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J Vet Pharmacol Ther. 2013 Apr;36(2):130-9. doi: 10.1111/j.1365-2885.2012.01396.x

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Preliminary results from the present study were communicated at the 11th EAVPT International Congress held on July 12–16, 2009 in Leipzig.

ABSTRACT

In this study, ex vivo assays were carried out in dairy cows to evaluate the anti-inflammatory effects of two nonsteroidal anti-inflammatory drugs: ketoprofen (KETO) and flunixin meglumine (FM). Twelve healthy Holstein dairy cattle were randomly allocated to two groups (n=6): group 1 received FM and group 2 received KETO at recommended therapeutic dosages. The anti-inflammatory effects of both drugs were determined by measuring the production of coagulation-induced thromboxane B2 (TXB2), lipopolysaccharides (LPS) (10 lg ⁄ mL)-induced prostaglandin E2 (PGE2), and calcium ionophore (60 lM)-induced leukotrien B4 (LTB4). Cytokine production was assessed by measuring tumor necrosis factor-a (TNF-a), interferon-c (IFN-c) and interleu-kin-8 (CXCL8) concentrations after incubation in the presence of 10 lg ⁄ mL LPS. The IC50 of FM and KETO was determined in vitro by determining the concentration of TXB2 and PGE2 in the presence of scalar drug concentrations (10)9–10)3 M). Both FM and KETO inhibited the two COX isoforms in vitro, but showed a preference for COX-1. FM and KETO showed similar antiinflammatory effects in the cow.

INTRODUCTION

Inflammation induced by Gram-negative bacteria is a cause of morbidity and mortality in bovines and can play an important role in the pathogenesis of septic shock with severe clinical signs in animals of all ages (Corl et al., 2010a). The prevalence rate is high among calves deprived of an adequate quantity of colostrum at neonatal age; however, even in adult bovines with an adequate immune system, sepsis caused by Gram-negative bacteria remains a serious clinical challenge for veterinarians (Gerros et al., 1995; Peek et al., 2008). Rapid, uncontrolled bacterial growth and consequent endotoxin release are generally considered responsible for the acute development of inflammatory and toxemic processes (Griel et al., 1975; Peek et al., 2008). Circulating lipopolysaccharides (LPS), released by the destruction of Gram-negative bacteria, form complexes in plasma with high-density lipoproteins or a unique plasma protein lipopoly-saccharides-binding protein (LBP). Bound LPS is cleared from plasma within a few minutes by fixed and circulating macrophages, in the bovine lung and liver, that recognize the LPS–LBP complex. The LPS–LBP complex binds to a membrane bound receptor (mCD14) on mononuclear cells and then attaches to a toll-like receptor 4 (TLR4) on the mononuclear cell membrane; the complex is then internalized and LPS is thought to be destroyed in the process (Bosshart & Heinzelmann, 2007; Heumann & Roger, 2002; Radostits et al., 2007; Sauter et al., 2007). Both TNF-a and CXCL8 play a pivotal role in the pathogenesis of inflammation and endotoxaemia; measurement of serum concentrations of these cytokines can be used as indicators to evaluate the efficacy of anti-inflammatory drugs such as

flunixin meglumine (FM) and ketoprofen (KETO). Activation of the TLR4 receptor leads to intracellular signal-ing, ultimately resulting in the production of COX-2 enzymes and a plethora of inflammatory mediators, including cytokines and eicosanoids (Sauter et al., 2007). Nonsteroidal anti-inflammatory drugs (NSAIDs) act as inhib-itors of COX enzymes, thus preventing the production of prostaglandins, resulting in anti-inflammatory, analgesic and antipyretic effects. COX is an endoperoxide synthase that promotes the conversion from arachidonic acid (AA), the fatty acid present in esterified form in cell membranes, into interme-diate endoperoxides, later transformed into prostaglandins (PGs) prostacyclins (PGI) by the same enzymes, and thromboxanes (TXBs) (Morrow & Jackson Roberts, 2001). At least two COX isoforms are known, having different functions: COX-1 (constit-utive isoform) mainly produces PGs and TXBs that regulate gastrointestinal, renal, vascular, and other physiological func-tions; COX-2 (inducible isoform expressed by cells after stimu-lation by mediators of inflammation such as LPS) that principally regulates production of PGs involved in inflammation, pain, and fever. Arachidonic acid is also a substrate of the lipoxygenase (LOX) pathway that leads to the release of leukotrienes (LTs), chemotactic factors responsible for the activation of leukocytes (Griswold & Adams, 1996; Laudano, 2001; Simon, 1999). The LPS released into the blood by bacteria initially trigger an acute nonspecific host response regulated by the production of tissue factors and cytokines, such as TNF-a and IFN-c, which cause gene expression that promotes the synthesis of various proteins responsible for inflammatory events (Ulmer et al., 2000). The principal cellular sources of CXCL8 are typically mono-cytes and macrophages. CXCL8 bears the primary responsibility for the recruitment of monocytes and neutrophils, the marker cells of acute inflammatory response. Cellular recruitment occurs through the development of a chemotactic gradient which causes the inflammatory cells to move toward an area of increased chemokine concentration (Apostolakis et al., 2009). In this study, ex vivo assays were carried out in dairy cows to evaluate the anti-inflammatory effects of two commonly used NSAIDs, KETO, and FM. Flunixin meglumine has claims for the control of pyrexia associated with various infectious diseases and the control of inflammation associated with endotoxemia (Wagner & Apley, 2004). The claim for the control of inflammation is based on the reduction in PGE2 and TXB2 production in a soft tissue model of inflammation in cattle (Lees et al., 2004b; Odensvik & Magnusson, 1996; Semrad, 1993). FM stops PGE2 production in vitro, requiring a concentration lower than aspirin and indomethacin (Myers et al., 2009). Experimental evidence in different species, including horses and cows, showed the beneficial effects of FM mediator production in both in vitro as well as ex vivo and in vivo models of endotoxemia (Jackman et al., 1994; Odensvik & Magnusson, 1996; Semrad, 1993; Semrad et al., 1987; Semrad & Moore, 1987). Ketoprofen, a propionic acid derivative, is believed to be a potent NSAID acting through relatively nonspecific inhibition of the two COX isoforms (Curry et al., 2005). Some authors have also investigated the antiendotoxemic effects of KETO (Jackman et al., 1994; Semrad, 1993). The aim of this study was to investigate the efficacy of FM and KETO in an ex vivo experimental model. The effects of FM and KETO on mediator synthesis were compared in a LPS-stimulated whole blood model. In addition, the potency and selectivity of both NSAIDs to inhibit the activity of COX-1 and COX-2 was investigated by measuring the IC50 values in vitro.

