NOTE *Internal Medicine*

Comparison of the Immunosuppressive Effects of Dexamethasone, Flunixin Meglumine and Meloxicam on the *In Vitro* Response of Calf Peripheral Blood Mononuclear Cells

Yousuke MAEDA[1)], Rie TANAKA[1)], Hiromichi OHTSUKA[1)]*, Keiici MATSUDA[2)], Taishi TANABE[1)] and Masaaki OIKAWA[1)]

[1)]*School of Veterinary Medicine, Kitasato University, Towada, Aomori 034–8628 and* [2)]*Chuou Veterinary Clinical Center, Miyagi Prefecture Federation of Agricultural Mutual Relief Association, Furukawa, Miyagi 989–6251, Japan*

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ABSTRACT. This study compared the immunosuppressive effects of dexamethasone (DEX), flunixin meglumine (FLU) and meloxicam (MEL) on the peripheral blood mononuclear cells (PBMCs) of seven healthy Holstein calves *in vitro*. DEX significantly inhibited lymphocyte proliferation and expression of interferon (IFN)-γ, interleukin (IL)-2 and IL-4 messenger RNA (mRNA) in comparison with FLU and MEL. FLU and MEL dose-dependently inhibited lymphocyte proliferation, but did not significantly reduce mRNA expression. Our *in vitro* study indicates that steroidal anti-inflammatory drugs (SAIDs) as well as nonsteroidal anti-inflammatory drugs (NSAIDs) have immunosuppressive effects on calf PBMCs. These findings are important for assessing the indications and complications of NSAIDs in calves.

KEY WORDS: calf, cytokine, dexamethasone, flunixin meglumine, meloxicam.

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Anti-inflammatory drugs (AIDs) are often used for treatment of diseases such as pneumonia, diarrhea and endotoxemia [3, 23]. Steroidal AIDs (SAIDs) have potent anti-inflammatory properties, but they are also potent suppressors of the immune system. SAIDs such as dexamethasone (DEX) exert antiproliferative effects on T cells by inhibiting cytokine expression, especially that of the T-cell growth factor interleukin (IL)-2 [1, 13].

Nonsteroidal AIDs (NSAIDs), including nonselective NSAIDs and cyclooxygenase (COX)-2 selective NSAIDs, also have an anti-inflammatory effect. COX, which is known to exist as COX-1 and COX-2 isoforms, is a key enzyme in prostaglandin synthesis. Although NSAIDs are generally accepted to be anti-immunosuppressive, there is growing evidence from human studies that some might have additional immunomodulatory properties. For example, Iñiguez *et al.* [9] reported that COX-2 inhibitors regulate T-cell activation. However, the effects of SAIDs and NSAIDs in the peripheral blood mononuclear cells (PBMCs) of calves have not been evaluated. The present study therefore evaluated the ability of three AIDs to suppress lymphocyte proliferation and the expression of cytokine messenger RNA (mRNA) in the PBMCs of calves *in vitro*. The compounds tested included DEX (a SAID), flunixin meglumine (FLU; a nonselective NSAID) and meloxicam (MEL; a COX-2 selective NSAID).

Seven healthy female Holstein calves, aged 3–4 months, were obtained from 2 dairy farms in Aomori, Japan. None of the animals had received any medical treatments involving the use of drugs since birth. The growth of the calves was within the range of the "Standard developmental growth curve of the Holstein heifer" published by the Japanese Holstein Association [22].

Peripheral blood samples were collected from the caudal vein in tubes containing heparin.

For lymphocyte proliferation, PBMCs were seeded in 96-well microplates at a density of 1×10^6 cells/well in a final volume of 200 μl/well of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). Each sample was cultured with 10 μg/m*l* phytohemagglutinin (PHA; Sigma, St. Louis, MO, U.S.A.) [24] alone, PHA and DEX (Sigma; 10, 15 or 20 μg/m*l*) [13, 20], PHA and FLU (Sigma; 10, 25 or 50 μg/m*l*) [2, 12] or PHA and MEL (Nippon Boehringer Ingelheim Co., Ltd., Tokyo, Japan; 10, 25 or 50 μg/m*l*) [14]. The mitogen PHA and three AIDs were used at concentrations based on those reported for previous *in vitro* experiments with cattle [11, 12, 19] and other animal species [2, 12, 14].

