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Abstract: Artemisinin, isolated from the Chinese herb Artemisia annua, is a sesquiterpene lactone used with very good results in treatment of malaria. Previous studies have shown that artemisinin may be effective in controlling poultry coccidiosis. Toxicity of artemisnin and its derivates was well documented in mammals, while in birds there are available few studies. The aim of this study was to determine the side effects of artemisinin on immune system in broiler chickens following morphometric changes in thymus, bursa and spleen, changes in concentrations of serum gamma globulins and changes in functional activity of the immune response (in vitro assays for cellular response and in vivo assays for humoral response). The lymphoid organs were negatively effected by chronic consuption of artemisinin; the most affected was bursa. Concentration of sera gamma globulins and antibodies titres against sheep red blood cells were higher in chickens exposed oraly to artemisnin comparing with unexposed chickens. In vitro, lymphocytes were stimulated by LPS but lower than in control group; artemisinin and concavalin A didn't stimulate the lymphocytes, causing a slightly immunosupresion. Spontaneous phagocytic activty was not affected by consumption of artemisinin, it was even significantly stimualted; when we added mitogens (concavalin A or artemisinin) to cultures the phagocytic activity was inhibited or slightly stimulated, respectively.In vitro assays revealed a slightly immunosupression, mainly for T lymphocytes, that was not supported in vivo (serum gamma globulins concentration and antibodies titres against SRBCs), even lymphoid organs were affected at structural level by artemisinin in high doses. To our knowledge this is the first study regarding immunotoxicity of artemisinin in poultry.

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Original Article

Effect of artemisinin on immune system in chickens

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Abstract

Artemisinin, isolated from the Chinese herb *Artemisia annua*, is a sesquiterpene lactone used with very good results in treatment of malaria. Previous studies have shown that artemisinin may be effective in controlling poultry coccidiosis. Toxicity of artemisnin and its derivates was well documented in mammals, while in birds there are available few studies.

The aim of this study was to determine the side effects of artemisinin on immune system in broiler chickens following morphometric changes in thymus, bursa and spleen, changes in concentrations of serum gamma globulins and changes in functional activity of the immune response (*in vitro* assays for cellular response and *in vivo* assays for humoral response). The lymphoid organs were negatively effected by chronic consuption of artemisinin; the most affected was bursa. Concentration of sera gamma globulins and antibodies titres against sheep red blood cells were higher in chickens exposed oraly to artemisnin comparing with unexposed chickens. *In vitro*, lymphocytes were stimulated by LPS but lower than in control group; artemisinin and concavalin A didn't stimulate the lymphocytes, causing a slightly immunosupresion. Spontaneous phagocytic activty was not affected by consumption of artemisinin, it was even significantly stimualted; when we added mitogens (concavalin A or artemisinin) to cultures the phagocytic activity was inhibited or slightly stimulated, respectively.

In vitro assays revealed a slightly immunosupression, mainly for T lymphocytes, that was not supported *in vivo* (serum gamma globulins concentration and antibodies titres against SRBCs), even lymphoid organs were affected at structural level by artemisinin in high doses. To our knowledge this is the first study regarding immunotoxicity of artemisinin in poultry.

Keywords: Antibodies, Artemisinin, Chickens, Lymphocytes, Toxicity

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 proving that it's main bioactive compound, artemisinin, can be used with very good results in treatment of malaria. This compound has also antiviral (Romero et al., 2006; Efferth et al., 2008) anti-tumor effects (Efferth, 2006) and antibacterial properties (Dhingra et al., 2000).

Due to emergence of chemoresistant strains of *Eimeria* to all known anticoccidials, the fact that vaccines are to expensive to be used on large scale in chickens coccidiosis and due to the increasing demands for organic production, the broiler industry is in need of an alternative for coccidiosis control.

Previous studies have shown that artemisinin may be one of the alternatives, this drug being effective in controlling poultry coccidiosis (Arab et al., 2006; Naidoo et al., 2008; del Cacho et al., 2010). It is also efficient on *Schistosoma, Babesia caballi*, *Toxoplasma gondii*, hepatic trematodes, trypanosomiasis and leishmaniosis (Utzinger et al., 2007; Kumar et. al., 2003; D'Angelo et al., 2009; Keiser and Utzinger, 2007; Mishina et al., 2007; Sen et al., 2010).