MATERIAL AND METHODS

Animals and treatments

For this study, 12 healthy Holstein cows from the same farm were selected to obtain two groups homogeneous for age, parity, physiological condition, general clinical condition, and lactation

stage. All animal studies were conducted in compliance with Italian animal welfare laws (D. Lgs 116/1992). The treatment protocol required 4 administrations; the first and second treatment were performed at a 12-h interval, the third and fourth every 24 h. Group 1 (cows 1–6) was given KETO (Comforion_, Janssen CilagJ SpA, Milan, Italy) intravenously at a dosage of 3 mg/kg for a total of four treatments; group 2 (cows 7–12) received FM (Finadyne_, Intervet Schering-Plough italia Srl, Milan, Italy) at a dosage of 2.2 mg/kg for a total of four treatments. Before treatment, a blood sample (T0) was collected from all subjects to obtain the basal values of PGE2, TXB2, LTB4, TNF-a, IFN-c, and CXCL8 (Table 1). All blood samples were collected from the coccigeal vein after having accurately cleaned the area with warm water and natural detergent and disinfected with Betadine_ (Meda Pharma Spa, Milan, Italy). Blood samples were collected into plastic tubes containing sodium citrate for the determination of cytokine concentrations. The blood samples to measure TXB2 concentration were placed into silicon-coated glass tubes, into sodium-heparinized tubes to measure PGE2 concentration, and into lithium-heparinized tubes to measure LTB4 concentration (Cuniberti et al., 2012; Giorgi et al., 2011). After each blood sampling, the whole-blood samples were immediately transferred to the laboratories of the Division of Pharmacology and Toxicology of the Department Animal Pathology, University of Turin. The time between blood collec-tion and testing was 30 min, during which the samples were kept at room temperature (Ray et al., 2006).

Ex vivo study

Measurement of TXB2 concentration.

Samples for measuring TXB2 concentration were treated according to the method described by Sidhu et al. (2005). Briefly, the samples were incubated at 37 LC for 90 min, and the formed clots were gently detached from the test tube walls using a glass Pasteur pipette. The samples were centrifuged at 900 • g for 10 min. Aliquots of 500 lL of serum were stored at)80 LC until analysis. The measurement of TXB2 concentrations was carried out in duplicate using 1:50 dilution.

Measurement of PGE2 concentration.

Plasma PGE2 concentration was measured according to the method described by Brideau et al. (2001). Briefly, aliquots (1 mL) of whole blood containing sodium heparin were incubated at 37 LC for 24 h in the presence of 10 lg LPS/mL (LPS of E. coli serotype 0111:B4; Sigma-Aldrich Co, Milan, Italy.) in phosphate-buffered saline solution (PBS). The blood samples collected at T0 were incubated for 24 h at 37 LC in the presence (K+) or absence (K), PBS only) of LPS and used as positive or negative control of basal PGE2 production, respectively. After incubation, the samples were centrifuged at 400 • g for 10 min to obtain plasma; aliquots (200 lL) of plasma samples were then collected and mixed with 800 lL of methanol (1:4 v:v) to precipitate proteins. After centrifugation at 3000 • g for 10 min, the supernatant was collected and stored at) 80 LC until PGE2 determination. Concentrations of PGE2 was measured in duplicate using a 1:10 dilution.

Measurement of LTB4 concentration.

Plasma LTB4 concentration was measured following the method described by Giorgi et al. (2011), but introducing some minor modification. Briefly, aliquots (300 lL) of whole-blood samples added with lithium heparin were incubated at 37 LC for 30 min in the presence of calcium ionophore A23187 (Sigma-Aldrich) at concentration of 60 lM to induce the release of LTB4 by neutrophils. Even in this case for T0 sample, aliquots (300 lL) of blood were treated in the presence (K+) or absence (K)) of calcium ionophore and used as positive or negative control of basal LTB4 production, respec-tively. After incubation, methanol (1:4 v:v) was added to induce protein

precipitation; the tubes were put in an ice bath for 20 min to stop the reaction. The samples were then ultra-centrifuged at 10 000 • g for 5 min at 4 LC and the supernatant was collected and stored at) 80 LC until LTB4 analysis. The measurement of LTB4 concentrations were carried out in duplicate using 1:5 dilution.

Concentrations of TXB2, PGE2, and LTB4 were measured using a commercial EIA kit validated for bovine species (TXB2, PGE2, LTB4 Biosource Europe S.A., Nivelles, Belgium). Coefficients for validation are reported in the Results Section.

Measurement of TNF-a, IFN-c, and CXCL8 concentrations.

To measure TNF-a, IFN-c, and CXCL8, we added to the whole-blood samples (with sodium citrate) a solution containing LPS diluted in phosphate buffer (PBS) to obtain a final concentration of 10 lg LPS ⁄ mL (Barbero et al., 2005). The LPS-stimulated samples, along with control samples to which we added a similar amount of PBS without LPS, were incubated in 5% CO2 modified atmosphere at 37 LC for 4 h as it was observed that a higher production of cytokines occurs after 3–6 h (Smith et al., 2004; Rontved et al., 2005). After incuba-tion, the tubes were centrifuged at 3000• g for 10 min at room temperature to obtain plasma samples which were immediately frozen at)80 LC until analysis. The measurement of TNF-a, IFN-c, and CXCL8 concentrations was carried out in triplicate after samples dilution of TNF-a (1:5). Concentrations of TNF-a, IFN-c, and CXCL8 were determined using a commercial EASIA kit validated for bovine species (CXCL8, IFN-c, TNF-a Biosource Europe S.A., Nivelles, Belgium). Coefficients for validation are reported in the Results Section. IFN-c kit was a semi-quantitative test.

