Effect of therapeutic doses of enrofloxacin on circulating lymphocyte subpopulations in pigs

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Abstract

Twenty pigs of similar genetics (PIC) were used. Pigs were randomly divided into two groups: experimental (ENRO, n = 10) and control (C, n = 10). From day 0 to day 4, pigs from ENRO group received enrofloxacin at the recommended therapeutic dose. Pigs from C group received PBS instead of enrofloxacin. Blood samples were collected on days 0 (before antibiotic administration), 2, 4 (during antibiotic therapy), 9, and 13 of the study (after enrofloxacin administration). Haematological examination and flow cytometry were used to establish the relative and absolute counts of various leukocyte subpopulations. Lymphocyte subpopulations were measured by fluorochrome-labelled antibodies according to following definitions: CD3+ (T cells), CD21+ (B cells), CD4+CD8- (helper T cells, Th), CD4-CD8+ (cytolytic T cells, CLT), CD4+CD8+ (cytolytic and memory T cells). The present study revealed the modulating effect of enrofloxacin on the composition of circulating lymphocytes in pigs. Concentration and percentage of CD8+ cells decreased significantly after treatment with enrofloxacin and as a result the absolute CD4/CD8 ratio increased significantly as compared to control group (P < 0.05). These findings should prompt further studies on the practical significance of the results obtained in terms of clinical implications. In view of the results, it cannot be excluded that enrofloxacin may also have immunomodulatory effects on host response to infection.

Keywords: swine, enrofloxacin, lymphocyte subsets, immunomodulation.

Introduction

Enrofloxacin is an anti-bacterial drug which belongs to fluoroquinolones, a popular class of antibiotics used in treatment of a range of infections in humans and animals (25). These agents are generally well absorbed from the gastrointestinal tract as well as from parenteral sites of injection (2). As a group, the fluoroquinolones are widely distributed throughout the body, including the kidneys, lungs, liver, uterus, bones, and inflammatory tissues (2, 23). Excretion of the fluoroquinolones is primarily through the kidneys, with secondary excretion through the liver (23). Beside antibacterial activity, the fluoroquinolones are also known to have various immunomodulatory properties (3, 4).

Enrofloxacin is used exclusively in veterinary medicine, in treatment of companion and farm animals, including pigs (2). Because of its pharmacokinetic properties, a broad spectrum of activity and low toxicity, enrofloxacin is frequently used in the field (2, 25). In pigs, enrofloxacin is used to treat gastrointestinal and respiratory tract infections. In vivo enrofloxacin is metabolised into pharmacologically active metabolite - ciprofloxacin, which is also known to have modulatory effect on the immune system (3, 4, 6, 24).

So far, the influence of enrofloxacin on the immune response in vivo has not been well documented. There are only a few reports about the effect of enrofloxacin treatments on the immune response in various hosts (7, 17, 21), but the effects of enrofloxacin on the subpopulation of circulating lymphocytes are unknown.

An in vitro study performed on phagocytes obtained from pigs showed that enrofloxacin may accumulate in phagocytes, and this way it may lead to an increase in their bactericidal properties (18). Khalifeh et al. (7) reported that enrofloxacin targeted the humoral immune response in chickens, and resulted
in a decrease in antibody production after vaccination during antibiotic therapy. Tokarzewski (21) found that enrofloxacin decreased the level of specific IgY in hens stimulated with live Salmonella organisms and lipopolisaccharide (LPS). The results of earlier studies also revealed the modulatory effects of fluoroquinolones, including ciprofloxacin, on secretion of a wide range of cytokines by various immune cells (1, 5, 8, 12, 13, 16, 18, 20).

To date, there is only limited information regarding the influence of fluoroquinolones, including enrofloxacin, on T-cell subpopulations in various lymphatic organs (19, 20). Szczypta and Obmińska-Mrukowicz (19) found that enrofloxacin decreases the frequency of CD4+ T cells in mouse spleen, while ciprofloxacin increases the percentage of CD3+ and CD4+ splenocyte T cells. Marbofloxacin has been found to decrease the percentage of CD4+ and CD8+ thymocytes (20). The results of previous experiments indicate that antibiotics from other classes (fenicols, cephalosporins, tetracyclines) can also change the frequency and absolute number of lymphocytes (9, 11, 22) in various lymphatic tissues and blood.

In view of potential immunomodulatory properties of enrofloxacin and its frequent use in pigs, the purpose of this study was to determine the effect of therapeutic doses of enrofloxacin on circulating lymphocyte subpopulations in these animals.

Material and Methods

Animals. Twenty 7 to 10-week-old pigs of similar genetics (PIC), of both sexes, were used in the study. The animals originated from the herd with a high health status. Only pigs that did not receive any antibiotics before experiment were used in the study.

Pigs were randomly divided into 2 groups: experimental (ENRO, n = 10) and control (C, n = 10). During the experiment, the pigs were housed in independent isolated units, one for each group. Feed and water were offered ad libitum. Before the study, the pigs were marked with numbers and were allowed a 7-d acclimation period.