The exact mode of action of artemisinin it's not yet known but was demonstrated that by reductive breaking of artemisinin's endoperoxide bridge, operated by iron complexes, reactive radicals are generated that would produce damage to the parasites (Galasso et al., 2007). Li et al. (2005) showed that artemisinin interacts with the electron transport chain leading to disruption of membrane potential, generates local free radicals and mithocondria damage. Artemisinin decreases production of nitric oxide in macrophages infected with *Leishmania donovani*, and causes an increase in IFN-γ and IL-2 in Leishmania-infected CD3+ T lymphocytes (Sen et al., 2010).

It was also shown that artemisinin inhibits SERCA (sarcoplasmic-endoplasmic reticulum calcium ATP-ase) in *Eimeria* macrogametes leading to altered secretion of the wall-forming bodies thus affecting the process of oocysts wall formation (del Cacho et al., 2010). Although artemisinin has been proven to be safe in therapeutic doses, some researchers have shown side effects of this compound. Embryolethality, morphological abnormalities during early pregnancy, hormonal imbalances were induced by treatment with artemisinin in mammals (Boareto et al., 2008). In broiler chickens, artemisinin causes decreases in red blood cells count and hematocrit, anemia, mild degeneration in renal tubules, hepatic degeneration with fatty changes, bile retention in liver, central chromatolysis in brain and neuronal necrosis (Arab et. al., 2009; Shahbazfar et. al, 2011).

Effect on immune system of artemisinin was studied in a mouse model. The study showed that topical administration of artemisinin could supress CHS response. Artemisinin had an inhibitory effect on IL-17 production, diminished the level of IL-6 and enhanced TGF-β (Li et al., 2012).

For assessing the effect on immune system in broiler chickens the following factors need to be taken in consideration: blood lymphocytes, T lymphocyte stimulation, phagocytosis activity, thymus (the site of T lymphocyte maturation) and bursa of Fabricious (the site of B lymphocyte maturation) weights (Kong et al., 2004).

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

The present study aimed to determine the effects of different doses of artemisinin on the immune system of broiler chickens following chronic oral intake.

Materials and methods

Animals

Two hundred one-day old chicken broilers, hybrid ROSS 308, were purchased from SC VISAVIS SA Vadul Crisului, Bihor county, Romania. Chickens were kept in cages till 17 days-old and were feed and water *ad libitum*. Lighting period was permanent. The diet consisted in starter feed with no anticoccidial drugs until the debut of the experiment.

Artemisinin

We used artemisinin (purity min. 98%) purchased from Intatrade Chemicals GmbH from Germany, catalog number NN04008. It was administrated in feed to 18 days-old chickens, for 28 days, continuously, in doses of 5, 50 and 500 ppm. Also, 0.113 mg of artemisinin was diluted in one millilitres of deionised water at 37C for *in vitro* assays.

Experimental design

At 18 days-old, chickens were weighted and randomly divided in 4 groups of 30 broilers, each group consisting in 3 replicates of 10 chickens. In the same day we introduced in their diet experimental feed (growing feed) with or without artemisinin for 28 days (46 days-old).

The experimental groups were: a control group (C_{1-3}) that received standard feed and three experimental groups that received 5, 50 and 500 ppm artemisinin in the feed ($Art5_{1-3}$; $Art50_{1-3}$ and $Art500_{1-3}$). Chickens from 1st and 2nd replicates were euthanized by cervical dislocation at the end of the experiment (day 28), and those from 3th replicate after 5 days (day 33) of withdrawal period.

The effect of artemisinin on immune system in chickens was assessed using the following criteria (Table 1): (1) morphometric changes in lymphoid tissues (bursa of

Fabricius, spleen, and thymus)- weight ratio, index and histopathology of lymphoid organs; (2) changes in concentrations of immunoglobulins - serum gamma globulins; and (3) changes in functional activity of the immune response (*in vitro* assays for cellular response and *in vivo* assays for humoral response) - phagocytosis assay (PhA), lymphocyte proliferation assay (LPA) and antibodies titres against sheep red blood cells (Dohms and Saif, 1984).

