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Original paper

Immune response of broiler chickens fed diets supplemented with artemisinin

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Abstract

The purpose of this study was to determine the side effects of artemisinin on the immune system of broiler chickens following chronic oral intake. Chickens were randomly divided into 4 groups: control group and three experimental groups that received 5, 50 and 500 ppm artemisinin in the feed. The effect of artemisinin on the chickens' immune system was assessed using the following criteria: morphometric changes in bursa of Fabricius, spleen, and thymus; changes in the concentrations of serum gamma globulins and functional activity of the immune response, lymphocyte proliferation assay and antibodies titres against sheep red blood cells. The lymphoid organs were negatively affected by the chronic consumption of artemisinin. Bursa was the most affected organ. The concentration of serum gamma globulins and antibody titres against sheep red blood cells were higher in the chickens exposed orally to artemisinin than in the control groups. The lymphocytes were stimulated by lipopolysaccharides, while artemisinin and concanavalin A caused a slight immunosuppression. The spontaneous phagocytic activity was significantly stimulated by the consumption of artemisinin and inhibited by the addition of concanavalin A. In vitro assays revealed a slight immunosuppression, mainly for T lymphocytes, even lymphoid organs were affected by high doses of artemisinin.

Keywords Artemisinin, chickens, immune system

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Introduction

Artemisinin is a sesquiterpene lactone isolated from the Chinese herb *Artemisia annua*. This plant has been used for centuries by herbalists to treat fever. In the 70's the scientists proved that its main bioactive compound, artemisinin, can be used with very good results in the treatment of malaria. This compound has also antiviral (M.R. ROMERO & al., 2006 [19]; T. EFFERTH & al. 2008 [9]), anti-tumor effects (T. EFFERTH & al. 2006 [8]) and antibacterial properties (V. DHINGRA & al. 2000 [7]).

The broiler industry is in need of an alternative to coccidiosis control and previous studies showed promising results for artemisinin (H.A. ARAB & al. 2006 [1]; V. NAIDOO & al. 2008 [17]; E. DEL CACHO & al. 2010 [5]).

Although artemisinin has been proven to be safe in therapeutic doses, researchers identified some side effects to this compound. Embryolethality, morphological abnormalities in early pregnancy, and hormonal imbalances occurred after treatment with artemisinin in mammals (A.C. BOARETO & al. 2008 [3]). In broiler chickens, artemisinin causes decreases in the red blood cells count and hematocrit, anaemia, mild degeneration in renal tubules, hepatic degeneration with fatty changes, bile retention in liver, central chromatolysis in the brain and neuronal necrosis (H.A. ARAB & al. 2009 [2]; A.A. SHAHBAZFAR &. al. 2011 [20]).

The effects of the *A. annua* extract and leaf powder on broiler chickens' immunity was studied in 2013 by Gholamrezaie & al. [13]. These authors showed that the weights of the thymus and bursa of Fabricius, the total anti-SRBC, the IgG titer and cellular immunity increased in chickens treated with *A. annua*.

This study attempts to determine the effects of different doses of artemisinin (5, 50 and 500 ppm) on the immune system of broiler chickens following chronic oral intake. The effects of artemisinin on the immune system in chickens was assessed using the following criteria: morphometric changes in lymphoid organs; changes in the concentrations of serum gamma globulins; and changes in the functional activity of the immune response – phagocytosis assay, lymphocyte proliferation assay and antibodies titres against sheep red blood cells.

Materials and methods

1. Experimental design

Eighteen days-old chickens (hybrid ROSS 308) were weighed and randomly divided into 4 groups of 30 broiler chickens, each group containing 3 replicates of 10 chickens. On the same day, we introduced in their diet the experimental feed with or without artemisinin for 28 days (until 46 days-old). The experimental groups were: a control group that received standard feed and three experimental groups that received 5, 50 and 500 ppm artemisinin in the feed. We used a 10 fold drug dose escalation in order to detect the dose – response relationship.

