Enrofloxacin decreases IL-6 and TNF-α production by lipopolysaccharide-stimulated porcine peripheral blood mononuclear cells

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Abstract

Introduction: The aim of the study was to explore the effect of enrofloxacin on production of selected cytokines by porcine peripheral blood mononuclear cells (PBMCs). **Material and Methods:** Twenty pigs (10 control and 10 experimental) were used in this research. Pigs from experimental group received enrofloxacin at therapeutic doses. Blood samples were collected before, during, and after treatment with enrofloxacin. PBMCs were incubated with or without lipopolysaccharide (LPS). Production of IL-6, IL-10, INF-γ, and TNF-α were determined by ELISA. **Results:** Administration of enrofloxacin to healthy pigs for 5 d induced a transient reduction of the PBMCs response to LPS in terms of IL-6 and TNF-α secretion. The concentration of IL-6 returned to the day 0 level shortly after treatment, while TNF-α production remained reduced for 10 d after the treatment. The production of IL-10 was not affected by enrofloxacin. The level of IFN-γ was below the detection limit of the tests. **Conclusion:** The results indicate that enrofloxacin administered *in vivo* in therapeutic doses has an immunomodulatory effect through its capacity to inhibit secretion of IL-6 and TNF-α by porcine PBMC stimulated by LPS.

Keywords: swine, fluoroquinolone, immunomodulation, lipopolysaccharide, cytokines.

Introduction

A wide range of antibiotics have been found to have immunomodulatory properties in addition to their antimicrobial activity (6, 17). It has been demonstrated that some fluoroquinolones can modulate host immune and inflammatory responses and cytokine production (1, 7-9, 14, 21). Khan *et al.* (9) have found that trovafloxacin suppresses the production of IL-1, IL-6, IL-10, and TNF-α by lipopolisaccharide (LPS)-stimulated monocytes. Ciprofloxacin and ofloxacin have been shown to reduce the production of TNF-α in LPS-stimulated monocytes (14). Araujo *et al.* (1) have demonstrated that moxifloxacin inhibited LPS-stimulated secretion of IL-1α, IL-6, IL-10, IL-12, and TNF-α. The evidence of the role of cytokines in the severity of infections as well as in the immune response indicate the importance of defining the immunomodulatory activity of antibiotics used to treat infections (15, 18).

Enrofloxacin is an antimicrobial agent which belongs to fluoroquinolones (25). Because of its pharmacokinetic properties, broad spectrum of activity, and low toxicity, enrofloxacin is frequently used by veterinarians to treat infections in swine (25). It is recommended for treatment of gastrointestinal and respiratory tract infections.

There are data on the effects of enrofloxacin treatments on the immune response in animals (8, 20, 25), but the influence of *in vivo* administration of enrofloxacin on the cytokine secretion profile has so far not been documented. However, the results of earlier studies revealed that ciprofloxacin, a pharmacologically active metabolite of enrofloxacin, has significant effects on the production of wide range of cytokines by various cells (2, 14, 24).

The evidence that fluoroquinolones have the capacity to modulate the production of cytokines by immune cells prompted us to study the effects of enrofloxacin given in therapeutics doses on the *ex vivo*

production of cytokines by porcine peripheral blood mononuclear cells (PBMCs).

Material and Methods

Animals. Twenty 8- to 20-week-old pigs, both sexes, originating from a herd with high health status, were used in the study. Only pigs which had not received any antibiotics before the experiment were involved in the trial. The animals were randomly divided into two groups: control (C; n = 10) and experimental (E; n = 10). During the study, the pigs were housed in isolated units, one for each group, and were subjected to a 7 d acclimation period. Food and water were offered *ad libitum*. Animal use and handling protocols were approved by the Local Ethical Commission (University of Life Sciences in Lublin, Poland).

Drug. The commercial product containing enrofloxacin was used (Enrobioflox 5% Injectio, 50 mg/ml, Vetoquinol Biowet, Poland).

