Comparative efficacy of flunixin on prostaglandin E2 synthesis in a bovine tissue cage model of acute inflammation

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SUMMARY

The efficacy of flunixin on PGE₂ synthesis in bovine inflammatory exudate was compared to that of meloxicam and of a placebo in a tissue cage model of acute inflammation. Twelve calves were randomly assigned in the study to three-treatment sequences. Two weeks prior to the first period, sterile hollow perforated polyethylene balls were surgically embedded in the subcutis at four distinct sites per animal. On the first day of each period, an aseptic inflammation was induced by injecting 0.5 mL of a 2% carrageenan solution. Treatment with either flunixin (2.2 mg/kg), meloxicam (0.5 mg/kg) or NaCl (0.25 mL/kg) immediately followed, 0.5 mL of exudate was collected prior to challenge, and at 2, 4, 8, 12, 24, 36 and 48 hours after. Exudate PGE₂ concentrations were measured using an ELISA kit. Mean PGE₂ concentrations displayed a post-challenge sharp increase, peaking 2 hours after treatment, and gradually decreasing, returning to baseline values within 48 hours. PGE₂ concentrations were consistently lower in animals treated with flunixin or meloxicam than in animals treated with the placebo. The most important reduction of PGE₂ concentrations was observed at +4 hours after treatment, in both treated groups by comparison with placebo. Differences with the placebo were significant throughout the post-treatment period for flunixin and until 12 hours after treatment for meloxicam. Pharmacokinetic modelling of area under the curve of PGE₂ showed significant difference of each treatment group with control group. The treated groups are not significantly different from each other. Decreases of exudate PGE₂ concentrations occurred in both NSAID groups, with the strongest effect occurring 4 hours after administration. The flunixin anti-inflammatory activity persisted up to 48 hours post-treatment.

Keywords: Tissue cage model, flunixin, meloxicam, cattle, prostaglandin E₂ (PGE₂), inflammatory exudate.

RESUME

L’efficacité de la flunixine sur la synthèse de PGE₂ dans l’exsudat inflammatoire bovin a été comparée à celle du meloxicam et d’un placebo dans un modèle de cage tissulaire d’inflammation aiguë. Douze bœufs ont été répartis aléatoirement dans l’une des 3 séquences de traitements. Deux semaines avant la première période de traitement, des sphères stériles et perforées en polyéthylène ont été chirurgicalement implantées dans le tissu sous-cutané au niveau de quatre sites distincts par animal. Le premier jour de chaque période, 0,5 ml de carraghénane (solution à 2%) a été injecté pour induire une inflammation aseptique du tissu sous-cutané. Le traitement avec la flunixine (2,2 mg/kg), le meloxicam (0,5 mg/kg) ou du NaCl (0,25 mL/kg), a été réalisé immédiatement après. 0,5 ml d’exsudat a été recueilli avant l’induction de l’inflammation et 2, 4, 8, 12, 24, 36 et 48 heures après. Les concentrations de PGE₂ ont été déterminées par une technique ELISA. Les concentrations moyennes de PGE₂ ont présenté une forte hausse après l’induction de l’inflammation, atteignant un maximum 2 heures après le traitement et diminuant ensuite graduellement, revenant aux valeurs de base dans les 48 heures. Les concentrations de PGE₂ étaient toujours plus faibles chez les animaux traités avec la flunixine ou le meloxicam que chez les animaux recevant le placebo. La réduction des concentrations de PGE₂, a été la plus importante à + 4 heures après le traitement dans les 2 groupes traités, en comparaison avec le groupe placebo. Les différences avec le placebo étaient significatives durant toute la période post-traitemen pour la flunixine et jusqu’à 12 heures après le traitement pour le meloxicam. La modélisation pharmacocinétique de l’aire sous la courbe de PGE₂, a montré une différence significative de chaque groupe de traitement avec le groupe contrôle. Les groupes de traitement ne sont pas significativement différents entre eux. Les concentrations de PGE₂ ont présenté une diminution dans les 2 groupes traités avec les anti-inflammatoires non-stéroïdiens, le plus fort effet survenant 4 heures après leur administration. L’activité anti-inflammatoire de la flunixine a persisté jusqu’à 48h après le traitement.