After incubation for 72 hr at 37°C, 5 μg/m*l* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to each well, and the plate was incubated for a further 2 hr at 37°C. After incubation, 100 μl of supernatant was removed from each well, and 100 μl of 50% dimethyl sulfoxide (DMSO) containing 0.7 M sodium dodecyl sulfate was added. After the dark-blue formazan crystals had dissolved, the optical density (OD) was measured using a microplate reader (Model 3550®; Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) at a wavelength of 595 nm (OD_{595}). The results were expressed as the stimulation index (SI), which was calculated according to the following formula:

* CORRESPONDENCE TO: OHTSUKA, H., Laboratory of Large Animal Internal Medicine, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034–8628, Japan.
e-mail: otsuka@vmas.kitasato-u.ac.jp

SI = (OD_{595} of stimulated sample – OD_{595} of control) / OD_{595} of control × 100.

For cytokine mRNA analysis, PBMCs were separated from blood with heparin, and 2×10^6 cells in 1 m*l* 10% FCS-RPMI were added to each well of 48-well plates. The concentrations of PHA and the three AIDs were as described above. PHA (10 μg/m*l*) [20, 24] was added to each PBMC culture, which was then mixed with DEX (10 or 20 μg/m*l*) [13], FLU (10 or 50 μg/m*l*) [2] or MEL (10 or 50 μg/m*l*) [12] and incubated for 12 hr at 37°C. PBMCs were then washed and re-suspended in TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) to extract RNA. Total RNA (2 μg) from each sample was used for synthesis of first-strand complementary DNA (cDNA) with oligo-dT primers (Invitrogen) and Superscript II Reverse Transcripts (Invitrogen) according to the manufacturer's protocols.

The real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed with SYBR Green Master Mix on an ABI prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, U.S.A.). The target DNA sequence was specifically amplified using primers previously designed for interferon (IFN)-γ, IL-2 and IL-4 [21]. The melting curve was determined for each PCR product, and the results were presented as cycle threshold (Ct) values, where ΔCt was the difference in threshold cycles between the target and β-actin as an internal control for cells [17]. ΔCT was calculated to normalize the amount of sample mRNA using the following formula:

ΔCT = Ct value of target cytokine – Ct value of β-actin.

The amount of each cytokine was calculated as $2^{-\Delta Ct}$.

Differences between means were evaluated using analysis of variance (ANOVA) together with Williams' Dunnett test as appropriate, according to the data normality. Data are presented as the mean ± standard error (SE). Differences between experimental groups were considered statistically significant at $P<0.05$.

DEX significantly inhibited lymphocyte proliferation in a dose-dependent fashion at concentrations of 15 and 20 μg/m*l* (67.7% and 84.2%, respectively, relative to PHA alone; $P<0.05$; Fig. 1a). By contrast, a significant suppressive effect of FLU on lymphocyte proliferation was observed at concentrations of 25 and 50 μg/m*l* (38.3% and 53.4%, respectively, relative to PHA alone; $P<0.05$; Fig. 1b), while MEL significantly inhibited lymphocyte proliferation at concentrations of 50 μg/m*l* (58.5% relative to PHA alone; $P<0.05$; Fig. 1c).

The effects of the 3 AIDs on the expression of cytokine mRNA in PBMCs stimulated with PHA and DEX (10 or 20 μg/m*l*), FLU (10 or 50 μg/m*l*) or MEL (10 or 50 μg/m*l*) are shown in Table 1. DEX markedly inhibited the mRNA expression of cytokines such as IFN-γ, IL-2 and IL-4 in comparison to FLU and MEL. FLU and MEL tended to decrease the mRNA expression of IFN-γ, but not IL-2 or IL-4. There was no difference in the effect between FLU and MEL.

