement was performed using a plate reader (MPT-120, Corona Electric, Ibaraki, Japan) at wave length of 570 nm. All samples evaluated by MTT assay were measured in triplicates.

Periods of COX-2 inhibition during osteogenic differentiation: The entire period for osteogenic differentiation in POS cells is 14 days (Fig. 1A). Each stage of osteoblast differentiation, including pre-osteoblastic stage, transitional stage and mature osteoblastic stage, is started with morphologic change of the cells such as proliferation with no nodule formation from day 0, non-calcified nodule formation from day 4 and calcified nodule formation from day 8, respectively. Carprofen (1 μ M) and meloxicam (1 μ M) were added to culture media for 72 hours during each osteogenic stage (Fig. 1B). Samples for the analyses of gene and protein expression were harvested on day 3, day 7, and day 11.

Analysis of gene expression: To perform relative quantification of gene expression, total RNA was

extracted using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instruction. Reverse transcription was performed with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT; Invitrogen) with oligo (dT)₁₅ as a primer. Quantitative real-time polymerase chain reaction (PCR) was carried out in Rotor-Gene Q (Qiagen, Hilden, Germany) with KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems, Boston, MA, USA). The primers used in this study were purchased from the external manufacturer (Hokkaido System Science Co.; Sapporo, Hokkaido, Japan). The nucleotide sequences of the primers were as follows: ALP forward, 5'-CCAAGCTCAACAGACCCTGA-3'; ALP reverse, 5'-GAGACACCCATCCCATCTCC-3'; osteocalcin forward, 5'-AGGAAGCTTACCAGCGC TTC-3'; osteocalcin reverse, 5'-TGACAAGGACC CCACACTTG-3'; GAPDH forward, 5'-AAGGTC ATCCCTGAGCTGAA-3'; GAPDH reverse, 5'-GAC CACCTGGTCCTCAGTGT-3'. The acquired data were analyzed with normalization to GAPDH as an internal control.

Intracellular ALP activity: Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with vigorous shaking and sonication for 30 minutes in 10 mM Tris-HCl solution (pH 7.4) containing 0.05% non-ionic surfactant, Triton X-100 (ICN Biomedicals Inc., Aurora, OH, USA). The supernatant of the cell lysates was separated by centrifugation at 20,000 $\times$ g for 30 minutes. The supernatants and p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA) were reacted for 10 minutes at 37°C in substrate solution (pH 10.5) which containing 1 mg/ml p-nitrophenyl phosphate, 100 mM glycine, 1 mM MgCl₂ and 1 mM ZnCl₂. Released p-nitrophenol was measured using plate reader (MPT-120) at wave length of 405 nm. Alkaline phosphatase activity was determined after normalization by the protein concentration measured by modified Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA)⁴⁾, and was