Mots-clés : Cage tissulaire, flunixine, meloxicam, bovin, prostaglandine E₂ (PGE₂), exsudat inflammatoire.

Introduction

Flunixin meglumine and meloxicam are non-steroidal anti-inflammatory drug (NSAID). Flunixin meglumine is classified as a carboxylic acid. Its mechanism of action is believed to be primarily via the inhibition of cyclooxygenase (COX) enzymes. This inhibition results in decreased formation of cyclooxygenase-derived eicosanoids involved in the pathophysiology of inflammation, such as thromboxanes and prostaglandins.

Meloxicam, used as comparative product in this study, belongs to the oxicam class which acts by inhibition of prostaglandin synthesis, thereby exerting anti-inflammatory, anti-exudative, analgesic and antipyretic effects. It reduces leukocyte infiltration into the inflamed tissue. To a minor extent it also inhibits collagen-induced thrombocyte
aggregation. Meloxicam also has anti-endotoxic properties because it has been shown to inhibit production of thromboxane B2 induced by E. coli endotoxin administration in calves, lactating cows and pigs [6].

Prostaglandins, and in particular prostaglandin E (PGE), are known to be present in large quantities in inflammatory exudates. The determination of PGE, concentration in in vivo exudate was used as an indicator of cyclooxygenase COX-2 activity [3, 4].

Flunixin is widely used for its anti-inflammatory and analgesic properties in equine and ruminant veterinary practice. There is a notably well-established rationale for using anti-inflammatory drugs as an adjunctive therapy to an antibiotic when treating infectious diseases in general. During the course of naturally occurring Bovine Respiratory Disease, it is recognized that inflammatory mediators may cause pulmonary lesions that may affect pulmonary function [13]. It is therefore important to lessen the excessive inflammatory response occurring in the lungs secondary to bacterial and viral infections [1, 3, 10].

Flunixin and meloxicam are both highly protein bond to plasma protein in cattle [2, 6, 18]. Flunixin (2.2 mg/kg) had a peak plasma concentration (Cmax) of 16.16 ± 5.28 µg/ml after intravenous administration [18] and of 1.27 g/ml after intramuscular administration [1]. Tmax after intravenous administration was of 0.49h. The apparent volume of distribution was 2.11 ± 0.37 L/kg after iv administration [11]. Flunixin showed an elimination half-life of 6.87 ± 0.49 hours (iv administration) [11, 18].

After a single subcutaneous dose of 0.5 mg meloxicam/kg in young cattle, meloxicam showed Cmax values of 2.1 µg/ml reached after 7.7 hours [6]. The bioavailability of meloxicam was 92% when compared with intravenous injection. The apparent volume of distribution was 193.94 ± 10.34 ml/kg after iv administration [11]. Flunixin showed an elimination half-life of 0.49 hours (iv administration) [11, 18].

Tissue cage studies have shown that flunixin achieves higher concentration in inflammatory exudate, exceeding corresponding plasma concentration. Lees et al. [13] reported a half-life for flunixin in inflammatory exudate of 23 hours in cattle. The accumulation of flunixin in, and the slow clearance from, inflammatory exudate is clinically advantageous because this is the site of therapeutic action and it explains the longer duration of action of flunixin than predicted by its relatively rapid plasma clearance. In piglet, meloxicam showed a lower AUC in exudate than in plasma (4479 ± 588 vs. 8034 ± 1075 h.ng/mL) [8].