Experimental design. From day 0 to day 4, pigs from group E received enrofloxacin intramuscularly at the recommended therapeutic dose (1 mL/10 kg b.w. per day). Pigs from group C were intramuscularly administered the appropriate volume of normal saline (1 mL/10 kg b.w. per day).

Blood samples were collected from the *vena jugularis* or *vena cava cranialis* to vacuum tubes, containing EDTA-K_3 as an anticoagulant (Medlab, Poland) at days 0 (before antibiotic administration), 2, 4 (during antibiotic therapy), and 6, 9, 14, 21, 35, 49, and 63 (after treatment with enrofloxacin).

Measurements. PBMCs were isolated from blood samples by centrifugation onto Histopaque 1.077 (Sigma, USA). The cells were counted in Bürker chamber and their viability was determined with trypan blue (Stem Cell Technologies, UK) staining. Cytokine (IL-6, IL-10, IFN-γ, and TNF-α) production by PBMCs was determined using cells cultured in plastic vials at a density of 1×10^6 viable cells/mL medium (RPMI 1640 containing 10% foetal bovine serum, 2 mM L-glutamine, and 1% of antibiotic-antimycotic solution) at 37°C and in a humidified 5% carbon dioxide atmosphere in the presence of 1 μg/mL lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4; Sigma, USA). Simultaneously, unstimulated PBMCs were cultured under the same conditions. All samples were analysed in quadruplicate. After 24 h, the culture media were harvested and centrifuged at 400 x *g* for 3 min, and the supernatants were collected and stored at -80°C until analysis.

To determine the concentrations of investigated cytokines in the culture supernatants, the ELISA kits specific for porcine IL-6, IL-10, IFN-γ, and TNF-α were used following the manufacturer's instructions (Invitrogen Corporation, USA; Abcam, UK). The absorbance was recorded at 450 nm using an ELISA plate reader (Multiskan RC, Labsystems, Finland).

Quantification was performed on the basis of a standard curve derived by dilution of the cytokine standards included in the kits. Concentrations of each cytokine were calculated from calibration curve using the FindGraph software. The detection limits of the kits were 2 pg/mL (IL-6), 2 pg/mL (IL-10), 2 pg/mL (IFN-γ), and 3 pg/mL (TNF-α).

Statistical analysis. Data were expressed as the mean and standard deviation (SD). The data from both groups were subjected to the W. Shapiro-Wilk's test of normality and the Levene's test of equal variances. Because of non-normal distribution of the data, comparisons between the groups at each time point were assessed using the U Mann-Whitney test. The significance of changes in continuous data was evaluated by nonparametric Friedman test. Differences with α < 0.05 were considered as significant. Calculations were performed with the use of Statistica 8.0 software (Statsoft, Poland).

Results

Treatment of PBMCs with LPS resulted in a significant (P < 0.05) increase in accumulation of IL-6, IL-10, and TNF-α in the cell culture supernatants when compared with unstimulated cells. The concentration of IFN-γ after LPS stimulation was always below the detection limit in both groups of pigs (C and E). No detectable levels of these cytokines were found in unstimulated cultures either.

There were no differences between the groups regarding IL-6 concentrations in supernatants at any time points (P < 0.05) after mock-stimulation (Fig.1). The maximal concentration of IL-6 did not exceed 34 pg/mL. In contrast, significant differences were found after LPS stimulation between IL-6 production by PBMCs isolated from pigs treated and not treated with enrofloxacin (P < 0.05). The concentrations of IL-6 in supernatants from LPS-stimulated cultures were significantly lower during and just after treatment with enrofloxacin (from day 2 to 6) in comparison with control animals (P < 0.05). At days 2, 4, and 6, IL-6 production was significantly decreased in enrofloxacin-treated pigs compared with day 0 level (P < 0.05). Starting from day 9, no significant differences were found in IL-6 production between both groups of animals (P > 0.05).