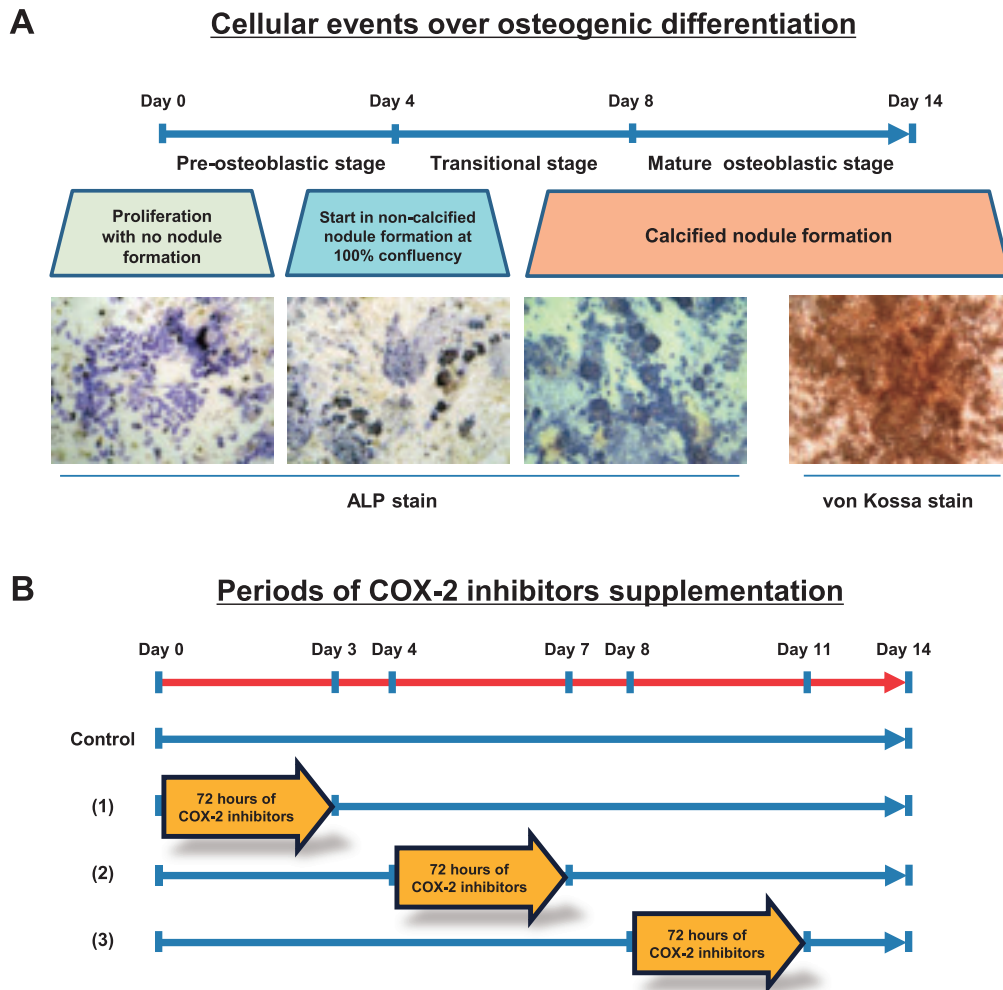


Fig. 1. Schematic representation of cellular events over osteogenic differentiation of canine POS osteosarcoma cell line (A) and periods for supplementation of COX-2 inhibitors (B). The observation of morphological changes of POS cells during osteogenesis was performed with conventional light microscope after ALP staining or von Kossa staining at each time point. The periods of drugs supplementation were designed for this study according to the three characteristic changes of morphology in each stage, including proliferation at 80% confluency from day 0 to 3 (1), non-calcified nodule formation from day 4 to 7 (2) and calcified matrix formation from day 8 to 11 (3).

expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein.

Morphological evaluation: Visual observation of effects of COX-2 inhibitors on non-calcified nodule formation of POS cells was performed using ALP staining after each 72 hours of the drug treatment on day 3, day 7, and day 11. Briefly, the cells were washed with PBS and fixed with 4% paraformaldehyde for 2 minutes. After washing, the cells were incubated in 0.1 M Tris-HCl buffer (pH 9.3) containing 0.25% (w/v) naphthol-AS-BI-phosphoric acid sodium salt

(Sigma-Aldrich) and 0.75% (w/v) fast blue RR salt (Sigma-Aldrich) at 37°C for 30 minutes.

To observe the effects of COX-2 inhibitors on calcium deposition in post-medication period, von Kossa staining was performed on day 14. Briefly, after fixation with 4% paraformaldehyde for 2 minutes, POS cells were treated with 2% silver nitrate (Wako) solution and placed under ultraviolet light at room temperature for 1 hour. After rinsing with distilled water, cells were bleached in 5% sodium thiosulfate (Wako) for 2 minutes. Results were observed using optical

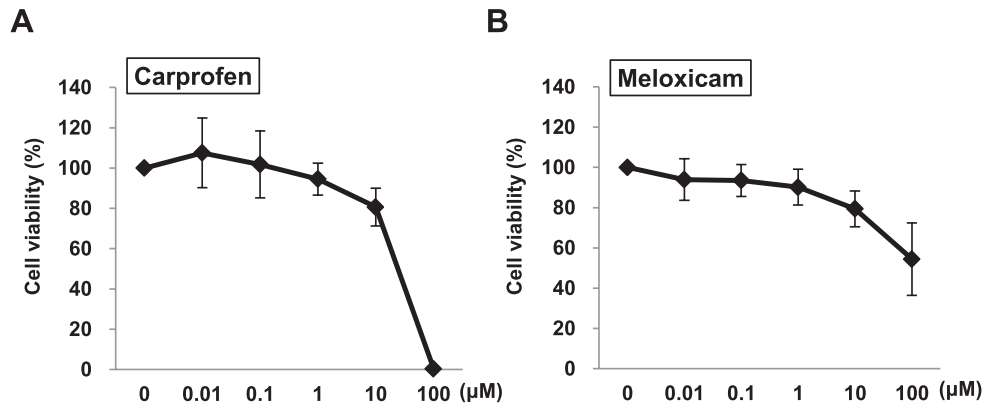


Fig. 2. Effects of carprofen and meloxicam on viability of canine POS osteosarcoma cell line. The cell viability determined by MTT assay after 48 hours of drug treatment. Carprofen (A) and meloxicam (B) decreased cell viability in a dose-dependent manner. Data are expressed as mean $\pm$ standard deviation from three independent experiments performed in triplicates.

microscope after the cells were rinsed with distilled water.