Blood and serum samples

Blood was collected aseptically in 1.5 ml eppendorf tubes, from ulnar vein, using a 2.0 ml syringe and 23G needle, on days 0, 14 and 28 of the experiment. We collected 0.5-1.0 ml of blood without additives from 1st and 3th replicates, in order to obtain serum, and 0.7 ml blood with heparin (50 UI/ml) to prevent coagulation from 2nd replicate (Table 1). Serum was obtained after blood coagulation at room temperature for 2 hours and centrifugation 5 minutes at 3000 rpm. Sera were stored at -20C until further analysis.

Weight ratio and index of lymphoid organs

Lymphoid organs were weighted (g) after removal of connective tissues. Atrophy was determined by calculating lymphoid organs to body weight ratio (WR) (lymphoid organ weight in grams/body weight in grams X 100) (Lucio and Hitchner, 1979) and their weight index (WI) (ratio of experimental ckicks/ratio of control chicks) (Thangavelu et al., 1998). An index of less than 0.7 was indicative of lymphoid organ atrophy (FAO, 1991).

Histopathology of lymphoid organs

After euthanasia complete necropsy was provided and multiple tissue sample including bursa of Fabricius, thymus, spleen were collected**,** trimmed in 5 mm thick samples and immediately fixed in 10% neutral-buffered formalin. After 72h of aldehydic fixation the

samples were dehydrated through successive baths of isopropyl alcohol (80%, 90%, 95%, and 100%), clarified in xylene, and embedded in high-melting temperature paraffin wax (Prophet et al., 1992). After solidification on the cold plate, the paraffin blocks were kept at room temperature until used.

Multiple tissue sections were cut from each paraffin block at 2-3 µm thickness with a rotary microtome (Leica RM2125 RT) and stored overnight at 37 °C on a thermostatically controlled slide warmer. Tissue sections were automatically stained (Sakura DRS 601 slide strainer) with hematoxylin and eosin (H&E) (Prophet et al., 1992) and examined under an Olympus BX41 microscope. The bright field microscopic images were collected with an Olympus UC30 camera and processed using Olympus Stream Basic image analysis software.

Serum gamma globulins

We analysed sera gamma globulins (SGGs) on days 0, 14 and 28 of the experiment, in 1^{st} replicate (Table 1). We used a turbidimetric method. Briefly, 5 µL of plasma were mixed with 500 µl of 18.5% Na_2SO_4 and then incubated 15 min at 37°C. We prepared in the same way the standard. After incubation, optic density was spectrophotometrically read (Spectrophotometre UV-VIS Screen Master Touch, Hospitex, Italy) at 546 nm. The concentration of SGGs is proportional with the intensity of turbidity, and it was expresed as g/dl.

Phagocytosis assay (PhA)

The activity of 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 in 3 equally controls: negative

(NC), positive (PC) and experimental (EC) controls (Table 2). The controls were incubated at 41C for 0 and 30 minutes. After each incubation time, 20 µl of controls were transferred in 266 µl of physiologic salt solution (PSS) and centrifuged 5 minutes at 800 rpm. The supernatants (200 µl) were read spectrophotometrically (Biorad 1100 reader plate) in a 96 well flat bottom plates at 540 nm. Because of phagocytic activity of the cells the absorbance decrease in time as carbon particle are phagocytized. The results were interpreted as the difference between optic densities (OD) read at 0 and 30 minutes after incubation ($OD_{0'} - OD_{30'}$).

Lymphocyte proliferation assay (LPA)

Three hundred forty microlitres of heparinized blood were mixed with 1.360.00 µl RPMI 1640 suplemeneted with peniciline/streptomycine (1000UI/1000µg/ml) and fetal bovine serum (5%) and with a pH adjusted at 7.2-7.4%. Then, 200 µl of mixture were distributed in 8 wells of a 96-sterile flat bottom well plate. For each sample, we used 4 experimental variants (2 wells/variant): (1) control culture, (2) concavalin A culture, (3) lipopolysaccharides (LPS) culture and (4) artemisinin culture. According to working variants, we added the mitogens for leukocytes (concavalin 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 culture supernatant/well was collected and mixed with its duplicate. Cell growth was quantified by means of the glucose consumption technique. Concentration of residual glucose (GC) in culture supernatant was measured spectrophotometrically using an enzymatic colorimetric CHOD-PAP method (GLUCOSE 4x250 ml kit, ref 4001325L, Hospitex Diagnostics, Italy) following manufacture instruction. The stimulation index (SI) was calculated as follows: GC of unstimulated cells (control culture)/GC of stimulated cells (concavalin A, lypopolizaharide and artemisinin cultures).