The effects of artemisinin on the immune system in chickens was assessed using the following criteria: (1) morphometric changes in the lymphoid organs (bursa of Fabricius, spleen, and thymus); (2) changes in the concentrations of serum gamma globulins; and (3) changes in the functional activity of the immune response – phagocytosis assay, lymphocyte proliferation assay and antibodies titres against sheep red blood cells (J.E. DOHMS & al. 1984 [6]).

Blood was collected aseptically from the ulnar vein on days 0, 14 and 28 of the experiment. Chickens from the 1st and 2nd replicates were euthanised by cervical dislocation at the end of the experiment, and those from the 3rd replicate after 5 days of withdrawal (day 33) in order to check for artemisinin residue in the meat and edible tissues (data not included).

We used the turbidimetric method with 18.5% Na_2SO_4 for serum gamma globulins (SGGs). The concentration of SGGs is proportional to the intensity of turbidity, and it was expressed as g/dl. Phagocytosis assay, lymphocyte proliferation assay and antibodies titres against sheep red blood cells were performed as previously described by M. SPÎNU & al. 1993 [21] and I.S. KHOKHLOVA & al. 2004 [14].

The veterinary requirements related to the protection of the animals used in this research have been observed according to the national standards and legislation. All experiments were approved by the Research Bioethics Commission of USAMV Cluj-Napoca (protocol no. 4/19.09.2013).

2. Morphometry of the lymphoid organs

Morphometric changes in the lymphoid organs were assessed by calculating weight ratio and index of the lymphoid organs and by histopathology.

The lymphoid organs were weighed (g) after removal of the connective tissues. Atrophy was determined by calculating the lymphoid organs/body weight ratio (WR) (B. LUCIO & AL. 1979 [16]) and their weight index (WI) (ratio of experimental chicks/ratio of control chicks) (A. THANGAVELU & al., 1998 [24]). An index of less than 0.7 was indicative of lymphoid organ atrophy (V. PALYA, 1991 [11]).

After euthanasia, we performed a full necropsy and collected multiple tissue samples including bursa of Fabricius, thymus, spleen**,** trimmed them into 5-mm thick pieces and immediately fixed them in 10% neutral-buffered

formalin. After 72 h of aldehydic fixation the samples were included in paraffin wax following a standard procedure (E.B. PROPHET & al., 1992 [18]), cut at 2-3 µm and automatically stained with hematoxylin and eosin (H&E).

3. Phagocytosis assay

The activity of the phagocytic cells was measured by a modified carbon particle inclusion test on days 0, 14 and 28 of the experiment. Phagocytic cells engulf inert particles such as carbon due to the defensive capacity of these cells (Spînu et al., 1993). First, each heparinized blood sample was mixed with China ink, and then divided into 3 equal controls: negative (NC), positive (PC) and experimental (EC). The controls were incubated at 41°C for 0 and 30 minutes. After each incubation time, 20 µl of the controls were transferred into 266 µl of physiologic salt solution (PSS) and centrifuged for 5 minutes at 800 rpm. The supernatants (200 µl) were read spectrophotometrically (Biorad 1100 reader plate) in a 96-well flat bottom plate at 540 nm. The results were interpreted as the difference between the optic densities (OD) read at 0 and 30 minutes after incubation ($OD_{0'} - OD_{30'}$).