Statistics: The statistical data analysis was carried out using one-way analysis of variance, followed by Tukey's Honestly Significant Difference test. All data were expressed as mean $\pm$ standard deviation. Values of $P < 0.05$ were considered to be statistically significant.

Results

Non-cytotoxic concentrations of carprofen and meloxicam existed within clinical doses

At 48 hours after carprofen or meloxicam treatment, cell viability showed more than 90% at 0.01, 0.1 and 1 μ M of each drug concentration (Fig. 2). Definitive cytotoxic effects of these drugs were observed at concentrations ≥ 10 μ M. Doses of carprofen and meloxicam used in this experiment were at the maximum concentration (1 μ M each) that has no effect on viability of POS cells. The maximum plasma concentration of the drugs in dogs presented by previous studies were 20.60 μ g/ml (76.29 μ M)¹⁸⁾ and 0.73 μ g/ml (2.09 μ M)⁶⁾ after injection of recommended clinical doses of carprofen (4.00 mg/kg/dose) and meloxicam (0.20 mg/kg/dose), respectively. These data indicate that the doses of the drugs supplemented in this

experiment were within the plasma concentration in dogs treated with clinical doses.

Carprofen and meloxicam had no effects on expression of mRNA and enzyme activity of ALP during osteogenic differentiation of POS cells

Expression of ALP, a marker of pre-osteoblast cells in early period of osteoblast differentiation, was continuously observed in control. This result indicates that POS cells constantly differentiated to pre-osteoblast-like cells during almost entire differentiation period. Expression of ALP mRNA (Fig. 3A) and ALP activity (Fig. 3B) showed no significant difference between control and drug-treated groups in all three periods examined. Meanwhile, expression of ALP mRNA tended to be more increased by meloxicam than by carprofen from day 4 to 7, and at the same period, enzyme activity of ALP tended to be more decreased by meloxicam than by carprofen.

Carprofen and meloxicam had limited effects on expression of osteocalcin mRNA in transitional stage of osteogenic differentiation of POS cells

Osteocalcin, a marker of mature osteoblast in late period of osteoblast differentiation, showed increase in its mRNA expression from the period between day 4 and day 7. Morphologically, non-calcified nodule formation

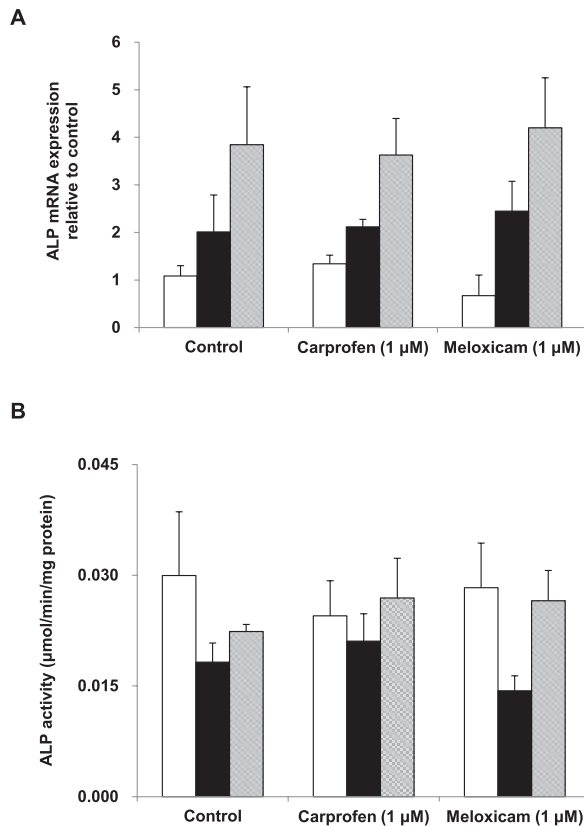


Fig. 3. Effects of carprofen and meloxicam on alkaline phosphatase (ALP) gene expression and ALP activity of canine POS osteosarcoma cell line in various stages of osteogenic differentiation. The level of mRNA expression of ALP was measured by quantitative real-time PCR. ALP activity was measured by colorimetric assay using *p*-nitrophenylphosphate. No significant difference was observed between control and experimental group in ALP gene expression (A) and ALP activity (B) over all stages. Data expressed as mean $\pm$ standard deviation ($n = 3$). (□, Day 0 to 3; ■, Day 4 to 7; ▨, Day 8 to 11)

