Original article

Modulation of lymphocyte subsets and humoral immune response by florfenicol administered to sheep red blood cell-immunized broiler chickens

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Abstract

Florfenicol is a broad-spectrum bacteriostatic antibiotic commonly used for the treatment of systemic infections in farm animals. The aim of this study was to determine the effect of florfenicol on the percentage of T lymphocytes (CD3$^+$, CD4$^+$, CD8$^+$, TCR$\gamma\delta^+$ cells) and B lymphocytes (Bu-1$^+$ cells) and on total serum anti - sheep red blood cell (SRBC) haemagglutinin titer in the peripheral blood of SRBC–immunized broiler chickens. The study included three groups of broiler chickens differentiated by weight (0.5, 1.2, 2.4 kg). Florfenicol was administered orally at a dose of 30 mg/kg. The drug was administered eight times at 24 h intervals. The chickens were immunized with SRBC 24 h after administration of the third dose of florfenicol. Florfenicol increased the percentage of CD3$^+$ blood lymphocytes with a corresponding decrease in the percentage of B lymphocytes in birds weighing 0.5 and 2.4 kg. Florfenicol reduced the production of total anti SRBC-haemagglutinins on day 5 after antigen injection in all three body weight groups of the broiler chickens. In conclusion, florfenicol exerted a modulating effect on the immune response of the birds and this should be taken into consideration when using this antibiotic for certain indications.

Key words: florfenicol, antibodies, bird, broilers, immunity, lymphocytes

Introduction

Antibiotics are widely used in the poultry industry to treat or prevent bacterial infections. The role of the immune system in the prophylaxis and treatment of such infections is also very important. Unfortunately, there are situations when antibiotics are used without a sufficient reason and/or not properly. A common practice in poultry farms is administration of the antibiotic without taking into consideration other factors, such as vaccination. Knowledge of the effect of particular drugs on the immune system is very often insufficient. Therefore, it is hard to conclude whether this practice brings more profit or damage to the function

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of the immune system. Florfenicol, a drug belonging to the group of fenicols, is a bacteriostatic antibiotic that acts by inhibiting the peptidyl transferase step of microbial protein synthesis by binding reversibly to the 50S subunit of the bacterial ribosome (Cannon et al. 1990). Florfenicol inhibits growth of most aerobic and anaerobic Gram-positive and Gram-negative bacteria. It is more potent than chloramphenicol and thiamphenicol due to the presence of the fluorine atom at the 3'carbon position that protects the drug from some mechanisms of bacterial inactivation. Idiosyncratic aplastic anemia in humans, the most serious adverse effect observed after administration of chloramphenicol, does not occur following treatment with florfenicol. This antibiotic possesses a sulfomethyl group instead of the *p*-nitro group (associated with this adverse effect) in its benzene ring. However, the drug may induce dose-dependent reversible bone marrow suppression (Dowling 2013). In the mid-1990s, the Committee for Veterinary Medicinal Products of the European Medicines Agency (EMA) approved florfenicol for use in veterinary medicine. The antibiotic is used for the treatment of diseases caused by susceptible bacteria in cattle, chickens, swine and fish. In the poultry industry, it is available in many preparations to treat respiratory and gastrointestinal system diseases caused by antibiotic-sensitive bacteria. The drug, administered orally, is effective in broiler chickens in the treatment and control of air sacculitis associated with *E. coli* susceptible to the antibiotic. The pharmacokinetic properties of florfenicol have been studied in many species, including birds (Świtała et al. 2007, Chang et al. 2010); ruminants (Ali et al. 2003, Palma et al. 2011, Sidhu et al. 2014), horses (McKellar and Varma 1996), pigs (Li et al. 2006), dogs (Park et. al. 2008), and rabbits (Koc et al. 2009). The bioavailability of florfenicol in broiler chickens after oral administration is almost complete. It was found to be 94% (Shen et al. 2003), and 87% (Anadón et al. 2008) after a single administration at a dose of 30 mg/kg and 20 mg/kg.

There are also some studies concerning the impact of florfenicol on the immune system (Bretzlaff et al. 1987, Paape et al. 1990, Lundèn et al. 1999, Caipang et al. 2009), but this knowledge is still incomplete.