Antibodies titres against sheep red blood cells

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

Chickens from 3rd replicate (10/group) were subcutaneusly stimulated 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 obtained aseptically. Heparinized blood (50 UI/ml) was centrifuged 10 min at 2500 rpm and then the sediment with red cells washed 3 times with PSS. Finally, red cells were resuspended in PSS to a concentration of 5% (vol/vol). Blood from chicknes to evaluate the titre of antibodies against sheep red blood cells (AbSRBC) was collected on days 0 (before first stimulation), 14 (before second stimulation) and 28 of the experiment.

In a round-bottomed 96-well plate we put 25 µl of PSS and then, in the first well of each row 25 µl of serum samples, followed by 4 serial dilutions 1/2. After sera dilution we added in each well 50 µl of 0.15% SRBC suspension and the plates were incubated at 37°C for 2 h. After incubation the plates were read. If the AbSRBCs were present a mat at the bottom of the well was observed, and the last diulution producing at least 50% SRBC agglutination was noted as agglutinant titre. In a negative case, the SRBC appear as a red pellet at the bottom of the well.

Statistical analysis

The data were statistically analysed in Excel 2007. Arithmetic mean and standard deviation was calculated for lymphoid oragans ratio, serum gamma globulines, phagocytosis and lymphocyte proliferation assays. Then, we checked the normal distribution of data and put out the outside and far-out values. We used F-test two-sample for variances. Finally, according to the results of F-test we performed t-Test two-sample assuming equal or unequal variance to evaluate the difference between control group and experimental groups.

In case of antibodies titres we calculated geometric mean (GM), geometric standard deviation (GSD) and quartile 1 and 3 (Q_1, Q_3). The difference between control group and experimental groups was assessed by Friedman's test (Reverberi, 2008). Differences were considered significant at a *p* (one-tail) value equal or lower than 0.05.

Results

Weight ratio and index of lymphoid organs

Globally, the lymphoid organs to body weight ratio (WR) was lower in treated groups than in control group, either at day 28 of experiment or even after withdrawal period of 5 days; the WR was significantly higher for all experimental groups (p = 0.01 Art5, Art 50; = 0.05 Art500) after withdrawal period for thymus (Fig. 1). This decrease of WR was statistically significant for all lymphoid organs but for different experimental groups: in group Art5 only thymus (p = 0.01); in group Art50 bursa (p < 0.0001) and thymus (p = 0.04). Interestingly, on day 28, in Art500 group the WR was higher in case of bursa and spleen (p = 0.05) than in control group; the picture remains during withdrawal period only for spleen, the WR for bursa turned significantly lower (p = 0.03) comparing with control group (Fig. 1).
The weight index (WI) followed the same pattern as WR, but it was lower (0.5) than 0.7 in case of chickens treated with 50 ppm artemisinin 28 days for bursa, suggesting bursal athrophy; after 5 days of withdrawal period WI increased at 0.954 (Fig. 1).

Histopathology of lymphoid organs

Histopathology of thymus revealed an important reduction in size of the cortex consisting in loosely arranged lymphocytes, focal infiltrations with heterophiles in medulla and multiple foci of cellular debris and tingible body macrophages in chickens treated with 50 and 500 ppm artemisinin for 28 days (**Fig. 2).**

At bursal level, we observed an increased amount of interfollicular fibrous stroma, in groups Art50 and Art500. In group Art50, we noticed also vacuolization of the cortical zone of the follicles and focal accumulation necrotic lymphocytes in the medulla (black arrow). In Art500 group, atrophied cortex and evident corticomedullary epithelium was observed (Fig. 3). As regarding the Art5 group a mild intercellular oedema (indicated by the arrow) was seen (Fig. 3).