4. Lymphocyte proliferation assay (LPA)

Three hundred forty microliters of heparinized blood were mixed with 1.360.00 µl RPMI 1640 supplemented with penicillin/streptomycin (1000UI/1000µg/ml) and fetal bovine serum (5%) and with a pH adjusted to 7.2-7.4%. Then, 200 µl of the mixture were distributed into 8 wells of a sterile 96-well flat bottom plate. For each sample, we used 4 experimental variants (2 wells/variant): (1) control culture, (2) concanavalin A culture, (3) lipopolysaccharides culture and (4) artemisinin culture. According to the working variants, we added the mitogens for leukocytes (concanavalin A, lipopolysaccharides from *Escherichia coli* and artemisinin) in a concentration of 0.5 µg/well. The cultures were incubated for 48 h at 41.0°C and 5% CO_2. After incubation, 30 µl of the culture supernatant/well was collected and mixed with its duplicate. Cell growth was measured by means of the glucose consumption technique. The concentration of residual glucose (GC) in the culture supernatant was measured spectrophotometrically using an enzymatic colorimetric CHOD-PAP method (GLUCOSE 4x250 ml kit, ref 4001325L, Hospitex Diagnostics, Italy) according to the manufacturer's instructions. The stimulation index (SI) was calculated as follows: GC of unstimulated cells (control culture)/GC of stimulated cells (concanavalin A, lipopolysaccharide and artemisinin cultures).

5. Antibody titres against sheep red blood cells

The humoral immune response was analysed following stimulation with sheep red blood cells and evaluation of the titre of antibodies (AbSRBC) by the haemagglutination assay.

Chickens from the 3rd replicate (10/group) were stimulated subcutaneously with 0.5 ml of sheep red blood cells (SRBC) suspension, two times, on days 0 and 14. Sheep red blood cells were prepared from whole sheep blood collected aseptically. The heparinized blood (50 UI/ml) was centrifuged 10 min at 2500 rpm and then the sediment with red cells was washed 3 times with PSS. Finally, the red cells were resuspended in PSS to a concentration of 5% (vol/vol). The blood from chickens used to measure the antibody titre against sheep red blood cells (AbSRBC) was collected on days 0, 14 and 28 of the experiment.

In a 96-well round-bottomed plate we placed 25 µl of PSS and then, in the first well of each row 25 µl of serum samples, followed by four 1/2 serial dilutions. After the serum dilution, we added 50 µl of 0.15% SRBC suspension in each well and the plates were incubated at 37°C for 2 h. After incubation, the plates were read. If AbSRBCs were present, and the last dilution produced at least 50% SRBC agglutination, it was noted as the agglutinant titre. In a negative case, the SRBC appear as a red pellet on the bottom of the well.

6. Statistical analysis

The data were statistically analysed in MedCalc software. The arithmetic mean and the standard deviation were calculated for lymphoid organs ratio, serum gamma globulins, phagocytosis and lymphocyte proliferation assays. Then, we checked the normal distribution of the data and excluded the outside and far-out values. We used F-test two-sample for variances. Finally, according to the results of the F-test we performed the t-Test two-sample assuming equal or unequal variance to measure the difference between the control group and the experimental groups.

In the case of antibody titres, we calculated the geometric mean (GM), the geometric standard deviation (GSD) and quartile 1 and 3 (Q_1, Q_3). The difference between the control group and the experimental groups was measured by means of Friedman's test. Differences were considered significant at a p (one-tail) value equal or lower than 0.05.

Results and discussion

The purpose of this study was to determine the side effects of artemisinin on the immune system in broiler chickens following chronic oral intake, by assessing the morphometric changes in lymphoid organs, the changes in concentrations of serum gamma globulins, and the changes in the functional activity of the immune response.

The toxicity of artemisinin and its derivates (artesunate, artemether, artenimol, arteether) in mammals is well documented and shown to induce neurotoxicity (R.I. ERICKSON & al. 2011 [2011]), hormonal imbalance in the absence of malarial parasite infection (E. FAROMBI & al. 2015 [12]), embryotoxicity (R.L. CLARK, 2009 [4]) and immunosuppressive activity (T. LI & al. 2013 [15]).

In birds, there are few studies regarding the side effects of artemisinin. It is hepatotoxic for turkeys in doses of 2600 mg/kg (I. THØFNER & al. 2013 [25]), while in poultry a single oral dose (1250 and 2500 mg/kg) or chronic oral

intake (17, 34, 68, and 136 ppm artemisinin for 36 days) cause microscopical lesions in the brain, liver and kidney (H.A. ARAB & al. 2009 [2]; A.A. SHAHBAZFAR & al. 2011 [20]).