In the last few years some studies were published describing the effects of florfenicol on the immune system of chickens (Khalifeh et al. 2009, Chrząstek et al. 2011, Hassanin et al. 2014). However, the reported results are inconsistent and the influence of florfenicol on the cell-mediated and humoral immune response in chickens is still unclear. Therefore, the aim of this study was to determine the effects of florfenicol on the subsets of T and B blood lymphocytes and on total anti-SRBC antibody titers in the serum of broiler chickens immunized with SRBC. In our previous studies, concerning the pharmacokinetic profile of florfenicol in chickens and turkeys, there was a correlation between the values of some pharmacokinetic parameters and body weight (Świtała et al. 2006, 2008). Therefore, the immunomodulating effect of florfenicol was determined depending on the bird body weight.

Materials and Methods

Animals

The study was carried out on Arian broiler chickens (obtained from a commercial breeding facility), weighing 0.5-0.6 kg (2.5 weeks of age), 1.2-1.4 kg (3.5 weeks of age), and 2.4-2.6 kg (5.5 weeks of age). The animals were kept in collective pens with straw bedding under conventional conditions and fed commercial food (containing no antimicrobial or anti-parasitic agents) and water *ad libitum*. Principles of laboratory animal care (NIH publication No. 86-23, revised 1985), as well as specific national laws on the protection of animals were followed. The study protocol was approved by the II Local Ethics Commission in Wroclaw, Poland (No. 85/2006).

The studies were performed on SRBC-immunized chickens. The animals were immunized intravenously with 0.5 ml of 5% SRBC suspension. The sheep blood was collected into Alsever's solution in a sterile manner and kept at 4°C for at least 3 days. The SRBC suspension was prepared ex tempore in phosphate buffered saline (PBS, Institute of Immunology and Experimental Therapy, Wroclaw, Poland).

Treatment and Measurements

Florfenicol (Vetos-Pharma, Bielawa, Poland) was administered orally via a tube into the crop at a dose of 30 mg/kg suspended in 2 ml of a starch jelly.

The study was divided into two experiments

In each experiment the drug was administered eight times at 24 h intervals in SRBC-immunized chickens. The chickens were immunized with SRBC 24 h after administration of the third dose of florfenicol.

Experiment I. The percentage of T lymphocytes (CD3^{+}, CD4^{+}, CD8^{+}, TCRγδ^{+}) and B lymphocytes (Bu-1^{+} cells) in peripheral blood was estimated 24 h after the last dose of florfenicol, i.e. on day 5 after SRBC immunization.

Experiment II. Total anti-SRBC haemagglutinin titers in the serum were determined 24 h after the last dose of the drug, i.e. on day 5 after SRBC immunization.

Experiment I

Table 1. Effect of florfenicol on the subsets of T and B peripheral blood lymphocytes in SRBC-immunized broiler chickens weighing 0.5, 1.2, and 2.4 kg. Mean values (n=8) and standard deviations are presented.

Weight	Group	% CD3$^+$	% CD4$^+$	% CD8$^+$	% TCRγδ$^+$	% Bu-1$^+$
0.5 kg	Control	81.5 ± 3.0	75.7 ± 3.5	25.4 ± 2.8	21.4 ± 4.8	18.5 ± 3.0
0.5 kg	SRBC	76.6 ± 5.3 ^	67.6 ± 7.1 ^	33.6 ± 7.8 ^	27.2 ± 2.9 ^	23.4 ± 5.3 ^
0.5 kg	Florfenicol 8 x 30 mg/kg + SRBC	83.1 ± 3.7 *	67.6 ± 7.1	36.1 ± 6.9	22.6 ± 5.9	16.9 ± 3.7*
1.2 kg	Control	79.7 ± 1.5	77.3 ± 9.3	21.3 ± 6.5	20.3 ± 3.8	20.3 ± 1.5
1.2 kg	SRBC	77.9 ± 4.3	76.3 ± 8.3	20.6 ± 6.6	14.5 ± 4.5 ^	22.1 ± 4.3
1.2 kg	Florfenicol 8 x 30 mg/kg + SRBC	81.6 ± 5.2	64.4 ± 6.7 *	35.2 ± 7.7 *	22.1 ± 2.7 *	18.4 ± 5.2
2.4 kg	Control	82.0 ± 3.0	69.1 ± 7.2	25.4 ± 2.5	32.8 ± 6.3	17.9 ± 3.0
2.4 kg	SRBC	76.5 ± 3.0^	75.3 ± 7.7	24.0 ± 6.7	35.3 ± 8.7	23.4 ± 3.0 ^
2.4 kg	Florfenicol 8 x 30 mg/kg + SRBC	87.6 ± 3.6 *	56.9 ± 6.7*	29.3 ± 4.5	33.5 ± 8.0	12.4 ± 3.6 *