The lesions in spleen were limited to a mild reduction in volume of the red pulp for chickens treated with 5ppm artemisinin. In higher doses (50 and 500 ppm) important reduction in the overall size of the white pulp, sever vacuolization of the marginal lymphoid tissue, morphologic changes also present in a reduced amount in the splenic red pulp, severe depletion of the peri-arteriolar lymphocyte sheath and multiple foci of cellular debries were observed (Fig. 4**).**

Sera gamma globulins

The concentration of sera gamma globulins (SGG) (g/dl) remained constantly for control group during experimental period of 28 days. In experimental groups it increased statistically significantly comparing with control group (Fig. 5); SGG increased on day 28 with 0.156 g/dl in Art5 group, 0.358 g/dl in Art50 group and with 0.239 g/dl in Art500 group.

Phagocytosis assay

Generally, spontaneous phagocytic activity (negative control) wasn't affected by consumption of artemisinin, even in high dose (500 ppm), being significantly increased in Art50 and Art500 groups (p = 0.001 - 0.04); as regarding Art5 group it was similar to control group to the debut of the experiment, higher after 14 days and lower after 28 days than in control group, but without statistical significance (Fig. 6). When the cultures were stimulated

with concavalin A or artemisinin we noticed a very low immunosupresion of phagocytic cells; the immunosupresion was statistically significant in chickens treated with 50 ppm artemisinin, after 28 days of consuption (p = 0.04 – 0.05) (Fig. 6).

Lymphocyte proliferation assay

Lymphocytes were stimulated *in vitro* by concavalin A after 14 days of treatment with artemisinin but statistically significant in case of group Art50 (p=0.01) comparing with control group. At the end of experiment (day 28) we noticed a decrease of stimulation index in experimental groups comparing with control group, suggesting immunosupresion wich was not statistically suported (p > 0.05) (Fig. 7). The same pattern was registered in case of stimulation with LPS and artemisinin (Fig. 7). The immunosupression on day 28 was supported statistically for LPS for all three experimental groups (p=0.01, p=0.005, p=0.05 for Art5, Art50 and Art500 respectively) and for artemisnin only for group treated with 50 ppm (p=0.01) (Fig. 7).

Antibodies titres against sheep red blood cells

Antibodies titre increased in all experimental groups, both after first and second stimulation with sheep red blood cells (SRBCs); generally it was higher in experimental groups than in control group (Fig. 8). The most important increase was noticed in group Art5 (p=0.05 on day 14^{th}; p=0.02 on day 28^{th}). The range of titres after 14 days from the first stimulation was as follows: control group 2-8 (GM 3); Art5 group 2-64 (GM 8); Art50 group 2-32 (GM 5); and Art500 group 2-4 (GM 3). After the second stimulation (day 28) the maximum titre was 32 for control group (GM 5) and 64 for all three experimental groups (GM: Art5 13, Art50 8, Art500 10).

At 14 days after first stimulation, there were 20 chicks without response, most of them from control group (n=7), followed by groups Art50 and Art500 (each n=5) and group Art5 (n=3); after second stimulation only 2 chicks did not respond, one from group Art5 and one from group Art500 (data not shown).

Discussion

Toxicity of artemisnin and its derivates (artesunate, artemether, artenimol, arteether) was well documented in mammals and revealed neurotoxicity (Erickson et al., 2011), hormonal imbalance in the absence of malarial parasite infection (Farombi et al., 2014), embriotoxicity (Clark, 2009) and immunosuppressive activity (Li et al., 2013).

In birds there are few studies regarding the side effects of artemisinin. It is hepatotoxic for turkeys in dose of 2600 mg/kg (Thøfner et al., 2013) and in poultry following single oral dose (1250 and 2500 mg/kg) or chronic oral intake (for 36 days 17, 34, 68, and 136 ppm artemisinin) causes microscopical lesions in brain, liver and kidney (Arab et al., 2009; Shahbazfar et al., 2011).