Drug. The commercially available product containing enrofloxacin was used (Enrobiolfox 5% Injectio, 50 mg/mL, Vetoquinion Biowet, Poland).

Experimental design. From day 0 to day 4 pigs from ENRO group received enrofloxacin intramuscularly, at the recommended dose of 1 mL/10 kg b.w. per day. Pigs from C group were injected with PBS instead of antibiotic.

Blood samples were collected from vena jugularis or vena cava cranialis to vacuum tubes, containing EDTA-K3 as an anticoagulant (Medlab, Poland) on days 0 (before antibiotic administration), 2, 4 (during antibiotic therapy), 9, and 13 of the study (after treatment with enrofloxacin).

Haematological examinations. Whole blood samples were analysed for total leukocyte (WBC) count as well as count and proportion of lymphocytes (LYM) on an Abacus Junior Vet 5 haematology analyser (Diatron, Hungary). Proportions of LYM were calculated as a percentage of leucocyte count. In order to produce consistently accurate results, haematology analyser was regularly calibrated and the results were compared with reference method results (microscopic review).

Flow cytometry. Lymphocyte subsets were measured by immunophenotyping of whole blood samples according to the previously described procedure (10).

A simultaneous dual-colour staining technique was used. In brief, 50 μL of blood were incubated with a mAb pair directed against molecules of interest. The cells were double stained for CD3/CD21 and CD4/CD8 using mAbs as follows: mouse IgG1x anti-pig CD3 (clone PPT), mouse IgG1x anti-pig CD21 (BB6-11C9.6), mouse IgG2a anti-pig CD24a (clone 74-12-4), and mouse IgG2a anti-pig CD8a (clone 76-2-11) (SouthernBiotech, USA). All the mAbs were labelled with PE or FITC respectively. FITC-conjugated mouse IgG1 anti-pig CD45 (CLONE: 1E4) (pan-leukocyte antigen) and RPE-conjugated mouse IgG2b anti-pig CD14 (clone: MIL2) (monocyte antigen) mAbs were used together for gating the lymphocytes (Antigenex America INC, USA). Respective isotype controls were also included in the assay.

Cells were incubated in the dark with saturating amounts of each antibody, at 4°C for 30 min. OptiLyse C (Immunotech, USA) lysing solution (250 μL) was added to the sample to lyse the erythrocytes, and then samples were incubated for the next 10 min under the same conditions. After incubation, the cells were washed twice in PBS with 2% inactivated horse serum (GIBCO, USA) and fixed in PBS with 0.5% paraformaldehyde prior to analysis with FACS Canto II flow cytometer (Becton Dickinson, USA).

Lymphocyte subpopulations were detected by fluorochrom-labelled antibodies according to following definitions: CD3+ (T cells), CD21+ (B cells), CD4+CD8− (helper T cells; Th), CD4+CD8+ (cytolytic T cells, CLT), and CD4+CD8+ (cytolytic and memory T cells). The results were expressed as percentages of gated lymphocytes as well as the absolute number of cells. The percentages of cells with respective phenotypes were converted to absolute cell numbers per a microlitre of blood according to counted lymphocytes in the WBC count. The results were analysed with the use of BD FACSDiva Software 6.0.

Statistical analysis. Data were expressed as the mean and standard deviation (SD). Data from both groups were subjected to the W. Shapiro-Wilk’s test (P < 0.05) of normality and the Levene’a test (P > 0.05) of equal variances. Comparisons between 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 $\alpha < 0.05$ were considered as significant. Calculations were performed with the use of Statistica 8.0 software (Statsoft, Poland).

**Results**

The number of WBC and LYM remained stable throughout the study period in pigs from both groups ($P > 0.05$), and did not differ significantly between the groups ($P > 0.05$) (Figs 1A and 1B). The number of WBC in the control pigs ranged from $16.28 \times 10^3/\mu L$ to $29.5 \times 10^3/\mu L$. In the pigs from ENRO group, the number of WBC ranged from $19.6 \times 10^3/\mu L$ to $28.37 \times 10^3/\mu L$. The mean number of LYM ranged from $6.91 \times 10^3/\mu L$ to $17.5 \times 10^3/\mu L$ in the control pigs and from $9.19 \times 10^3/\mu L$ to $18.26 \times 10^3/\mu L$ in the pigs treated with enrofloxacin. No significant changes and differences were found with regard to the percentage of LYM in both experimental groups ($P > 0.05$) (data not shown).

The number of CD3$^+$ and CD21$^+$ cells was stable during the study in both groups and did not differ significantly ($P > 0.05$) (Fig. 2). The only significant difference was observed in the number of CD8$^+$ CD4$^-$ cells, which decreased significantly after treatment with enrofloxacin (from day 9 to the end of the study) ($P < 0.05$). At the same time, there was also a significant increase in the CD4/CD8 ratio (calculated from the absolute size of lymphocyte subsets) in enrofloxacin-treated pigs compared with control ($P < 0.05$) (Fig. 3).