^ p<0.05 SRBC group compared to the control group
* p<0.05 florfenicol group compared to the SRBC group

Experiment II

Table 2. Effect of florfenicol on total haemagglutinin titer in SRBC-immunized broiler chickens weighing 0.5, 1.2, and 2.4 kg. Mean values (n=8) and standard deviations are presented.

Group	Anti-SRBC hemagglutinins (log$_2$ titre)		
	0.5 kg	1.2 kg	2.4 kg
Control	9.1 ± 1.6	11.1 ± 0.9	11.5± 0.7
Florfenicol 8 x 30 mg/kg	8.0 ± 1.5*	9.6 ± 1.8*	10.0±1.6*

* p<0.05 as compared to the control group

Control groups were conducted simultaneously in each experiment. The chickens in the control groups received a starch jelly instead of florfenicol. Each control and experimental group consisted of eight birds.

Determination of lymphocyte subsets in peripheral blood

Peripheral blood samples (1 ml) were collected from the wing vein of each bird. The blood was taken on heparin (1 ml of blood per 50 units of heparin) (Heparinum® WZF, Polfa Warszawa S.A., Poland). Each blood sample was diluted with 1 ml of sterile ice-cold PBS and centrifuged (1200 x g, 30 min, 4°C) on a layer of Ficoll-400® (Pharmacia, Fine Chemicals AB, Uppsala, Sweden)/Uropolinum® 75% (sodium diatrizoate and meglumine diatrizoate, Polpharma S.A., Starogard Gdański, Poland) at a 1:3 ratio and a density of 1.076. After centrifugation, the lymphocytes were collected from the interphase and suspended in the medium containing sterile PBS supplemented with 1% bovine serum albumin (BSA®, Sigma, USA) and washed twice (375 x g, 7 min, 4°C). After the second rinsing, the cells were suspended in PBS with 1% BSA

at a concentration of 10 x 10^6 cells. The viability of each tested cell suspension was 95 to 100% according to the trypan blue (Sigma, USA) exclusion assay. The samples (100 µl) of each lymphocyte suspension prepared this way were stained with proper monoclonal antibodies (incubation for 30 min at 4°C). The following monoclonal antibodies were used: Mouse anti-chicken® CD3-FITC (cat. no. 8200-02), Mouse anti-chicken® Bu-1-RPE (cat. no. 8395-02), Mouse anti-chicken® CD8α-RPE (cat. no. 8390-09), Mouse anti-chicken® CD4-FITC (cat. no. 8210-02,) and Mouse anti-chicken® TCRγδ-FITC (cat. no. 8230-02). These monoclonal antibodies were used in the concentrations recommended by the producer (SouthernBiotech, Birmingham, AL, USA). After incubation, the cells were rinsed three times in ice-cold PBS. After the last wash, they were suspended in 0.5 ml of PBS. Fluorescence was read using a flow cytometer (FACS Calibur, Becton Dickinson Biosciences, USA), a total of 10 000 events were collected. The data were analyzed using CellQuest 3.1f software.

The data (percentage of T and B blood lymphocytes) were calculated according to the formula published by Bowen et al. (2009). Total lymphocyte

percentage was estimated by adding the percentage of T lymphocytes and B lymphocytes. T lymphocytes were estimated by dividing CD3$^+$ cells by total lymphocyte percentage and multiplying by 100. B lymphocytes were estimated by dividing Bu-1$^+$ cells by total lymphocyte percentage and multiplying by 100. CD4$^+$, CD8$^+$ and TCR γδ$^+$ were estimated by dividing the proper subpopulation by CD3$^+$ cells and multiplying by 100.

Determination of total anti-SRBC antibodies in the serum

Total anti-SRBC haemagglutinin titers were determined on day 5 after SRBC immunization. The blood samples were taken from the wing vein. The sera were obtained by blood centrifugation and inactivated at 56°C for 30 min. Total serum haemagglutinin titers were defined by an active haemagglutination test carried out on 96-well microplates (Hudson and Hay 1980). The results were expressed as a value of log$_2$ of haemagglutinin titers.