started with expression of osteocalcin mRNA, indicating this period was transitional stage for POS cells to mature into functional osteoblast-like cells. Expression of osteocalcin mRNA was significantly suppressed by carprofen and meloxicam in transitional stage ($P < 0.001$), but no significant suppression was observed in mature osteoblastic stage (Fig. 4). While, mRNA expression of osteocalcin in meloxicam-treated group tended to be more increased than that in carprofen-treated group, as the expression of ALP mRNA did, suggesting the cellular responses to each COX-2 inhibitor were somewhat different.

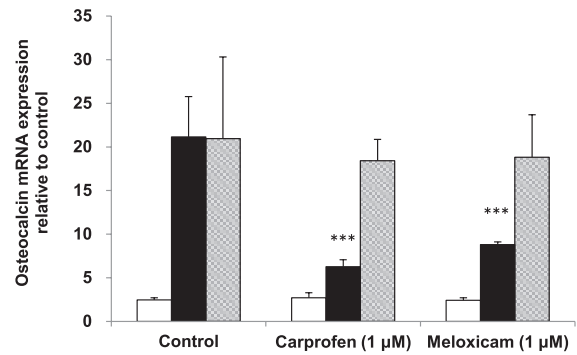


Fig. 4. Effects of carprofen and meloxicam on osteocalcin gene expression of canine POS osteosarcoma cell line in various stages of osteogenic differentiation. The level of mRNA expression of osteocalcin was measured by quantitative real-time PCR. Osteocalcin gene expression was significantly decreased in transitional stage by carprofen (1 μ M) and meloxicam (1 μ M). Data expressed as mean $\pm$ standard deviation (***) $P < 0.001$; relative to control; $n = 3$). (□, Day 0 to 3; ■, Day 4 to 7; ▨, Day 8 to 11)

Carprofen and meloxicam delayed non-calcified nodule formation of POS cells in transitional stage of osteoblast-like differentiation, reversibly

The results of ALP staining revealed delay in start of non-calcified nodule formation by carprofen and meloxicam from day 4 to 7 (Fig. 5). However, from day 8 to 11, nodule formation showed no suppression by the COX-2 inhibitors. These results were consistent with the differentiation stage-dependent suppression in expression of osteocalcin occurred by effects of the COX-2 inhibitors. While, on day 14, no difference was observed in calcification level between control and all groups of drug treatment by von Kossa staining regardless of treatment period (Fig. 6). The retarded nodule formations during transitional stage were also recovered with further differentiation after the discontinuation of the drugs. These results support that the transitional stage-limited suppressive effects of carprofen and meloxicam was reversible.

Discussion

In the present study, POS cells showed continuous proliferation of ALP-positive cells,