To our knowledge, this is the first study on the immunotoxicity of artemisinin in poultry.

Overall, the lymphoid organs/body weight ratio (WR) was lower in the treated groups than in the control group, both at day 28 of the experiment and even after 5 days of withdrawal. After 5 days of withdrawal period, the WR of thymus was significantly higher for all experimental groups ($p = 0.01$ Art5, Art 50; $= 0.05$ Art500) (Table 1). The weight index (WI) followed the same pattern as the WR, but the bursa WI was lower (0.5) than 0.7 in the case of chickens treated with 50 ppm artemisinin for 28 days; after 5 days of withdrawal, the WI increased to 0.954 (Table 1).

The thymus histopathology revealed an important reduction in the size of the cortex, consisting of loosely arranged lymphocytes, focal infiltrations with heterophils in the medulla and multiple foci of cellular debris and tingible body macrophages in the chickens treated with 50 and 500 ppm artemisinin for 28 days (Fig. 1).

In the bursa of Fabricius, we observed an increased amount of interfollicular fibrous stroma, in groups Art50 and Art500. In group Art50, we also noticed vacuolization of the cortical zone of the follicles and focal accumulation of necrotic lymphocytes in the medulla (black arrow). In the Art500 group, atrophied cortex and evident corticomedullary epithelium were observed (Fig. 1). The Art5 group also showed a mild intercellular edema (indicated by the arrow) (Fig. 1).

Spleen lesions were limited to a mild reduction in the red pulp volume in the chickens treated with 5ppm artemisinin. An important reduction in the overall size of the white pulp, severe vacuolization of the marginal lymphoid tissue, slight morphologic changes in the splenic red pulp, severe depletion of the *periarteriolar lymphocyte sheath and* multiple foci of cellular debris were observed for higher doses (50 and 500 ppm) (Fig. 1).

Overall, lymphoid organs (thymus, bursa and spleen) were affected negatively by the chronic consumption of artemisinin. Bursa was the most affected lymphoid organ, the weight index suggesting atrophy, finding supported by the histopathology evaluation.

In previous studies, researchers have not found any lesions in the spleen after a single oral dose or even chronic oral intake kidney (H.A. ARAB & al. 2009 [2]; A.A. SHAHBAZFAR & al. 2011 [20]). The doses used by these researchers were 10, 50, 250, 1250 and 2500 mg/kg for the study of a single oral dose and 17, 34, 68, and 136 ppm for 36 days in the study of chronic oral intake. We used 5, 50 and 500 ppm for 28 days and could report moderate and severe spleen lesions in the chickens treated with 50 and 500 ppm artemisinin.

Moreover, morphometric data and histopathology also revealed lesions in the cortical and medullary region of the bursa, and the concentrations of serum gamma globulins and antibody titres against sheep red blood cells were higher in the chickens exposed orally to artemisinin than in unexposed chickens. (Table 1).

Antibody titre increased in all experimental groups, both after the first and the second stimulation with sheep red blood cells (SRBCs). Generally, it was higher in the experimental groups than in the control group (Table 1). The most important increase was noticed in group Art5 ($p=0.02$-0.05). The range of titres 14 days after the first stimulation was as follows: control group 2-8 (GM 3); Art5 group 2-64 (GM 8); Art50 group2-32 (GM 5); and Art500 group 2-4 (GM 3). After the second stimulation (day 28) the maximum titre was 32 for the control group (GM 5) and 64 for all three experimental groups (GM: Art5 13, Art50 8, Art500 10). Fourteen days after the first stimulation, there were 20 chickens without response, the majority from the control group (n=7), followed by groups Art50 and Art500 (each n=5) and group Art5 (n=3); after the second stimulation only 2 chickens did not respond, one from group Art5 and one from group Art500 (data not shown). It is worth mentioning that the highest and most statistically significant antibody titres were registered in the chickens treated with 5 ppm artemisinin, where lesions were limited to a mild intercellular edema. There are studies that reported suppression of humoral responses with *A. annua* and its derivative (A.F. TAWFIK & al. 1990 [23]; X.Z. SUN 1991 [22]). Based on our findings, we can conclude that chronic oral intake of a low dose of artemisinin (5 ppm) was immunostimulatory for humoral responses and higher doses (50 and 500 ppm) were immunotolerant compared to unexposed chickens.