The reaction product was measured by reading the absor-bance at 450 nM using a spectrophotometer (Power Wave X; Bio-Tek Instruments, Highland Park, Winooski, Vermont, USA).

In vitro COX-1 ⁄ COX-2 assays

The IC50 of FM and KETO was determined in vitro by measuring the concentration of coagulation and LPS induced of TXB2 and PGE2, respectively, in the presence of scalar drug concentrations (10)9–10)3 M) using the method previously described by Bri-deau et al. (2001) with some minor modifications. The results are expressed as mean ± SEM. The sigmoidal dose–response curves were analyzed using GraphPad Prism version 4.00 (GraphPad Software Inc., San Diego, CA, USA) plotting the data in a nonlinear curve fitting model using the four parameter logistic equation:

% Inhibition ¼ I0 þ ðImax _ I0Þ=1 þ 10½ðLogIC50 _CÞ_nH &

where % inhibition is the inhibition of prostanoid (TXB2 and PGE2, respectively) production expressed as a percentage of the control value; C is the test compound concentration after logarithmic transformation; IC50 (potency) is the test compound concentration that resulted in 50% inhibition of COX-1 (IC50 COX-1) and COX-2 (IC50 COX-2); Imax is the maximum inhibition (drug efficacy); I0 is the baseline inhibition; nH is the Hill coefficient (sensitivity) that results in the slope of the concen-tration–response curve. Selectivity for FM and KETO was determined by calculating the COX-1:COX-2 ratio. We used the IC50 value of potency ratio that is acceptable if COX-1 and COX-2 inhibition curves of a drug are parallel, because the potency ratio will be independent of the particular IC value selected (Lees et al., 2004a).

Statistical analysis of data

Data obtained, expressed by mean ± SEM, were statistically analyzed using a computer program (GraphPad Prism version 4.00; GraphPad Software Inc), and the analysis of the distribu-tion of data within each group was performed with the Kolmogorov–Smirnov test. The TXB2, PGE2, LTB4, TNF-a, IFN-c, CXCL8 concentrations of FM vs. KETO were analyzed at the different sampling times using Student's t-test for unpaired data with Welch's correction. The variations of the same parameters in different experimental times were analyzed with the Two-way ANOVA followed by Bonferroni posttest. $P < 0.05$ was considered as statistically significant.

RESULTS

Ex vivo study

Coefficient of variance values of the intra- and inter-assay were 1.6% and 6.2% for TXB2, 17.5% and 3.9% for PGE2, and 5.9% and 5.0% for LTB4, respectively ($R2 > 0.90$).

Measurement of TXB2 concentrations.

In Fig. 1, the effects of FM and KETO on TXB2 production are illustrated. Student's t-test showed statistically significant differences between FM and KETO at T2, T3, and T4 (*$P < 0.05$) (Fig. 1A). The ANOVA test showed a significant difference between the TXB2 concentrations in the samples taken at T0 and those taken at T1, T2, T3, and T4 in both treatment groups (Fig. 1B,C). Interestingly, FM, but not KETO, still showed significant inhibition of TXB2 production 25 h after the last administration of the drugs ($P < 0.05$; Fig. 1B,C).

Measurement of PGE2 concentrations.

Figure 2A Significant differences ($P < 0.05$) were noted between T0 and all other experimental times (T1, T2, T3 and T4) in both groups (Fig. 2B,C). From T2, a gradual decrease in inhibition up to T5 could be observed. Only in the KETO-treated group, there was a significant decrease ($P < 0.05$) in PGE2 inhibition at T5 when compared with T1, T2, T3, and T4 (Fig. 2C).

Measurement of LTB4 concentrations.

There were no significant differences in plasma LTB4 concentrations between the two drugs in the samples collected in the different sampling times. There is only one significant difference ($P < 0.05$) between K+ (stimulated) and K) (not stimulated) (Fig. 3A,B,C). Coefficient of variance values of the intra- and inter-assay were 4.4% and 9.0% for TNF-a; 3.5% and 7.9% for IFN-c and 4.2% and 9.0% for CXCL8, respectively ($R2 > 0.90$).

Measurement of TNF-a concentrations.

Figure 4 shows the plasma TNF-a concentrations. Student's t-test did not show any statistically significant differences ($P < 0.05$) between FM and KETO (Fig. 4A). ANOVA indicated significant differences ($P < 0.05$) between T0 and all other experimental times (T1, T2, T3, T4 and T5) (Fig. 4B,C).

Measurement of IFN-c concentrations.

Figure 5 shows the plasma IFN-c values. No significant difference between the two treat-ment groups was observed at T0, T1, T3, T4, and T5 sampling time (Fig. 5A). A statistically significant

difference ($P < 0.05$) in the effects induced by the two drugs was observed only at T2, where the values of FM were higher than those of KETO. ANOVA indicated significant differences ($P < 0.05$) between T0 and other experimental times (T1, T2, T3, T4 and T5) for FM and KETO (Fig. 5B,C). The values of T4 and T5 of KETO and FM were significantly decreased ($P < 0.05$) when compared with T1, T2, and T3, while FM values of T2 and T3 were significantly increased compared with T1 ($P < 0.05$).

Measurement of CXCL8 concentrations.

Figure 6 shows that there were no difference in CXCL8 plasma concentrations between the two treatment groups (Fig. 6A). However, there were significant differences ($P < 0.05$) for both drugs between T0 and the experimental times T1, T2, T3, and T4 (Fig. 5B,C). The effect of inhibition decreased gradually to T5. Only FM T5 value was significantly different ($P < 0.05$) when compared with T1, T2, T3, and T4, while KETO T5 value was significantly different ($P < 0.05$) only compared with T1 and T2.