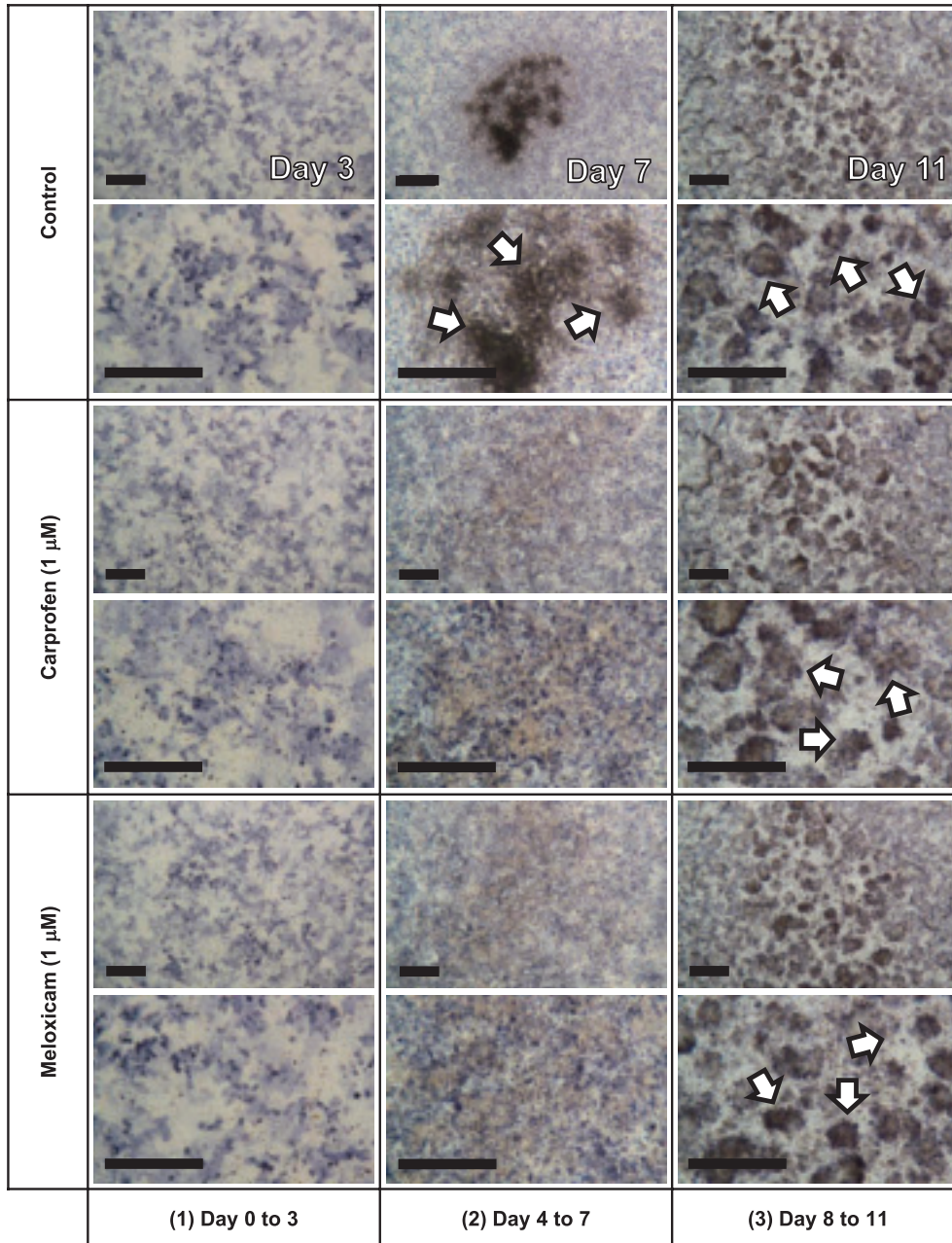


Fig. 5. Morphological analysis of effect of carprofen and meloxicam on canine POS osteosarcoma cell line by alkaline phosphatase (ALP) staining in various stages of osteoblast differentiation. Non-calcified nodule formation (white arrow) was delayed by carprofen (1 μ M) and meloxicam (1 μ M) during transitional stage. Each bar represents 1 μ m.

nodule formation and matrix calcification (Fig. 1A). Short-term treatment of carprofen and meloxicam limitedly inhibited osteoblast-like differentiation of POS cells at the transitional stage. However, no suppressive effect was observed in fully mature osteoblast-like cells by

the COX-2 inhibitors. Delay of non-calcified nodule formation during transitional stage was recovered after withdrawal of the drugs. These data represent that, while transitional stage of osteoblast differentiation was the most vulnerable for COX-2 inhibitors, short-term use

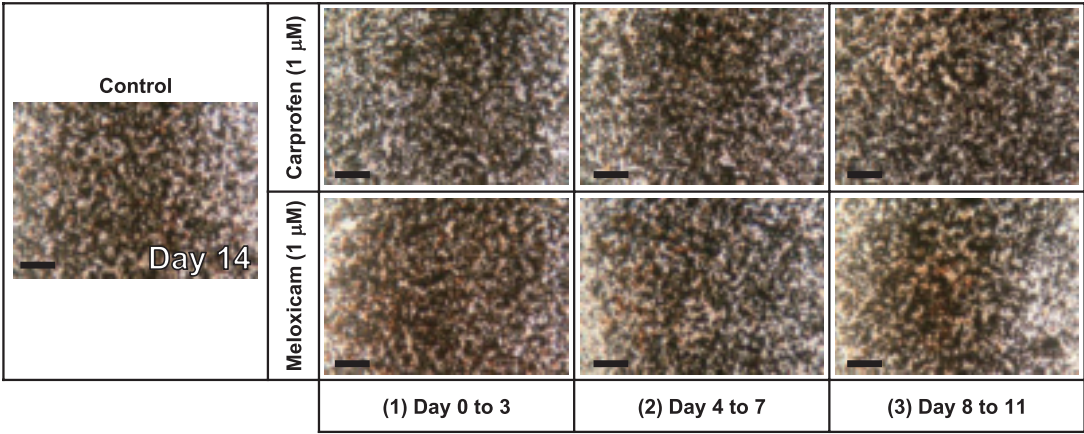


Fig. 6. Morphological analysis of effect of carprofen and meloxicam on calcified matrix formation in canine POS osteosarcoma cell line by von Kossa staining on day 14 after further osteogenic induction with withdrawal of drug treatments. No significant difference was observed between control and experimental group in calcification level. Each bar represents 1 μm.

of COX-2 inhibitor unaffected calcified matrix formation.