Statistical analysis

The data obtained in the study were analyzed statistically using t-Student test. The differences were considered significant at p<0.05.

Results

It was found that SRBC injected to broiler chickens (experiment I) weighing 0.5 kg reduced the percentage of CD3$^+$ and CD4$^+$ blood lymphocytes but heightened the percentage of CD8$^+$, TCRγδ$^+$ and Bu-1$^+$ blood lymphocytes. As shown in Table 1, florfenicol administered eight-fold orally at a dose of 30 mg/kg to SRBC - immunized broiler chickens decreased the percentage of B lymphocytes but increased the percentage of CD3$^+$ lymphocytes. A similar effect of immunization and florfenicol on blood lymphocytes was observed in the birds weighing 2.4 kg. SRBC injection lowered the percentage of CD3$^+$ cells but raised the percentage of B blood lymphocytes. Administration of florfenicol altered this effect of immunization. The drug increased the percentage of CD3$^+$ lymphocytes and decreased the percentage of CD4$^+$ and Bu-1$^+$ lymphocytes (Table 1). The effect of SRBC on the percentage of T and B blood lymphocytes was not observed in the chickens weighing 1.2 kg (except for a decrease in the percentage of TCRγδ$^+$ lymphocytes). However, florfenicol administered to the immunized broiler chickens weighing 1.2 kg reduced the percentage of CD4$^+$ cells and raised the percentage of CD8$^+$ and TCRγδ$^+$ lymphocytes as compared to the SRBC group (Table 1).

Moreover, exposure to eight doses of florfenicol, irrespective of the body weight, discontinued the production of total anti-SRBC haemagglutinins on day 5 after the antigen administration (Experiment II, Table 2).

Discussion

Results of the study carried out in SRBC-immunized Arian broiler chickens showed modulating effects of florfenicol on T and B blood lymphocytes subsets and humoral immune response. The antibiotic administered at a therapeutic dose of 30 mg/kg increased the percentage of T blood lymphocytes with a corresponding decrease of the percentage of B lymphocytes. These effects were observed in the birds weighing 0.5 kg and 2.4 kg. Moreover, florfenicol administered eight times suppressed humoral immune response in the chickens (in all three body weight groups), resulting in lowering of the total anti-SRBC haemagglutinin titer. These outcomes are consistent with our earlier studies in mice (Lis et al. 2011), and the studies of other authors (Khalife et al. 2009, Chrząstek et al. 2011, Guan et al. 2011, Shuang et al. 2011). In our previous paper, we reported that florfenicol administered six times orally at the same dose of 30 mg/kg to non-immunized mice increased the percentage of immature CD4$^-$CD8$^-$ thymocytes and the absolute count of mature CD4$^+$ and CD8$^+$ cells in the thymus as well as the percentage and absolute number of CD3$^+$, CD4$^+$, and CD8$^+$ lymphocytes in mesenteric lymph nodes. At the same time, the antibiotic decreased the percentage of B lymphocytes in the mesenteric lymph nodes. We also demonstrated a suppressive effect of four and seven doses of florfenicol on humoral immune response in mice that was manifested by a reduced number of plaque forming cells (PFC) and production of anti-SRBC antibodies. The immunosuppressive effect of florfenicol on the immune response to ovalbumin in mice was reported by Shuang et al. (2011). Florfenicol administered orally for 10 days at 50, 100 and 200 mg/kg decreased the percentage of B cells (CD19$^+$) in a dose-dependent manner, as well as T cells (CD3$^+$) at high doses and reduced OVA-specific IgG, IgG1, and IgG2b titers. The antibiotic also suppressed ConA-, LPS- and OVA-induced splenocyte proliferation in vitro and in vivo (Shuang et al. 2011). Similar outcomes were achieved by Guan et al. (2011) in mice immunized with foot-and-mouth disease virus (FMDV) serotype O antigen. In another study (Chrząstek et al. 2011), florfenicol administered orally five times, once a day, at a dose of 30 mg/kg to 2-day-old chicks decreased the percentage of bursal Bu-1$^+$ cells and the number of Bu-1$^+$ cells in the medulla of this organ. These effects of the anti-

biotic indicated that florfenicol probably affected younger forms of B cells by inhibiting their proliferation, and a lower release of B cells into the circulation could be a reason for the increase in the number of these cells in the cortex of the bursa of Fabricius (Chrząstek et al. 2011). A negative impact of florfenicol on B lymphocytes may be associated with its effect on the production of the cytokine that affects or promotes B cell activity. Xinxin et al. (2011), in their study on a murine asthma model, showed that florfenicol administered orally four times at 5, 25, and 100 mg/kg, once a day, reduced the concentration of IL-4, IL-5 and IL-13 in a dose-dependent manner in bronchoalveolar fluid. IL-4 and IL-13 play a role in the proliferation and differentiation of B cells (Matthews and Callard 1998, Mire-Sluis 1998).

