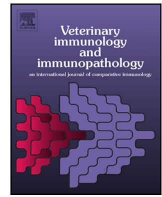
Veterinary Immunology and Immunopathology 171 (2016) 28–37

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



# Immunomodulatory properties of gamithromycin and ketoprofen in lipopolysaccharide-challenged calves with emphasis on the acute-phase response

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ARTICLE INFO

*Article history:*
Received 17 July 2015
Received in revised form 24 January 2016
Accepted 25 January 2016

*Keywords:*
Acute-phase response
Bovine
Gamithromycin
Immunomodulation
Ketoprofen
Lipopolysaccharide

ABSTRACT

Macrolide antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to be modulators of the innate immune response, irrespectively of their antimicrobial and anti-inflammatory actions. Therefore, it was our objective to evaluate whether the macrolide gamithromycin (GAM) and the NSAID ketoprofen (KETO) attenuate the acute-phase response in calves, and whether their combined administration is beneficial due to synergistic and/or additive effects. To this end, both drugs, as well as their combination, were studied in a previously developed inflammation model, i.e., the induction of an acute-phase response by an intravenous lipopolysaccharide (LPS) challenge (0.5 μg/kg body weight). Sixteen 4-week-old Holstein–Friesian calves were randomized into 4 groups: a positive control (+CONTR) group, receiving LPS but no pharmacological treatment ($n = 4$) and a GAM ($n = 4$), a KETO ($n = 4$) and a GAM–KETO ($n = 4$) group, receiving the respective drugs 1 h prior to LPS administration. Clinical scoring and blood collection were performed at regular time points until 72 h post LPS challenge. Plasma concentrations of the selected cytokines (tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6)), acute-phase protein (serum amyloid A (SAA)) and prostaglandin $E_2$ ($PGE_2$) were subsequently quantified. Pre-treatment with GAM had no effect in the inflammation model compared to the +CONTR group. KETO, on the other hand, completely inhibited depression, anorexia and fever. This remarkable influence was associated with a significant reduction of $PGE_2$ synthesis by KETO, while the effect on TNF-α, IL-6 and SAA was not straightforward. The combined administration of GAM and KETO provided no synergistic or additive effects in this model, neither clinically nor regarding the studied inflammatory mediators. In conclusion, KETO entirely inhibited $PGE_2$ synthesis, fever development and depression, while GAM did not exert any effect in this model. These results promote the concomitant use of an antimicrobial drug and a NSAID in the treatment of calf diseases associated with LPS, both to enhance clinical recovery and to improve animal welfare.

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## 1. Introduction

Bacterial infections are highly common in bovine veterinary medicine, with LPS playing a key role in the pathogenesis of several of these clinical entities. Currently, the treatment of acute bacterial infections in cattle is mostly restricted to the administration of antimicrobial drugs (Edwards, 2010; Pardon et al., 2012). However, both from the animal welfare and economic perspective, the concomitant use of an anti-inflammatory drug is advantageous as it decreases the severity of clinical symptoms and limits the negative consequences of inflammation (Francoz et al., 2012). In this respect, the choice between a non-steroidal anti-inflammatory

*Abbreviations:* +CONTR, positive control; AUC, area under the curve; BRD, bovine respiratory disease; BW, body weight; COX, cyclooxygenase; DEX, dexamethasone; GAM, gamithromycin; HR, heart rate; KETO, ketoprofen; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; $m/z$, mass-to-charge ratio; $N_2$, nitrogen; NF-κB, nuclear factor κB; NSAID, non-steroidal anti-inflammatory drug; p.c., post LPS challenge; PG, prostaglandin; $PGE_2$-*met*, 13,14-dihydro-15-keto $PGA_2$; RR, respiratory rate; RT, rectal body temperature; SAA, serum amyloid A; TX, thromboxane; UPLC, ultra-performance liquid chromatography.

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http://dx.doi.org/10.1016/j.vetimm.2016.01.007
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drug (NSAID) and a corticosteroid remains a controversial topic in cattle practice (Lekeux and Van de Weerdt, 1997). Although immunosuppression is a well-known side effect of corticosteroids, these drugs are still commonly preferred by practitioners for their potent anti-inflammatory properties (Sustronck et al., 1997; Wagner and Apley, 2004; Hewson et al., 2011; Sipka et al., 2013). Indeed, a fast clinical effect on depression and anorexia is highly appreciated by farmers. Nevertheless, in our previous study in LPS-challenged calves, dexamethasone (DEX) did not inhibit the onset of respiratory distress and fever (Plessers et al., 2015a). A faster recovery of the calves, on the other hand, was observed as well as reduced levels of the pro-inflammatory cytokines TNF-α and IL-6.

From this point of view, NSAIDs might exert a more pronounced influence on the LPS-induced acute-phase response in comparison with corticosteroids. Indeed, cyclooxygenase (COX)-inhibitors directly block the enzymatic production of prostanoids. As a result, the production of prostaglandins (PGs) and thromboxanes (TXs) ceases, consequently resolving fever and pulmonary effects (Lekeux and Van de Weerdt, 1997; Lees et al., 2004). In addition to these COX-dependent actions, certain NSAIDs have been established to modulate the innate immune response, including the inhibition of nuclear factor-κB (NF-κB)-related gene transcription (Tegeder et al., 2001; Bryant et al., 2003). In bovine veterinary medicine, ketoprofen (KETO) is a commonly used NSAID, exerting anti-inflammatory, antipyretic and analgesic effects (Lees et al., 2004; Pardon et al., 2012).