Lipopolysaccharide treatment of PBMCs isolated from control and enrofloxacin-treated animals resulted in a significant (P < 0.05) increase in accumulation of IL-10 in the cell culture supernatants when compared with non-stimulated cultures. *Ex vivo* secretion of IL-10 by LPS- or mock-stimulated PBMCs was not affected significantly (P > 0.05) by *in vivo* treatment with enrofloxacin (Fig. 2.)

Fig. 3 shows that TNF-α production in supernatants from mock-stimulated PBMCs did not differ between groups (P > 0.05) and was stable during the period of study (P > 0.05). In contrast, TNF-α production by LPS-

stimulated PBMCs was significantly (P < 0.05) higher in control group from day 2 to 14 of the study in comparison to enrofloxacin-treated pigs. Inhibition of *ex vivo* secretion of TNF-α by PBMCs isolated from pigs treated with enrofloxacin was observed during antibiotic therapy and up to 10 d after treatment. Starting from day 21, no significant differences in LPS-induced production of this cytokine were noted between both groups (P > 0.05).
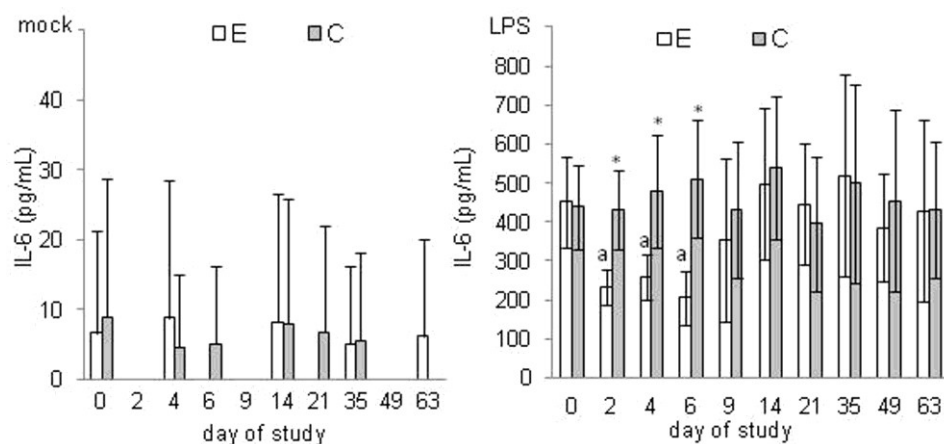

Fig. 1. IL-6 *ex vivo* secretion by porcine PBMCs. The columns represent the mean (±SD) concentration of IL-6 in culture supernatant in unstimulated cultures (mock) and cultures stimulated with lipopolysaccharide (LPS). In the case of non-detectable level of this cytokine, the 0 pg/mL value was taken for calculation.
E - pigs treated with enrofloxacin at therapeutic doses for five consecutive days; C - control pigs
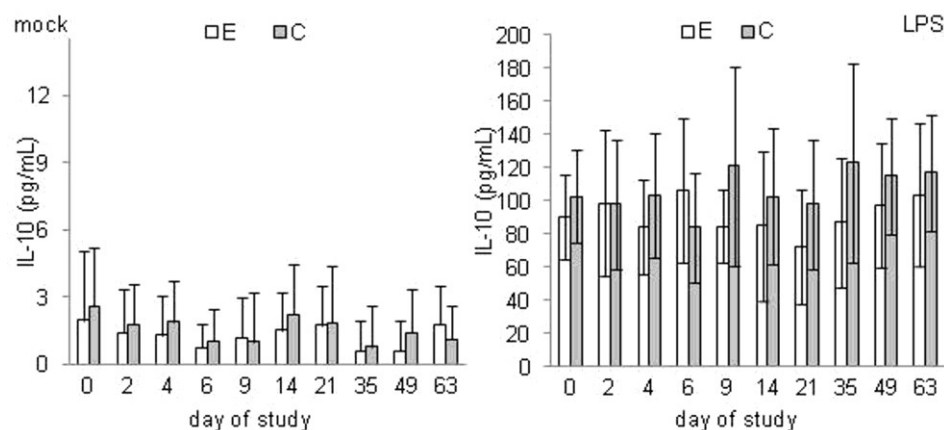* - P < 0.05 as compared with group E; a - P < 0.05 as compared with day 0 level