In vitro COX-1 / COX-2 assays

Figures 7 and 8 show the dose–effect relationship for FM and KETO, respectively.

For FM, the resulting COX-1 / COX-2 ratio was 0.26. For KETO, the resulting COX-1 / COX-2 ratio was 0.41 (Table 2).

DISCUSSION AND CONCLUSIONS

Bovine mastitis induced by coliform bacteria is considered a relevant pathology because of its acute, severe and sometimes fatal course and because it is difficult to control with current intra-mammary antibiotic formulations (Griel et al., 1975; Peek et al., 2008). An adequate response is necessary to expedite bacterial clearing (Corl et al., 2010b). Long used as therapeutic agents to control inflammatory response, NSAIDs are inhibitors of COX-1 and COX-2 activity, and the inhibitor selectivity of NSAIDs is species dependent (Brideau et al., 2001). The evaluation of anti-inflammatory activity in our ex vivo model shows that there is no significant difference between the two NSAIDs considered. In fact, FM and KETO are able to inhibit TXB2 and PGE2 production (indicative of drug action against COX-1 and COX-2, respectively) without any signifi-cant difference. The results obtained agree with those pub-lished by Semrad (1993). In this case, the dosage was 1.1 mg / kg of body weight for FM and 2.2 mg / kg for KETO. Odensvik and Magnusson (1996) also observed a decrease in PGs baseline concentration after oral administration of FM in heifers and this action lasted longer than that after IV administration of FM. Concerning the drugs activity on the LOX pathway, their mechanism of action does not include inhibition of 5-LOX, in fact, no statistically significant differences were observed by either comparing their activity individually or analyzing their activity at different sampling time. In fact, there is only a significant difference between K+ (stimulated) and K) (not stimulated) for both drugs (Fig. 3). Our results suggest that both FM and KETO can significantly reduce cytokine concentrations (TNF-a, CXCL8 and IFN-c) in an ex vivo model in dairy cows and that there is no significant difference in the efficacy of the two drugs. After an increase in LPS concentrations in the body, the production of TNF-a by lymphocytes and macrophages increases considerably, causing the synthesis of other endogenous medi-ators (e.g., IL-1, IL-6, CXCL8 and IFN-c) which play a key role in the pathological process (Sordillo & Peel, 1992). As shown in Fig. 4A, the TNF-a concentrations in the LPS-stimulated samples at T0 were significantly higher than in the corresponding unstimulated

samples. At all later sampling times (T1 – T5), the exposure to therapeutic doses of FM and KETO significantly reduced ($P < 0.05$) the TNF-a concentrations with statistical differences compared with the basal stimulated (K+) values. These results agree with Myers et al. (2009), who suggest that FM may inhibit TNF-a expression in cattle, and Yazar et al. (2007), who indicate that FM may reduce pro-inflammatory cytokine TNF-a concentrations in endotoxemia in mice. It has been shown that cytokine release can be induced by various different bacteria or their products, both in vivo and in vitro in leukocyte cultures. Evidence suggests a critical role for IFN-c in toxinfections (Bienek et al., 1998). IFN-c is said to be important for regulating nonspecific host defenses. It increases killer cell activity and antibody-dependent cell cytotoxicity and cytotoxic activity of T-lymphocytes. In addition, it can increase the phagocytic and bactericidal action of macrophages and neutrophils (Sordillo & Peel, 1992). In our study, we noted significant differences between the LPS-stimulated samples and the corresponding unstimulated control samples at T0 (Fig. 5). The measurements at later sampling times (T1–T5) highlight that treatment with KETO or FM significantly reduced ($P < 0.05$) the increase in plasma concentrations of IFN-c generated by LPS in both groups. The biological effects of CXCL8 are mediated through the binding of CXCL8 to two cell surface G-protein-coupled receptors that share considerable structural similarity and induce a nearly identical range of biological activities (Apostolakis et al., 2009). The role of CXCL8 in changes within the neutrophil population has been demonstrated in different animal species. (Gangur et al., 2002). Furthermore, KETO is reported to be a potent and specific noncompetitive inhibitor of CXCL8-induced human polymorphonuclear neutrophils chemotaxis (Allegretti et al., 2005). CXCL8 is produced early in the inflammatory response, but remains active for a prolonged period of time, even days and weeks (Apostolakis et al., 2009). As illustrated in Fig. 6, there was a significant difference between the basal values (T0) and those obtained after LPS stimulation, thus suggesting a signif-icant increase in the production of the mediator. At the two subsequent sampling times (T1 and T2), anti-inflammatory drug treatment reduced the production of CXCL8 to the extent of annulling the difference between the stimulated samples and the corresponding unstimulated controls. These results agree with data reported in previous papers, but concerning other species, in which KETO reduces CXCL8 production (Wang et al., 1997) and the inhibition of CXCL8 chemotaxis contribute to the anti-inflammatory activity of NSAIDs (Bizzarri et al., 2001). However, a lack of inhibition of CXCL8 release from T3 to T5 was observed in the two groups, but with a significant difference between basal values and the sampling times T1–T5. As a result, data obtained showed a significant decrease in drugs inhibitory effect toward TXB2, PGE2, and CXCL8 at T5; presumably, the reduced inhibition at T5 can be explained by the lowered concentration of NSAIDs in the blood. Similar result is not observable for TNF-a and IFN-c, where the inhibitory effect still persists 24 h after the last treatment. The CXCL8 concentration variations are difficult to explain when dealing with anti-inflammatory treatment, although this occurred in both treatment groups. A similar behavior of CXCL8 production has been observed in other different experimental models of diseases. In this cases, the authors believed that there are other activation ⁄ regulation mechanisms to stimulate CXCL8 production, preferably after having previously administered an anti-inflammatory drug (Wu et al., 1993; Opre´e & Kress, 2000). In humans, some COX-2 selective NSAIDs inhibited the production of IFN-c and TNF-a, while nonselective NSAIDs inhibited production of IFN-c, but not interleukins expression (Dolhain et al., 1995; In˜iguez et al., 1999). In a study about calves, the authors found that FM and some NSAIDs inhibited lymphocytes proliferation in a dose-dependent manner in vitro and tended to decrease the expression of IFN-c, but not interleukins (Maeda et al., 2005). Furthermore, experimental evidence in a in vivo model demonstrated that some NSAIDs exert their effects inducing down-regulation of pro-inflammatory cytokines such as TNF-a and IFN-c by a inhibitory effects on nuclear factor-jB (NF-jB) and COX-2 expression. They also observed that this down-regulation remains for several hours until this way is