Subcutaneously implanted tissue cages have been extensively used in many animal species since the 1960s to study the mechanism and time course of the acute non-immune inflammatory response, and also the pharmacokinetics and pharmacodynamics of antimicrobials [9]. Tissue cages are constituted of hollow perforated devices which, after subcutaneous implantation, become surrounded by, and partially filled with, infiltrating highly vascularised granulation tissue. The remainder of the chamber becomes filled with a fluid that is similar in composition to interstitial fluid. The intra-caveal injection of a mild irritant, e.g. carrageenan, allows the production of inflammatory exudate and measurement of inflammatory mediators [7].

This acute inflammation model was used in the study presented hereafter to compare the action of two NSAIDs (flunixin and meloxicam) on the synthesis of PGE, in bovine inflammatory exudate.

Materials & Methods

ANIMALS

The study was conducted on 12 healthy calves of both sexes (8 females and 4 males), aged from 5 to 11 months and weighing 183.3 ± 39.1 kg at study initiation (range 128 to 247 kg). Three different dairy or dual-purpose breeds were represented: Normande (8 animals), French Brune (3) and Holstein (1). The animals did not receive any treatment within one week preceding study initiation.

HUSBANDRY

The animals were housed in Good Laboratory Practice-compliant experimental facilities (accreditation D37-109-2). During the 2-week post-implant stabilisation period, they were housed in 4 pens of 3 animals located in a thermo-regulated bovine unit. From the first challenge onwards, animals of all groups were commingled in two open barns within the same building. The animals had free access to water, hay and an antibiotic-free commercial feed.

SURGICAL PROCEDURE

Two weeks before study initiation, four sterile spherical perforated polypropylene tissue cages were surgically embedded in the subcutaneous space (sub-cuts) at four distinct sites per animal; two on each side of the animal’s flank. The animals were sedated by intravenous injection of xylazine (Rompun® 2%, 0.25 ml/100 kg) keeping them standing and weight-bearing. The hair over the implantation sites was clipped and the skin steriley prepped. Local anesthesia of the skin and subcutaneous tissue with xylocaine was provided at the incision site and laterally to it by small anesthetic blebs of Xylovet®. The skin was incised and the subcutaneous tissue extensively dissected in order to implant one tissue cage cranial to the incision site. Once implanted, the skin was closed by suture of the sub-cutis and skin. Post-operative antibiotic and anti-inflammatory treatments were administered (Injectyl®, 10 ml/100kg sid during 3 days, and Tolfine®, 1 ml/20 kg once). A 2-week post-implant stabilization period was then allowed to elapse before beginning the first study period.
Efficacy of Flunixin on Prostaglandin E2 Synthesis in Bovine Inflammatory Exudate

Experimental Design and Model of Inflammation

A three period, three sequences, three treatments, Latin square crossover design was used, such that each calf received flunixin, meloxicam or the negative control (placebo) sequentially. The animals were randomly allocated on D0 to one of the 3 treatment sequences. Body weight, sex and age were statistically similar between groups at study initiation (ANOVA or Chi-square test, p > 0.05).

During each of the 3 study periods, 4 calves were injected with flunixin intramuscularly at 2.2 mg/kg (Finadyne® 50 mg/mL); 4 calves received meloxicam subcutaneously at 0.5 mg/kg (Metacam® 20 mg/mL) and 4 calves were administered the placebo intramuscularly at 0.25 mL/kg (0.9% NaCl). All injections were made at time zero of each period, immediately after the intra-caveal injection of 0.5 mL 2% sterile carrageenan solution. Three-week intervals (washout periods) were implemented between each period of the crossover experiment.

Sampling and Sample Processing Procedures

Exudate was collected from 3 tissue cages per animal prior to injection of the carrageenan solution (time zero), as well as at 2, 4, 8, 12, 24, 36 and 48 hours after time zero. Each 0.5 mL sample was transferred into individual blood collection tubes containing lithium heparinate anticoagulant and indomethacin at 10 μg/mL of exudate to prevent in vitro PGE₂ production. Samples were placed on ice. At each collection time point, all 3 samples from one animal were pooled, mixed and centrifuged. The pooled exudate was subsequently transferred into duplicate labelled vials and frozen at -80°C pending assay.