In human studies, SAIDs were shown to modulate cytok-

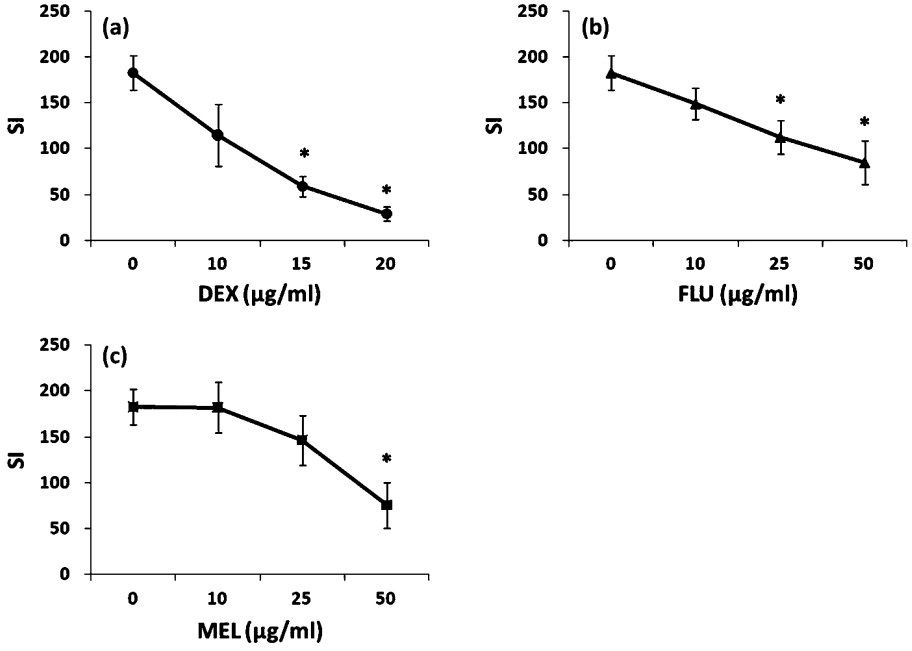

Fig. 1. Comparison of the effects of AIDs on lymphocyte proliferation in calf PBMCs. (a) DEX. (b) FLU. (c) MEL. Results are expressed as the SI. Data represent the mean ± SE. An asterisk denotes a significant difference ($P<0.05$) from PHA alone.

Table 1. The influence of DEX, FLU and MEL on cytokine mRNA expression in the calf PBMCs

Groups	IFN-γ/β-actin	IL-2/β-actin	IL-4/β-actin
Control	0.051 ± 0.021	0.022 ± 0.015	0.007 ± 0.002
PHA	0.183 ± 0.129	0.086 ± 0.026	0.087 ± 0.030
PHA+DEX (10 μg/ml)	0.016 ± 0.003	0.015 ± 0.002	0.038 ± 0.016
PHA+DEX (20 μg/ml)	0.027 ± 0.009	0.011 ± 0.002	0.003 ± 0.000
PHA+FLU (10 μg/ml)	0.116 ± 0.064	0.173 ± 0.038	0.168 ± 0.059
PHA+FLU (50 μg/ml)	0.046 ± 0.007	0.222 ± 0.033	0.058 ± 0.009
PHA+MEL (10 μg/ml)	0.156 ± 0.082	0.187 ± 0.060	0.164 ± 0.065
PHA+MEL (50 μg/ml)	0.067 ± 0.046	0.166 ± 0.338	0.126 ± 0.048

The ratio of each cytokine transcription was compared to the β-actin mRNA expression. Date represent the mean ± SE (n=7).

ine expression by a combination of genomic mechanisms, including the inhibition of genes encoding cytokines IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8 and IFN-γ, with the most important being IL-2 [5]. The immunosuppressive properties of SAIDs have also been reported for bovine PBMCs [18], in which 1 *n*g/m*l* to 10 μg/m*l* DEX suppressed the mRNA expression of cytokines such as IL-1β and tumor necrosis factor-α (TNF-α) in a dose-dependent manner *in vitro* [13], and inhibited the production of IL-2, IFN-β, IL-4, and transforming growth factor-β (TGF-β) *in vivo* [19]. The diminished level of IL-2 also caused fewer T-lymphocyte cells to be activated [10], which reduced T-cell proliferation [16]. In the present study, DEX significantly inhibited lymphocyte proliferation and markedly inhibited cytokine mRNA expression, which was similar to previously reported findings.

An earlier study in calves demonstrated that a combination of FLU or MEL with an antibiotic did not exert an immunosuppressive influence and had a therapeutic effect superior to an antibiotic alone *in vivo* [4]. Other clinical studies in fattening cattle have reported no adverse drug effects related to FLU or MEL treatments [8]. However, we found that both FLU and MEL inhibited lymphocyte proliferation in a dose-dependent manner *in vitro*. In humans, COX-2-specific inhibitors such as NS398 (3.1–31 μg/m*l*) or celecoxib (3.8–38 μg/m*l*) severely diminished T-cell activation, including IL-2, TNF-α and IFN-γ production, and cell proliferation [9], while the nonselective NSAID tenidap inhibited the production and mRNA expression of IL-2 and IFN-γ in T lymphocytes [7].

In our present study, lymphocyte proliferation was reduced by FLU and MEL stimulation and tended to decrease the mRNA expression of IFN-γ, but not IL-2 or IL-4. There are many examples of differences in cytokine expression between animal species [15, 25]. This variation might be caused by different reactivities or sensitivities of animal species to NSAIDs. Further studies are needed to clarify this issue. Nonetheless, FLU and MEL clearly weakly control immune function, although they inhibit lymphocyte proliferation.

In conclusion, our *in vitro* study indicates that not only SAIDs but also NSAIDs exert an immunosuppressive effect on the PBMCs of calves. These findings are important for assessing the indications and complications of NSAIDs in calves. Recent findings in humans suggest that COX-2-selective NSAIDs act as immunosuppressants and could have applications in anti-inflammatory therapy [6, 9]. We believe that it is necessary to reexamine the usage of NSAIDs in the treatment of calves.

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