restored. (Vaish & Sanyal, 2011). The immune-suppressive effect of NSAIDs on lymphocytes proliferation and the down-regulation of NF-jB and COX-2 expression could be the explanation of the different effects on INF-c and TNF-a respect CXCL8 production and of the persistence of inhibitory effects for 24 h after last treatment observed in the present study (Figs 4, 5 & 6). In vitro experiments show that neither drug has a stronger selectivity toward one of the two isoforms as no significant differences between the two drugs were noted in their ability to inhibit the two isoforms, although a preferential activity vs. COX-1 was shown by both drugs (COX-1 / COX-2 ratio < 1). In conclusion, the results obtained in the present studies show that in dairy cows, as observed in the horse, KETO and FM, at therapeutic doses, have a similar anti-inflammatory action. In our experiment, we observed that they are able to inhibit the production of CXCL8, IFN-c, and TNF-a. Moreover, both drugs showed comparable ability to inhibit both COX isoforms in vitro, with a preference for inhibition of COX-1 (COX-1 / COX-2 ratio < 1). Finally, in the present study, both FM and KETO demonstrated that they are not able to significantly inhibit LOX activity, and thus, they should not be considered as 'dual inhibitor' NSAIDs.

ACKNOWLEDGMENTS

This work was supported by grants from the Regione Piemonte, Italy (Ricerca Sanitaria Finalizzata 2009), from the University of Turin (ex-60%) Italy and from Janssen Animal Health, Italy. Particular thanks to Dr Riccardo Data for kind support.

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Figures & Tables

	Day 1	Day 2	Day 3	Day 4	Day 5
Treatments	Treatment 1 6 PM	Treatment 2 6 AM	Treatment 3 6 AM	Treatment 4 6 AM	–
Sample collections	Blood sample 1 5 PM (T_0) (basal values – 1 h before treatment 1) Blood sample 2 7 PM (T_1) (1 h after treatment 1)	Blood sample 3 7 AM (T_2)	Blood sample 4 7 AM (T_3)	Blood sample 5 7 AM (T_4)	Blood sample 6 7 AM (T_5)

Table 1. Schedule of the experimental protocol. Group 1 (n = 6 dairy cows) 3 mg ketoprofen ⁄ kg bodyweight intravenously; group 2 (n = 6 dairy cows) 2.2 mg flunixin meglumine ⁄ kg body weight intravenously

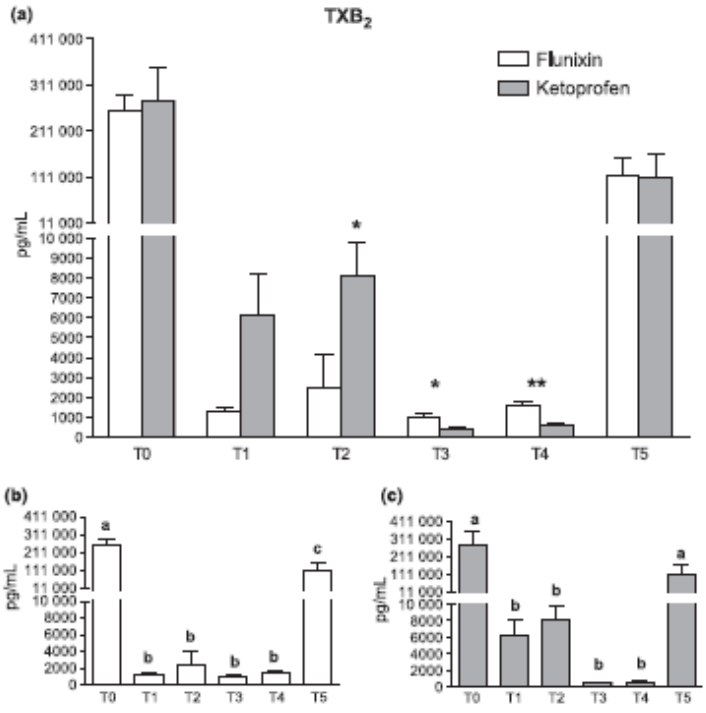

Fig. 1. Effect of flunixin meglumine (n = 6; white bars; 2.2 mg ⁄ kg BW) and ketoprofen (n = 6; gray bars; 3 mg ⁄ kg BW) on TXB2 production. The measurements of TXB2 con-centrations were performed after incubation of whole blood at 37 LC for 90 min. Concentra-tions of TXB2 (mean ± SEM) were statistically analyzed using Student's t-test with signifi-cant differences for *P < 0.05; **P < 0.001. (A) and two-way ANOVA followed by Bonfer-roni posttest with significant differences for a, b, c = P < 0.05 (B, C) (GraphPad Prism).