Analytical Procedure

PGE₂ exudate concentrations were measured by immunoassay in an independent laboratory, under blinded conditions (samples not identified with the treatment group). The commercial kit PGE₂, High Sensitivity Colorimetric Competitive ELISA (R&D Systems, Inc. Minneapolis, USA) was used (sensitivity < 8.25 pg/mL; range of dosage 7.8–1000 pg/mL).

Statistical Analysis

A 6-sequence, 3-treatment and 3-period crossover Latin Square design was used for this study with 3-week washout period between two consecutive periods. Twelve animals were randomly assigned to the 6 sequences. Individual exudate PGE₂ concentrations were obtained at each time point (0, 2, 4, 8, 12, 24, 36 and 48 hours post-treatment) in each period. Comparison of periods was performed at each time point: a linear mixed model was used for the analysis including treatment, period, and treatment*period as fixed effects, and animal and animal*period as random effects.

Least squares means of exudate PGE₂ concentrations were used for comparisons of the period effect. Pooled data were analysed by a linear mixed model with repeated measures was used for the analysis including treatment, period, treatment*period, hour and treatment*hour as fixed effects, and animal, period*animal as random effects. Animal was the subject where the repeated measures were taken. Exudate PGE₂ concentrations at time zero were used as covariate. Least squares means of exudate PGE₂ concentrations for treatment groups were used for comparisons. A two-sided t-test was used at significance level α =0.05.

The percentages of reduction in concentration of each treated group relative to the placebo were calculated using the formula:

Arithmetic and geometric means of area under the curve (AUC) were calculated for each group and each period. AUC was analyzed using a mixed linear model including treatment and period as fixed effects. AUC for the three treatment groups were compared at significance level α=0.05.

Results

Exudate PGE₂ Concentrations

Mean exudate PGE₂ concentrations followed the same trend for all 3 treatments during all 3 periods. Pre-challenge exudate PGE₂ concentrations were considered close to 0 as they were inferior to the quantification limit of the ELISA kit (table I). Exudate PGE₂ concentrations increased sharply after challenge, reaching a peak at 2 hours and decreased gradually to reach baseline levels 48 hours after challenge. Mean exudate PGE₂ concentrations in the flunixin and meloxicam groups were significantly lower than those in the placebo group at every time point up to 36 hours. Exudate PGE₂ concentrations in the flunixin group were also significantly lower than those in the placebo group at 48 hours as opposed to the meloxicam group (table II). The mean exudate PGE₂ concentration in the flunixin group was not significantly lower than the exudate PGE₂ concentration in the meloxicam group at all time-points.

In line with the crossover nature of the design, and as pair-wise comparison among the three periods did not show any significant difference (p ≥ 0.0914), PGE₂ concentrations of all three periods were pooled per group. Results per period and statistical p-values for the pooled results are presented in tables I and II, respectively.

Pooled PGE₂ Concentrations

Post-challenge, mean exudate PGE₂ concentrations were numerically lower in the flunixin or meloxicam groups compared to placebo group (table I). Differences between both NSAIDs and the placebo groups were highest at 4 hours post challenge and lowered gradually after.
Reduction of exudate PGE$_2$ concentrations in the flunixin group by comparison to placebo varied from 47% (+2h) to 30% (+48h), with a peak at +4h (59%). In the meloxicam group, the percentage of reduction was of 43% (+2h) to 14% (+48h), with a peak also at +4h (48%). Throughout the study, percentage of reduction of exudate PGE$_2$ concentrations was numerically higher in flunixin group than in the meloxicam group.

**RATIO OF EXUDATE PGE$_2$ CONCENTRATION RELATIVE TO BASELINE VALUES**

Differences in the pre-dose PGE$_2$ levels among calves were accounted for by dividing the exudate PGE$_2$ concentration after dosing by the pre-dose exudate PGE$_2$ concentration for each calf. The ratio of exudate PGE$_2$ concentrations relative to baseline values decreased in all groups, sharply over the first 12 hours, and then slightly from 12 to 48 hours post-challenge (figure 1).