Hassanin et al. (2014) studied the effect of different doses of florfenicol on the immune response in broiler chickens after Newcastle disease virus (NDV) vaccination and infection with *E. coli*. They reported that florfenicol administered at a dose of 60 mg/kg for 5 days to the group of birds infected with *E. coli* upregulated the post-vaccinal immune response against NDV showing a higher haemagglutination inhibition (HI) response and a higher level of IgG compared with florfenicol at a dose of 30 mg/kg. Furthermore, the authors observed no significant differences in the post-vaccinal (NDV) humoral immune response in the *E. coli* infected groups of birds – non-treated and treated with florfenicol at a dose of 30 mg/kg. This observation suggested that the antibiotic at that dose (30 mg/kg) did not exert a negative effect on the humoral immune response in the broiler chickens. However, the regulatory effect of 60 mg/kg florfenicol on the humoral immune response could be attributed to an efficient therapeutic effect of the antibiotic on *E. coli* infection (Hassanin et al. 2014), as it was reported that *E. coli* infection negatively affected post-vaccinal immune response against NDV vaccine (Hegazy et al. 2010). Another effect of florfenicol was a dose-dependent up-regulation in the level of the interferon-α (IFN-α) pathway related genes (IRF 7, 2'-5'OAS) (Hassanin et al. 2014). The results reported by Hassanin et al. (2014) concerning humoral immune response are inconsistent with ours and those of other authors. Khalife et al. (2009) also showed a negative impact of florfenicol on humoral immune response in chickens. The authors concluded that florfenicol at a dose of 20 mg/kg administered orally 5 times significantly decreased the antibody titer (measured using the HI method) in the ND-vaccinated groups of birds 28 and 42 days after the beginning of the antibiotic treatment. However, this effect was not detected on the 18th day after the beginning of the antibiotic treatment. In the same study, a similar negative impact of florfenicol on anti-NDV IgG production using the ELISA test was shown on the 28th day. It was also reported that antibiotics including florfenicol markedly increased IFN-γ production by splenocytes stimulated in vitro with Con A and NDV antigen. This may indicate that florfenicol exerts beneficial effects on the cell mediated immune response (Khalife et al. 2009). Khalife et al. (2009) claimed that although florfenicol exerted a negative impact on humoral immune response, it did not affect the protection outcome of birds. Therefore, the administration of the drug at the time of vaccination should not affect the bird immunological status.

Most studies published so far reported a negative impact of florfenicol on humoral immune response in mice and birds. The authors described also positive effects of florfenicol on cell-mediated immune response. It was shown (in vitro and in vivo in mice) that florfenicol reduced the production of IL-6 (Zhang et al. 2008, 2009), an important factor in the development of antigen specific humoral response (Richards 1998). Although there are no studies concerning the effects of florfenicol on the synthesis of IL-6 in chickens, it may be assumed that also in birds the negative impact of the antibiotic on humoral immune response may be connected with attenuated production of that cytokine.

In conclusion, our study showed that florfenicol at a therapeutic dose (30 mg/kg) increased the percentage of T blood lymphocytes and decreased percentage of B lymphocytes and humoral immune response in the broiler chickens. The body weight of the chickens reflected their age. The modulating effect of florfenicol on the lymphocyte subsets varies between the weight groups, and it may be concluded that it depends on bird body weight/age. However, the impact of the antibiotic on the humoral immune response observed in all three groups of chickens indicates that it might be independent of the bird's age. Therefore it can be stated that the effect of florfenicol is differential depending on the evaluated parameters of the immune system and bird age. The knowledge of the effect of florfenicol on the function of the immune system may be valuable information which can help in choosing the optimal therapy. For this reason the results obtained in this study may be useful not only for scientists but also for practising veterinary surgeons.

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