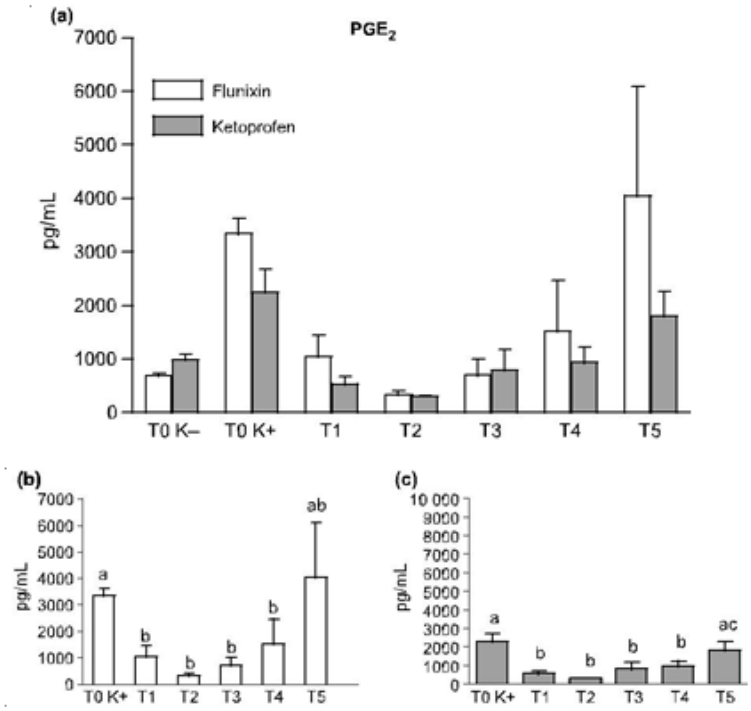

Fig. 2. Effect of flunixin meglumine (n = 6; white bars; 2.2 mg ⁄ kg BW) and ketoprofen (n = 6; gray bars; 3 mg ⁄ kg BW) on PGE2 production. The measurements of PGE2 con-centrations were performed after incubation of whole blood (with sodium heparin) at 37 LC for 24 h in the presence of 10 lg LPS ⁄ mL. Concentrations of PGE2 (mean ± SEM) were statistically analyzed using Student's t-test (A) and two-way ANOVA followed by Bonferroni posttest with significant differences for a,b,c = $P < 0.05$ (B, C) (GraphPad Prism).

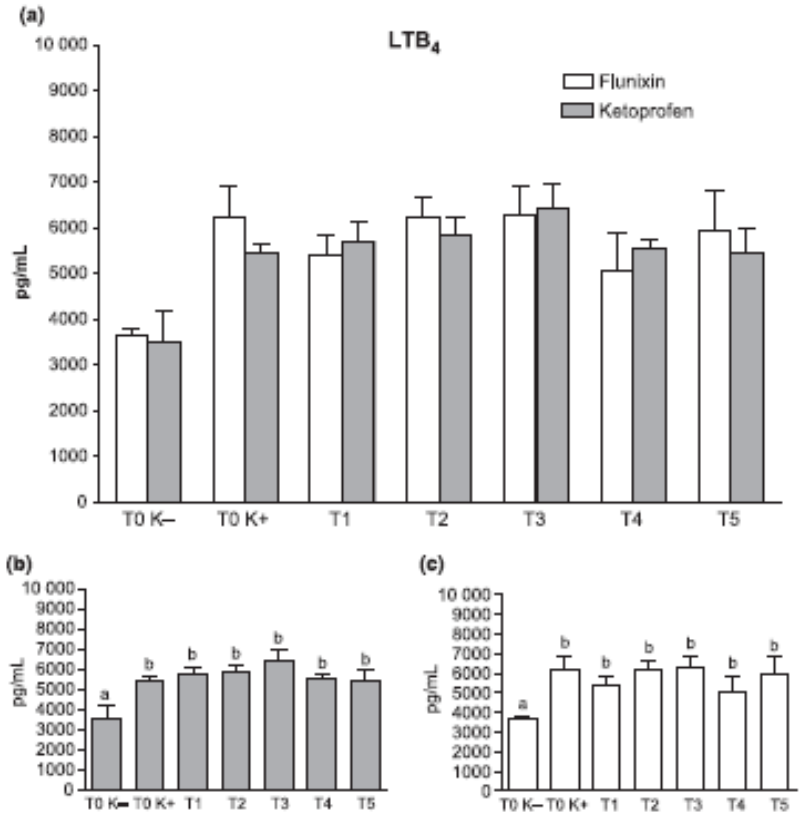

Fig. 3. Effect of flunixin meglumine (n = 6; white bars; 2.2 mg ⁄ kg BW) and ketoprofen (n = 6; gray bars; 3 mg ⁄ kg BW) on produc-tion. The measurements of LTB4 concentra-tions were performed after incubation of whole blood (with lithium heparin) at 37 LC for 30 min in the presence of calcium iono-phore A23187 at concentration of 60 lM. Concentrations of LTB4 (mean ± SEM) were statistically analyzed using Student's t-test with (A) and two-way ANOVA followed by Bonferroni posttest with significant differences for a,b = $P < 0.05$ (B, C) (GraphPad Prism).