Fig. 2. IL-10 *ex vivo* secretion by porcine PBMCs. The columns represent the mean (±SD) concentration of IL-10 in culture supernatant in unstimulated cultures (mock) and cultures stimulated with lipopolysaccharide (LPS)
E - pigs treated with enrofloxacin at therapeutic doses for five consecutive days; C - control pigs

Fig. 3. TNF-α *ex vivo* secretion by porcine PBMCs. The columns represent the mean (±SD) concentration of TNF-α in culture supernatant in unstimulated cultures (mock) and cultures stimulated with lipopolysaccharide (LPS)
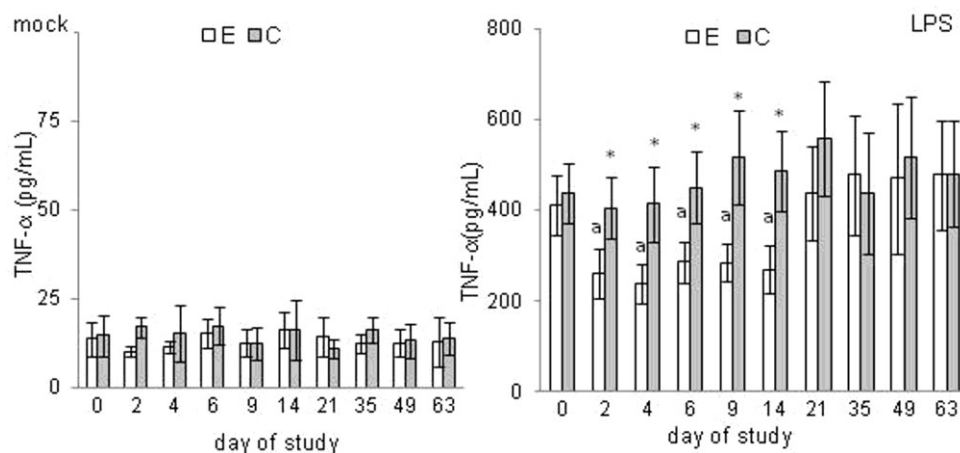E - pigs treated with enrofloxacin at therapeutic doses for five consecutive days; C - control pigs
* - P < 0.05 as compared with E group; a - P <0.05 as compared with day 0 level

Discussion

Intramuscular administration of enrofloxacin to healthy pigs for five consecutive days induced a transient reduction of *ex vivo* response of PBMCs to LPS in terms of IL-6 and TNF-α secretion. The concentration of IL-6 returned to the day 0 level shortly after the end of treatment, while the TNF-α production remained reduced longer, up to 10 d after the end of treatment. The production of IL-10, an anti-inflamatory cytokine, was not affected by the enrofloxacin treatment. The supression of IL-6 and TNF-α secretion occurred in all animals treated with enrofloxacin. At the same time the spontaneus production of these cytokines (unstimulated cells) was not modified. The results described above revealed that enrofloxacin can reduce production of IL-6 and TNF-α porcine immunocompetent cells, which suggests that, in addition to its antimicrobial effects, enrofloxacin may limit the immune and inflamatory reaction occurring in the course of certain infections.

The role of cytokines investigated in the present study is significant in the immune response to various pathogens. IL-6 is a cytokine of innate immunity and regulates inflammation and transition from innate to adaptive immune responses. It is also important in haematopoiesis and acute phase response. The early secretion of this cytokine may constitute an important line of defence against pathogens (11). IL-10 is a cytokine secreted by various immune cells. It has pleiotropic effects in immunoregulation and inflammation. IL-10 can down-regulate major histocompatibility complex class II expression, synthesis of other cytokines, and its own production, and is able to inhibit the functions of the natural killer (NK) cells, lymphocytes and monocytes (13, 15). TNF-α, produced mainly by activated macrophages, CD4+ lymphocytes and NK cells, can stimulate neutrophil activation, synthesis of acute phase proteins, and apoptosis of cells. It is also involved in T-cell activation. Bacterial endotoxins, a range of molecules of pathogens, and other cytokines are believed to be principal stimuli for TNF-α release from cells (3). The reduction of the TNF-α production may affect neutrophil activation and modulate the immune response against pathogens.