![Figure 1: Mean ratios of PGE$_2$ to baseline concentration for each treatment](image-url)
In the placebo group, the mean exudate PGE$_2$ concentrations ranged from 2 to 76 times the pre-dose exudate PGE$_2$ concentration (at +48h and +2h respectively). The values in the flunixin group ranged from 47 to 2 times the baseline value and those of meloxicam group from 45 to 2 times that the baseline value.

MEAN AREA UNDER THE CURVE (AUC)

Results of pharmacokinetic modelisation of area under the curve (AUC) of PGE$_2$ are presented in table III. The flunixin and meloxicam groups are significantly different from the NaCl group (p-values=0.0005 and 0.006, respectively). The flunixin and meloxicam groups are not significantly different from each other (p-value=0.3322).

### Discussion

Inflammation is an essential defence mechanism of the body to injury. The prostaglandins and related metabolites are end products of a chain reaction starting with injury to the cell and its membranes, triggering the release of unsaturated fatty acids. The prostaglandins and related metabolites are copious in inflammatory exudates. NSAIDs have varying degrees of potency, mainly by inhibiting prostaglandin synthesis by cyclooxygenases. In particular, prostaglandin E$_2$ is shown to be a mediator of acute inflammation, and in vivo exudate PGE$_2$ concentration is therefore used as an indicator of COX-2 activity [3].

This study investigated the activity of two NSAIDs, flunixin and meloxicam on synthesis of PGE$_2$ in bovine...
inflammatory exudate in a tissue cage model of acute inflammation. The tissue cage model is proven to be a valuable interstitial tissue model, as the liquid collected from the tissue cages is considered similar to interstitial fluid [17]. The carrageenan solution induces an acute aseptic and non-immune inflammatory reaction, which remains limited by using spherical chambers (relatively short time course of acute inflammation). Sequential sampling is easy, and the time course of inflammation can be followed.

In this study, three breeds and both sex of cattle were represented. Animal bias due to this variability was controlled by following a crossover design. Bias due to environmental conditions was minimal as environmental conditions were similar during all three periods.

Only one cage out of the 48 implanted was rejected over the 9-week study period proving the excellent tolerance of the implants and validating the surgical procedure followed for implantation.

Near zero pre-challenge exudate PGE$_2$ concentration values showed that both the post-implant period and the period of time allowed to elapse between challenges were long enough for inflammation due to surgery or challenge to resolve. Furthermore, NSAID activity was considered null at that time. The exudates collected from three cages were pooled to minimize any bias due to sampling. The validity of the model is further assessed by similarity of PGE$_2$ concentration over all three periods.

The inhibition of PGE$_2$ synthesis observed in this study can be compared to those obtained in other studies using the tissue cage model in cattle. The duration of the inflammatory response was similar to the one observed in the study conducted by Espinasse et al. [7], although the peak occurred earlier at 2 hours versus 12 hours. Landoni et al. [11] showed a higher decrease of PGE$_2$ concentrations in bovine exude after administration of flunixin (between 60 and 90% up to 24 hours). However, differences in the experimental conditions and assay method influence pharmacodynamics and pharmacokinetics of anti-inflammatory drugs. It should be noted that the rate and extent of penetration of any drug into intra-caveal fluids depends in part on its serum concentration-time profile, and also on whether the granulation tissue within the cage depends in part on its serum concentration-time profile, indicating an inhibitory activity of both agents on cyclooxygenase. Percentage of inhibition after treatment was highest at +4 hours after challenge with carrageenan. As opposed to meloxicam, the flunixin anti-inflammatory activity, demonstrated by a significant decrease in exudate PGE$_2$ concentrations, persisted up to 48 hours post-treatment.

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