Recently, effects of carprofen on fracture healing have been reported in dog fracture model³⁰, suggesting long-term treatment (120 days) of carprofen could delay bone healing with clinical dosage. Effects of meloxicam on bone healing in rabbit fracture model¹⁷ showed low therapeutic dose for short-term administration (5 days) of meloxicam would show no major complications in bone healing. These results imply that retardation of bone healing, which may be occurred by use of COX-2 inhibitors with very long-term and high dose, would not be permanent, but could be reversible by withdrawal of medication.

No significant decrease observed in expression of ALP by carprofen or meloxicam genetically, biochemically and morphologically, implying PGE₂ production suppressed by these drugs could be restored by POS cell itself. Thereby, pre-osteoblast-like cells could be normally induced in every stage of osteoblast-like differentiation. Basically PGE₂ increases ALP activity osteoblastic cells in a differentiating stage-dependent manner^{15,42}. Our preliminary experiment shown that ALP activity of POS cells in control reached highest level on day 4, when cell density became 100% confluency, then it

decreased as cells mature. This pattern of expression of ALP was consistent with the data from other studies^{1,3,24}. As osteoblast-like cells mature, level of mRNA expression of osteocalcin on day 7 increased eight times higher than that on day 3, and this high expression level was continuously observed until day 11. Osteocalcin is a matrix protein that is secreted by mature osteoblasts^{7,19}. In this study, not only gene expression of osteocalcin, but also non-calcified nodule formation was reduced by carprofen and meloxicam in transitional stage. Other studies using bone marrow cells in mice also demonstrated down-regulation of osteocalcin expression by COX-2 inhibitors in mature osteoblasts²⁷. However, in POS cells, mRNA expression of osteocalcin as well as nodule formation was not suppressed by the drugs during mature osteoblastic stage. It is speculated that compensatory capacity to restore PGE₂ would increase with differentiation of the cell.

Reversible suppressive effect of COX-2 inhibitors on osteogenic differentiation was confirmed by callcification levels that showed no difference from control at the end of osteogenesis. Several long-term treatment study in rat fracture-healing models histologically demonstrated suppressive effects of selective COX-2 inhibitors on bone union^{5,12,36,37}. Expression of COX-2

mRNA was up-regulated in the callus during first two weeks of fracture healing relative to un-fractured bone¹³⁾. Moreover, PGE₂ levels were highly detected from one day of fracture and gradually decreased until thirty-five days¹²⁾. The functions of COX-2 on bone healing via deriving PGE₂ in this period would be important for differentiation of osteogenic cells. This might also explain why long-term use of COX-2 inhibitor from early of fracture healing could be one of the reasons for delaying the bone healing in *in vivo*. While, one of these studies demonstrated that decreased PGE₂ production by COX-2 selective or non-selective inhibitor within the fracture site was rebounded after a week of drug withdrawal¹²⁾. That response from the body would also assist osteogenic cells in continuing bone healing. These results indicate that, although the ideal level of PGE₂ would be required in certain period of bone healing to induce differentiation of osteoprogenitor cells, short-term use of COX-2 inhibitor minimally affect bone healing because of their reversible effect on COX-2 inhibition.

Canine POS osteosarcoma cell line used in the present study was not pure osteoblast cells and had somewhat different life cycle due to its origin of tumors. Although there are limitations to reflect these data directly to the normal physiological phenomena in bone metabolism, it is assumed that short-term treatment of COX-2 inhibitors, such as carprofen and meloxicam, for pain control in fracture patients would be more positive to patient owing to its minor effects on osteogenesis. Further studies will be needed, using normal osteogenic cell source, to clearly determine the effects of various classes of NSAIDs on osteogenic differentiation.

In conclusion, short-term treatment of carprofen and meloxicam would reversibly suppress osteogenic differentiation, suggesting that short-term treatment of carprofen and meloxicam in bone fracture patients could be allowed.

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