Particularly with respect to the treatment of bovine respiratory disease (BRD), the combination of an antimicrobial agent with a NSAID (including carprofen, flunixin meglumine, KETO and meloxicam) has been demonstrated to be superior to the administration of an antimicrobial alone (Lockwood et al., 2003; Elitok and Elitok, 2004; Friton et al., 2005). In this context, gamithromycin (GAM) is a recently developed azalide which has been approved for the treatment of BRD (Huang et al., 2010). To date, studies comparing the efficacy of NSAIDs and corticosteroids in cattle are rather limited, whereas research regarding their possible synergistic and additive effects with GAM is lacking.

Therefore, the aim of this study was to investigate the immunomodulatory properties of KETO in a previously developed inflammation model in calves (Plessers et al., 2015c). The combined administration of GAM and KETO was included in the study to test for synergistic and/or additive effects. Besides the effect on TNF-α, IL-6, the acute-phase protein serum amyloid A (SAA) and clinical signs, emphasis was placed on the impact on prostanoids, i.e., $PGE_2$.

## 2. Materials and methods

### 2.1. Animal experiment

Sixteen healthy male Holstein Friesian calves, with a mean age of 21.1 ± 4.0 days, were obtained from local farms. Upon arrival at the Faculty of Veterinary Medicine, the calves were housed and treated following a similar protocol as described in Plessers et al. (2015c). Briefly, the animals were housed in individual pens on straw with ad libitum access to hay and fresh water. The calves were fed milk replacer three times a day, receiving a total of 5 L daily. After the morning feeding, 50 g of starter mix was given to the calves. In order to evaluate the calves' clinical condition, and to habituate the animals to human presence and contact, as well as to experimental manipulations, a one-week acclimatization period was set.

The calves were weighed the day before the start of the experiment (56.1 ± 6.9 kg), after which a 14 G indwelling catheter (Cavafix, B. Braun, Diegem, Belgium) was placed aseptically in the right jugular vein. A recovery period of at least 12 h was respected. After this period, the 4-week-old calves were randomly divided into four groups: a positive control (+CONTR) group ($n$ = 4), a GAM group ($n$ = 4), a KETO group ($n$ = 4) and a GAM–KETO group ($n$ = 4). Negative control animals were not included in the present study, as our previous research demonstrated that these animals showed no changes regarding clinical condition, nor with respect to cytokine and acute-phase protein concentrations (Plessers et al., 2015c). The clinical condition at 0 h was evaluated by determination of the rectal body temperature (RT) and visual inspection of the faeces. A RT ≥ 39.5 °C was handled as an exclusion criterion for the experiment at this time.

Reference venous blood samples (0 h; 7.5 mL) for cytokines and SAA were drawn from the catheter and transferred into EDTA-containing tubes. Additionally, reference blood samples for $PGE_2$ determination were collected by adding 1 mL of blood into Eppendorf tubes previously coated with 10 μg indomethacin (Sigma–Aldrich, Diegem, Belgium) and containing 10 IU of sodium heparine (Heparine LEO, Leo Pharma, Lier, Belgium). The indomethacin was added in order to prevent ex vivo artefactual eicosanoid generation (Pelligand et al., 2012). Briefly, 100 μL of the stock solution of indomethacin (0.1 mg/mL in ethanol (VWR, Leuven, Belgium)) were added per tube, and evaporated to dryness under a gentle stream of nitrogen ($N_2$) at 35 °C. According to the group, the calves were subsequently either not treated (+CONTR group), treated with 6 mg/kg body weight (BW) GAM (Zactran, Merial, Diegem, Belgium) s.c. in the neck region, treated with 3 mg/kg BW KETO (Ketofen 10%, Merial, Diegem, Belgium) i.m., in the neck region (cervical ventral serratus muscle), or treated with the combination of both drugs (6 mg/kg BW GAM s.c. and 3 mg/kg BW KETO i.m.). Following a one-hour interval (at 8 AM), all calves were i.v., challenged with 0.5 μg/kg BW ultrapure LPS (500 units/kg BW, *Escherichia coli* serotype O111:B4, LPS-EB Ultrapure, InvivoGen, Toulouse, France) via the catheter. Blood samples (7.5 mL) were collected at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 18, 24, 28, 32, 48, 54 and 72 h post LPS challenge (p.c.). At all mentioned sampling points, RT, respiratory rate (RR) and heart rate (HR) were recorded. Additionally, animals were clinically scored during the first 9 h of the experiment, including the evaluation of the presence of dyspnoea, coughing, breathing sounds, mental state, position and appetite. Based on these observations, the appearance of the previously described behavioral phases (respiratory, depression and recovery phase) following LPS administration was recorded (Plessers et al., 2015c). If systemic shock symptoms would occur, the respective calf would be humanely euthanized, and subsequently necropsied. The investigators were not blinded to the treatment groups.

All blood samples were mixed by gentle inversion and placed on ice until centrifugation at 1,000 × $g$ for 15 min. Subsequently, plasma was harvested and stored in aliquots at ≤−70 °C for future analysis, with a maximum of 10 months.

All procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Faculty of Bioscience Engineering of Ghent University (EC 2012/189).

### 2.2. Sample analyses for TNF-α, IL-6 and SAA

All plasma samples were analysed in duplicate for cytokines (TNF-α and IL-6) and the acute phase protein (SAA) using commercially available ELISAs. As their suitability for plasma samples was not guaranteed by the manufacturer, the assays for cytokines (Bovine TNF-α DuoSet, R&D Systems Europe, Abingdon, UK; and Bovine IL-6 Screening Set, Thermo Fisher Scientific, Rockford, IL, USA) were validated prior to use (Plessers et al., 2015c). The SAA assay (Phase SAA Assay, Tridelta Development Ltd., Maynooth, Ireland) was performed according to the manufacturer's protocol.

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