Generally, the spontaneous phagocytic activity wasn't suppressed by consumption of artemisinin; with high doses (50 and 500 ppm) it increased significantly ($p = 0.001$ - 0.04) (Table 1). When the cultures were stimulated with concanavalin A or artemisinin we noticed a very low immunosuppression of the phagocytic cells, statistically significant in chickens treated with 50 ppm artemisinin ($p = 0.04 – 0.05$) (Table 1).

In vitro, lymphocytes were stimulated ($SI > 1.0$) by LPS (mitogen that stimulates B lymphocytes) in different timeframes, after 14 and 28 days of consumption of artemisinin - significantly higher, respectively lower than in the control group. On the other hand, artemisinin did not stimulate ($SI \leq 1.0$) lymphocytes, causing a slight *in vitro* immunosuppression in any dosage (Table 1). Considering that concanavalin A (mitogen that stimulates T lymphocytes) did not stimulate ($SI \leq 1.0$) lymphocytes, we can conclude that artemisinin produced immunosuppression mainly for the T lymphocytes, affirmation supported also by the histopathological findings in the thymus. Despite it beneficial effects in treating malaria and different forms of cancer, as well as of its antibacterial potential (D. MILITARU & al., 2015 [26]), many studies reported the immunosuppressive activity of artemisinin and its derivates against T lymphocytes both *in vitro* and *in vivo* (J.X. WANG & al. 2007 [27]; T. LI & al. 2013 [15]).

Table 1. Response of the lymphoid organs and the immune system in broiler chickens following daily chronic oral intake (28 days) of artemisinin

	Control	**Artemisinin 5 ppm**	**Artemisinin 50 ppm**	**Artemisinin 500 ppm**
Ratio of bursa	0.170±0.058	0.145±0.058	0.092±0.042***	0.194±0.063
Index of bursa		0.853	0.544	1.145
Ratio of thymus	0.398±0.110	0.321±0.075**	0.333±0.104*	0.369±0.069
Index of thymus		0.806	0.838	0.928
Ratio of spleen	0.168±0.041	0.161±0.036	0.153±0.032	0.195±0.060*
Index of spleen		0.958	0.909	1.164
Sera gamma globulins (g/dl)	0.549±0.110	0.628±0.188	0.821±0.194***	0.815±0.034***
Phagocytosis assay (UDO)				
Negative control	-0.006±0.018	-0.020±0.038	0.012±0.014**	0.010±0.020*
Concavalin A	0.015±0.008	0.011±0.007	0.008±0.008*	0.010±0.009
Artemisinin	0.015±0.005	0.016±0.012	0.010±0.007*	0.018±0.011
Lymphocyte proliferation assay (stimulation index)				
Concavalin A	1.013±0.048	1.010±0.033	1.004±0.040	0.997±0.027
LPS	1.071±0.042	1.024±0.044**	1.010±0.049**	1.032±0.053*
Artemisinin	1.038±0.100	0.978±0.059	0.943±0.058**	1.004±0.019
Haemagglutination assay titres $^{\#}$GM±GSD (Q_1-Q_3)	5±2 (2-6)	13±4 (6-64)*	8±4 (2-32)	10±4 (2-64)

*p < 0.05; **p < 0.01; ***p < 0.001.

Conclusions

The present study showed that "in vitro" high dosages of artemisinin caused a slight immunosuppression, mainly for the T lymphocytes. This finding was not supported "in vivo" when serum gamma globulins concentration and antibody titres against SRBCs were not affected by chronic intake of artemisinin, even the lymphoid organs were affected at structural level by high doses of artemisinin (50 and 500 ppm for 28 days in the feed).