No significant differences were found with regard to the mean percentage of CD4$^+$ CD8$^-$ lymphocytes ($P > 0.05$) (Fig. 4). A significantly lower percentage of CD8$^+$ CD4$^-$ cells was observed from day 9 to day 13 of the study in the pigs from ENRO group as compared to the control pigs ($P < 0.05$). On the same days, significant differences ($P < 0.05$) in the percentage of CD8$^+$ CD4$^-$ cells were observed in the pigs from ENRO group as compared to the values from day 0. Moreover, the percentage of double positive cells in the ENRO group pigs was significantly lower on day 13 compared to the pigs not receiving enrofloxacin ($P < 0.05$).

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**Fig. 1.** The mean (±SD) white blood cell (A) and lymphocytes (B) counts in the peripheral blood of pigs from control (C) and experimental (ENRO) groups

**Fig. 3.** Absolute CD4/CD8 ratio in pigs from control (C) and experimental (ENRO) group

* - $P < 0.05$ as compared to C group
Fig. 2. The mean (±SD) number of CD3+, CD21+, CD4-CD8+, CD4+CD8- and CD4+CD8+ cells (×10^3/µL) within circulating lymphocyte T subpopulations in pigs from control (C) and experimental (ENRO) group.

* - P < 0.05 as compared to C group,

a - P < 0.05 as compared to day 0 value
Discussion

In the present study, treatment with a normal dose of enrofloxacin has been used in pigs to investigate its influence on circulating lymphocyte subpopulations. The study revealed the modulating effect of enrofloxacin on the composition of circulating lymphocytes. Both counts and percentage of CD8+ cells decreased significantly after treatment with enrofloxacin and, as a result, the absolute CD4/CD8 ratio was also significantly elevated as compared to control group (P < 0.05). In agreement with the current findings, a significant decrease in the relative number of CD8+ cells was also reported in mice after administration of marbofloxacin in therapeutic doses (20). In addition, the reduction of the CD8+ cells by
marbofloxacin led to an increased CD4+/CD8+ ratio in mice.

The higher CD4/CD8 ratio in ENRO group on days 9 and 13 of the study resulted mainly from the reduction of CD4+CD8+ cell counts with a relatively stable number of CD4+CD8- cells. At the same time, the number of WBC as well as the number and percentage of lymphocytes did not change significantly. Furthermore, no significant differences regarding the mentioned parameters were found between the groups (P > 0.05).

In contrast to our results, Szczypka and Obmińska-Mrukowicz (19) reported that in mice exposed to enrofloxacin a significant decrease in the percentage of T-helper cells was observed. However, the authors studied the subsets of T lymphocytes in the thymus, spleen, and mesenteric lymph nodes, but not in blood. The authors also found that other fluoroquinolones (flumequine, norfloxacin, ciprofloxacin – an active metabolite of enrofloxacin) also modulated the expression of CD3+, CD4+ and CD8+ markers on thymocytes, splenocytes, and lymphocytes of the mesenteric lymph nodes. Flumequine decreased frequency of CD4+CD8+ cells (immature thymic cells) and increased frequency of mature CD4+ and CD8+ cells. Ciprofloxacin increased the percentage of CD4+ splenocytes and CD8+ mesenteric lymph node cells. These results suggest that the same antibiotic can exert different effects on lymphocyte subpopulations in various organs and probably in blood.

The significant reduction of CD4-CD8+ T-cell number indicates that enrofloxacin possesses the immunomodulatory effects, since this population of T-cells includes cells with spontaneous cytolytic activity against cancer cells and cytolytic T lymphocytes (CTLs) directed against cells infected with intracellular pathogens (14, 15). CTLs are an important part of cell-mediated immunity against viral infections because they can directly attack host cells that have foreign antigen presented on the surface of MHC class I molecules (14). The question remains whether the observed changes in circulating lymphocyte subsets in pigs are specific to enrofloxacin or would they also be found if pigs were treated with other fluoroquinolones (e.g. marbofloxacin, ciprofloxacin). It has been previously reported that immunomodulating effect of fluoroquinolones depends on various factors: the chemical structure of drugs, host’s immunological status, dose and route of administration (20). Earlier studies with the use of antibiotics from other groups (e.g. cephalosporins, tetracyclines) revealed that doxycycline and cefodizime also can influence the amount and/or composition of circulating lymphocytes (11, 22), but to date the mechanisms by which these drugs interact with immune system have not been explained.

In summary, results of the present study indicate that enrofloxacin, in addition to its antimicrobial properties, produces significant immunomodulatory effects in vivo and may alter the immune response in pigs. The mechanisms of interference with the immune system have not been identified and are beyond the scope of this article; however, the influence of enrofloxacin on the secretion of various cytokines, as it was reported previously for other fluoroquinolones, may be considered as a possible cause (1, 5, 8, 12, 13, 16, 18, 20).

The knowledge on interactions of antibiotics with the immune system is of great importance. The presented results should prompt further studies on the practical significance of recent observations in terms of clinical implications. Moreover, in view of the current results, it cannot be excluded that enrofloxacin may also have immunomodulatory effects on the host response to infection.

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References