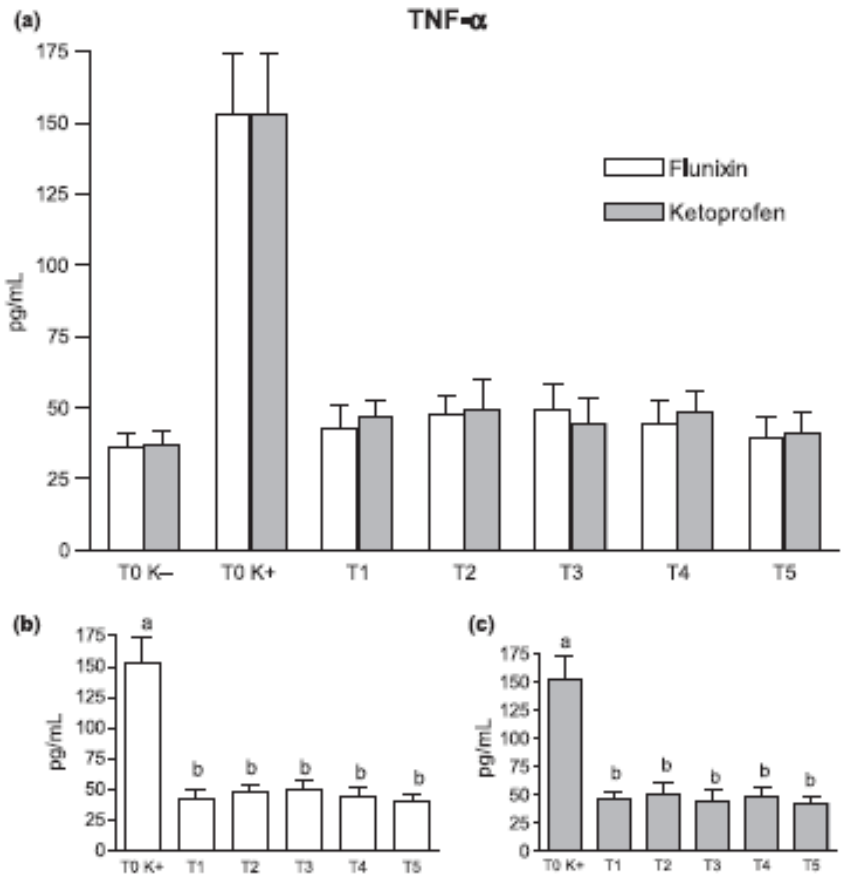

Fig. 4. Effect of flunixin meglumine (n = 6; white bars; 2.2 mg ⁄ kg BW) and ketoprofen (n = 6; gray bars; 3 mg ⁄ kg BW) on TNF-a production. The measurements of TNF-a concentrations were performed after incuba-tion of whole blood (with sodium citrate) at 37 LC for 4 h in the presence of LPS at 10 lg ⁄ mL. Concentrations of TNF-a (mean ± SEM) were statistically analyzed using Student's t-test (A) and two-way ANOVA followed by Bonferroni posttest with signifi-cant differences for a,b = $P<0.05$ (B, C) (GraphPad Prism).

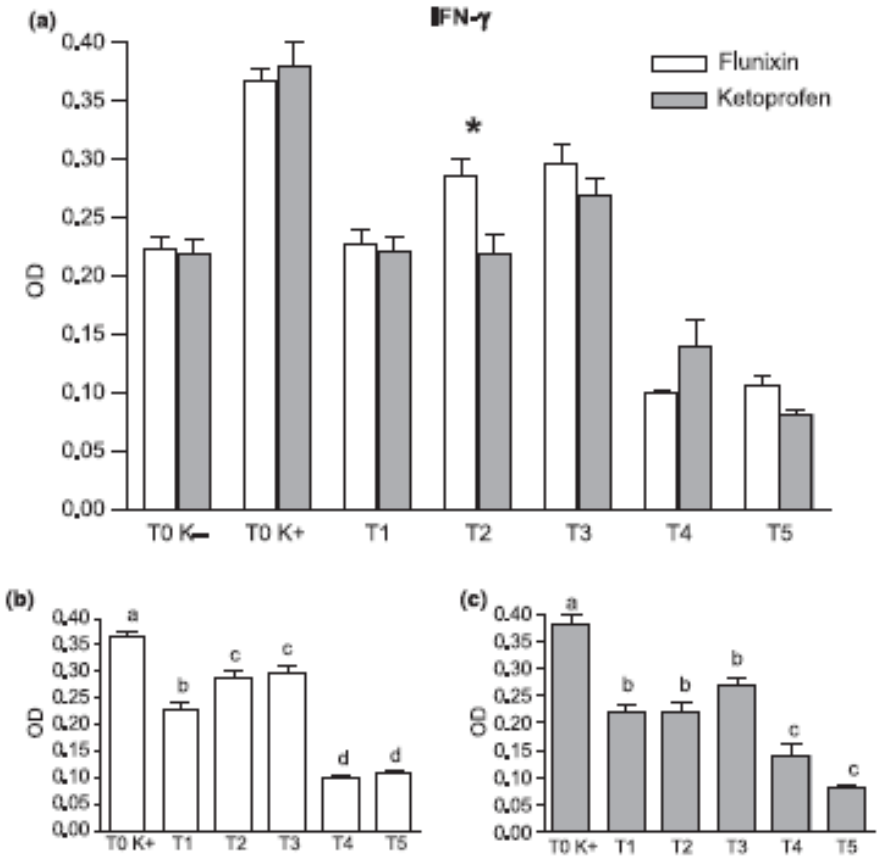

Fig. 5. Effect of flunixin meglumine (n = 6; white bars; 2.2 mg ⁄ kg BW) and ketoprofen (n = 6; gray bars; 3 mg ⁄ kg BW) on IFN-c production. The measurements of IFN-c con-centrations were performed after incubation of whole blood (with sodium citrate) at 37 LC for 4 h in the presence of LPS at 10 lg ⁄ mL. Concentrations of IFN-c (mean ± SEM) were statistically analyzed using Student's t-test with significant differences for *P < 0.05 (A) and two-way ANOVA followed by Bonferroni posttest with significant differences for a, b, c, d = P < 0.05 (B, C) (GraphPad Prism).