Previously, other fluoroquinolones have been evaluated for their effect on the cytokine production *in vitro* or *ex vivo* (1, 9, 19, 22). Our results regarding the significant reduction in LPS-induced IL-6 and TNF-α secretion are in agrement with previous findings from various *in vitro* studies (1, 9, 19, 22). Khan *et al.* (9) have found that trovafloxacin, an antimicrobial drug belonging to fluoroquinolones, suppressed the secretion of various cytokines, including IL-6, IL-10, and TNF-α, by monocytes cultivated *in vitro* after LPS stimulation. The data presented by Tsivkovskii *et al.* (22) have demonstrated that levofloxacin also reduces LPS-induced IL-6 levels in cultured human airway epithelial cells. In contrast, Bailly *et al.* (2) have found that ciprofloxacin treatment *in vivo* in humans increases the *ex vivo* capacity of LPS-stimulated human monocytes to produce IL-6 and TNF-α. However, in the same study the extracellular production of IL-6 was reduced after treatment with ciprofloxacin (2).

Our results revealed that stimulation of porcine PMBCs with LPS resulted in a significant increase in the production of most of the investigated cytokines in supernatants of the cultured PBMCs isolated from both enrofloxacin treated and non-treated pigs, when compared with unstimulated cells. Similar results were reported by Araujo *et al.* (1) who investigated the *in vitro* influence of moxifloxacin on secretion of cytokines by human monocytes stimulated with LPS. They have also found that moxifloxacin, similarly as enrofloxacin, significantly inhibited secretion of TNF-α.

In the present study only the level of IFN-γ was below detection limit in supernatants of the LPS-stimulated as well as unstimulated PBMCs cultured *in vivo*. The lack of detectable levels of IFN-γ in the culture supernatants after LPS-stimulation is not surprising since this cytokine is produced mostly by activated T lymphocytes and NK cells (5, 23). LPS stimulates mainly, but not solely, B lymphocytes, monocytes, and macrophages, the cells that express the LPS receptor (23). There is also evidence that activated human, but not murine, CD8+ T cells can secrete high concentrations of IFN-γ in response to LPS (10). It is important to note that LPS sensitivity differs considerably among species (23). To date there is no information regarding reactivity of porcine T cells upon LPS stimulation. However, the results of the present study suggest that porcine T lymphocytes, similarly to murine T lymphocytes, do not respond with significant IFN-γ production after LPS stimulation.

In conclusion, our results indicate that enrofloxacin has an immunomodulatory effect through its capacity to inhibit *ex vivo* secretion of IL-6 and TNF-α by porcine PBMC after LPS stimulation. Because of the important role of these cytokines in the host immune response, this effect may influence the course of infection in a manner that is independent of the drug's antimicrobial activity. The inhibiting effects of enrofloxacin on the production of IL-6 and TNF-α in response to LPS may be beneficial through modulation of the inflammatory response to the invading pathogens, especially during bacteraemia, which may lead to massive production of cytokines (12, 15, 19). Overproduction of proinflammatory cytokines (including IL-6 and TNF-α) may result in a range of pathological conditions, including septic shock (4). It is worth noting that the beneficial effects of *in vivo* treatment with ciprofloxacin, an active metabolite of enrofloxacin, were previously reported by Purswani *et al.* (19), who found that ciprofloxacin can prevent endotoxin-mediated death in mice and alter early host cytokine responses.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The experiment was approved by Local Ethics Commission (University of Life Sciences in Lublin, Poland).

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