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Conflict of Interest Statement

The authors state that there are no conflicting financial interests that could compromise the scientific integrity of the report.

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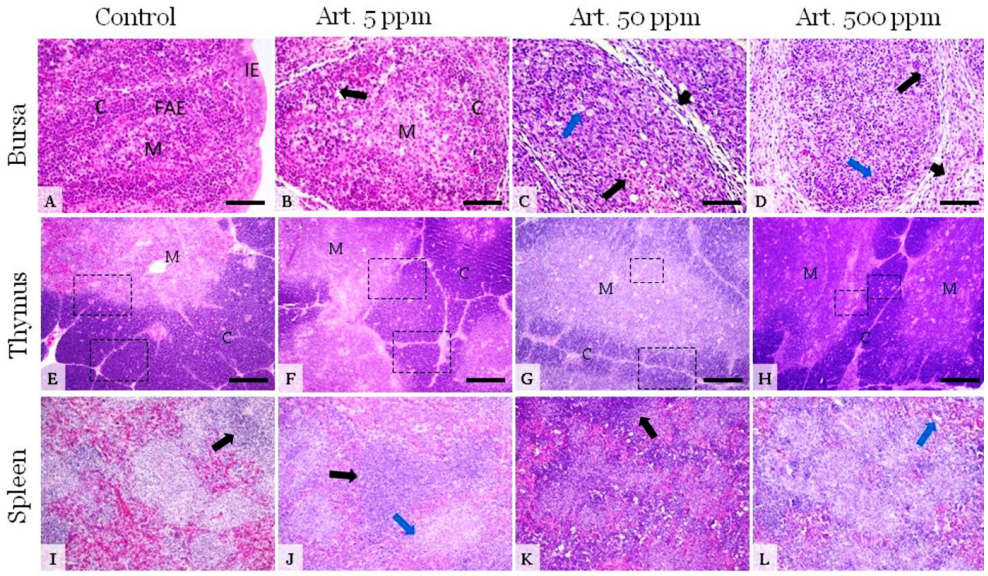
Figure 1. Histopathological images of the lymphoid organs following chronic oral intake of 5, 50 and 500 ppm of artemisinin in comparison with the control group.



A-D: High power images (obx40) of the bursal follicles. A - control presenting a normal morphology (C-follicular cortex; M-follicular medulla; IE-interfollicular epithelium; FAE- corticomedullary epithelium). B – a mild intercellular edema (indicated by the arrow). C - a slight increase of interfollicular fibrous stroma, vacuolization of the cortical zone of the follicles (blue arrow) and focal accumulation of necrotic lymphocytes in the medulla (black arrow) of follicles. D - increased amount of interfollicular fibrous stroma (short arrow), atrophied cortex (black arrow) and evident corticomedullary epithelium (blue arrow) *(*H&E stain, scale bar=50µm).

E-H: Low magnification images (obx10) *of the* thymus, assessing the overall morphology and the ratio between cortex (C) and medulla (M); an important reduction in the size of the thymic cortex is observed in image G (artemisinin 50 ppm) and image H (artemisinin 500 ppm) (H&E stain, scale bar=200µm).

I-L: Images (ob x20) of the *white and red* splenic *pulp* presenting the size and cellularity of the *peri-ellipsoid lymphocyte sheath (PELS) (indicated by blue arrow in image I), periarteriolar lymphocyte sheath (PALS, indicated by blue arrow in Image J) and the germinal centers (GS, indicated by the black arrows in images I and J).* The normal structure of the previously mentioned structures is presented in image I. In image K a severe vacuolization of the marginal lymphoid tissue can be observed, the morphologic change which is also present at a low level in the splenic red pulp. Image L shows the severe depletion of the PALS (marked by blue arrow); the vacuolar aspect is present in both white and red pulp; *(*H&E stain, scale bar=100µm).

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