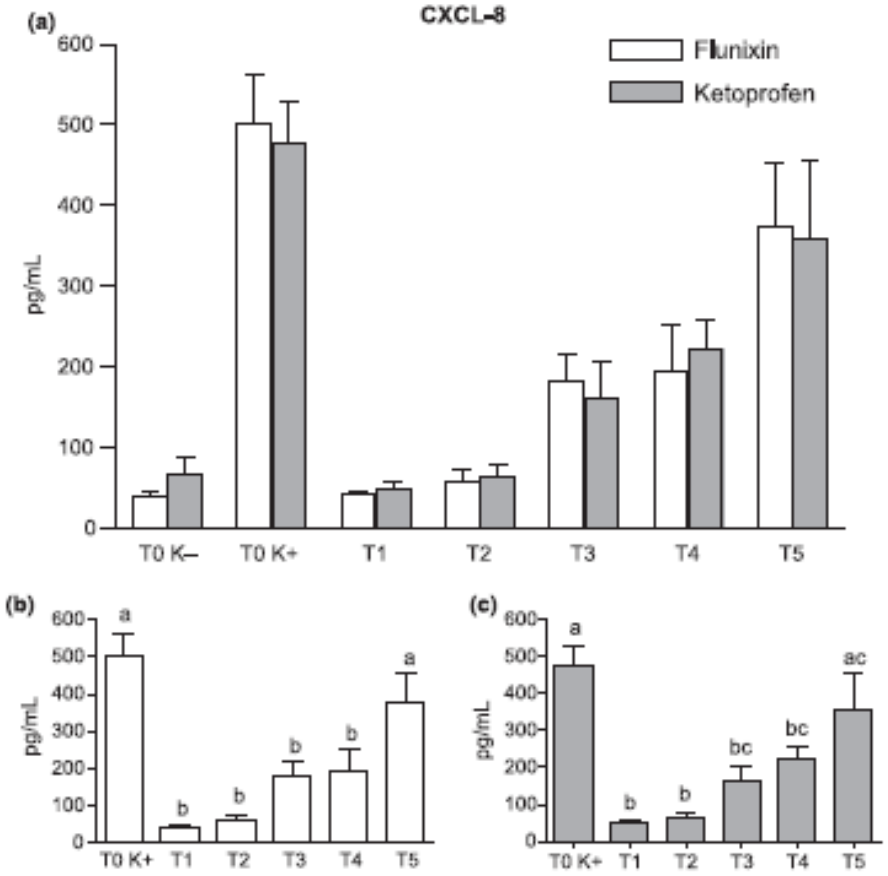

Fig. 6. Effect of flunixin meglumine (n = 6; white bars; 2.2 mg ⁄ kg BW) and ketoprofen (n = 6; gray bars; 3 mg ⁄ kg BW) on CXCL8 production. The measurements of CXCL8 concentrations were performed after incubation of whole blood (with sodium citrate) at 37 LC for 4 h in the presence of LPS at 10 lg ⁄ mL. Concentrations of CXCL8 (mean ± SEM) were statistically analyzed using Student's t-test (A) and two-way ANOVA followed by Bonferroni posttest with significant differences for a, b, c = $P < 0.05$ (B, C) (GraphPad Prism).

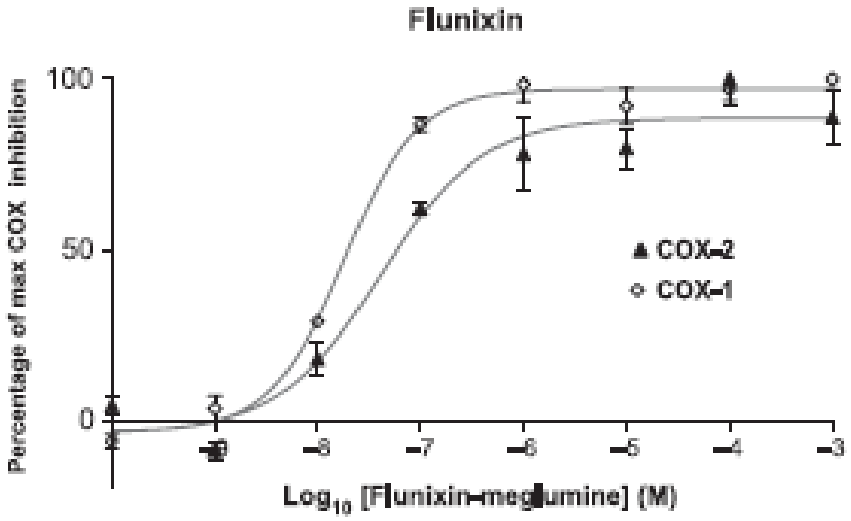

Fig. 7. The IC50 of flunixin meglumine (FM) was determined in vitro by measuring the concentrations of TXB2 and PGE2 in the presence of increasing drug concentrations (10)9–10)3 M). Percentage inhibition of COX-2 ( ) and COX-1(e) by FM (n = 6) was expressed as mean ± SEM and the sigmoidal dose–response curves were analyzed using GraphPad Prism.

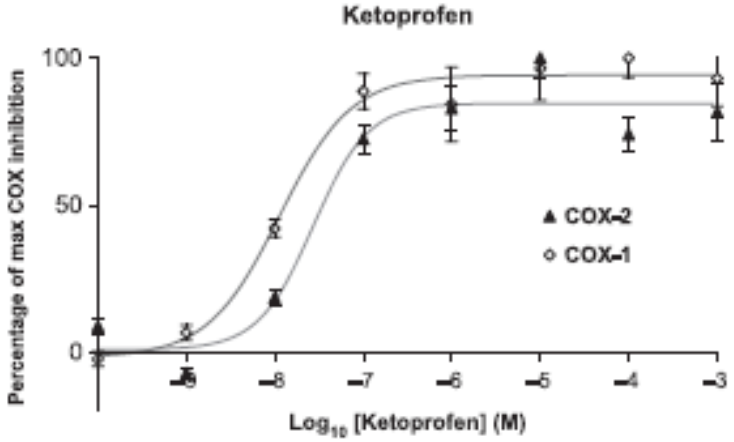

Fig. 8. The IC50 of ketoprofen (KETO) was determined in vitro by measuring the concentration of TXB2 and PGE2 in the presence of increasing drug concentrations (10)9–10)3 M). Percentage inhibition of COX-2 ( ) and COX-1(e) by KETO (n = 6) was expressed as mean ± - SEM and the sigmoidal dose–response curves were analyzed using GraphPad Prism.

FM	KETO
IC_{50} COX-1 = 1.083 · $10^{)8}$ M	IC_{50} COX-1 = 1.127 · $10^{)8}$ M
IC_{50} COX-2 = 4.066 · $10^{)8}$ M	IC_{50} COX-2 = 2.703 · $10^{)8}$ M

Table 2. Concentrations of flunixin meglumine, and ketoprofen which inhibits 50% of the maximum COX isoform activity. Concentrations of TXB2 and PGE2, expression of COX-1 and COX-2 activity, respectively, were determined using commercial EIA kits (Biosource Europe S.A., Nivelles, Belgium). Statistical analysis (Student's t-test; $P < 0.05$) was performed using GraphPad Prism

FM, flunixin meglumine; KETO, ketoprofen.