To our knowledge this is the first study regarding immunotoxicity of artemisinin in poultry. We used the artemisinin in feed, for 28 days in three concentrations: 5, 50 and 500 ppm. The side effects on immune system were assessed following morphometric changes in lymphoid tissues (thymus, bursa and spleen), changes in concentrations of immunoglobulins (serum gamma globulins) and changes in functional activity of the immune response (*in vitro* assays for cellular response and *in vivo* assays for humoral response) (Dohms and Saif, 1984). Based on lymphoid organs to body weight ratio, all three lymphoid organs (thymus, bursa and spleen) were negatively effected by chronic consuption of artemisinin; this was reversibilly for thymus after a withdrawal period of 5 days. The most affected lymphoid organ was bursa at 50 ppm dose, weight index (0.5) suggesting bursal athrophy. This finding was suported by

histopathology that revealed increment of interfollicular fibrous stroma, vacuolization of the cortical zone of the follicles and focal accumulation of necrotic lymphocytes in the medulla. In previous studies the authors didn't find any lesions in spleen even after single oral dose or chronic oral intake (Arab et al., 2009; Shahbazfar et al., 2011). The doses used by these authors were 10, 50, 250, 1250 and 2500 mg/kg for single oral dose study and 17, 34, 68, and 136 ppm for 36 days in case of chronic oral intake study. We used 5, 50 and 500 ppm for 28 days and moderate and severe lesions in spleen were noticed in chickens treated with 50 and 500 ppm artemisinin.

Even morphometric data and histophatology revealed lesions to the cortical (vacuolization and atrophy) and medular (focal accumulation of necrotic lymphocytes) levels of bursa, concentration of sera gamma globulins and antibodies titres against sheep red blood cells were higher in chickens exposed oraly to artemisnin comparing with unexposed chickens. To notice that the highest antibodies titres and statistically significant were registered in chickens treated with 5 ppm artemisnin where the lesions were limited to a mild intercellular oedema. There are studies that revealed suppression of humoral responses by *A. annua* and its derivative (Tawfik et al., 1990; Sun, 1991). Based on our data, we conclude that chronic oral intake of low dose of artemisinin (5 ppm) was immunostimulative for humoral responses and higher doses (50 and 500 ppm) were immunotolerante, comparing with unexposed chickens.

In vitro, lymphocytes were stimulated (SI > 1.0) by LPS (mitogen that stimulate B lymphocytes) in a different manner on time, after 14 and 28 days of consumption of artemisinin; significantly higher, respectively lower than in control group. On the other side, artemisinin didn't stimulate (SI $\leq$ 1.0) the lymphocytes, causing a slightly immunosupresion *in vitro* in any doses used. Taking in consideration that concavalin A (mitogen that stimulate T lymphocytes) didn't stimulate (SI $\leq$ 1.0) the lymphocytes we can conclude that artemisinin

produced immunosupresion mainly for T lymphocytes. Histopathology of thymus revealed changes in chickens exposed to 50 and 500 ppm artemisnin: reduction in size of the thymic cortex with loosely arranged lymphocytes, focal infiltrations with heterophiles in medulla and multiple foci of cellular debris and tingible body macrophages. Many studies revealed immunosuppressive activity of artemisnin and its derivates against T lymphocytes both *in vitro* and *in vivo* (Wang et al., 2007; Li et al., 2013).

Spontaneous phagocytic activty was not affected by consuption of artemisinin, it was even significantly stimualted; when we added mitogens (concavalin A or artemisinin) to cultures the phagocytic activity was inhibited or slightly stimulated, respectively.

Conclusions

In vitro assays (phagocytosis and lymphocyte proliferation assays) revealed a slightly immunosupression, mainly for T lymphocytes, that was not supported *in vivo* (serum gamma globulins concentration and antibodies titres against SRBCs), even lymphoid organs were affected at structural level by artemisinin in high doses (50 and 500 ppm for 28 days in feed). We added the artemisinin when chickens were 18 days-old; in control of coccidiosis drugs (coccidiostats) are added to day-old chickens and it will be important to find the changes in this case.

Conflict of interest statement

There is no conflict of interest. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi: …

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525 **Table 1**

526 Criteria and methods used to evaluate the effect of artemisinin on immune system in chickens

527

Replicates[(a)]	Morphometric changes in lymphoid tissues[(b)]	Changes in concentrations of immunoglobulins[(d)]	Changes in functional activity of the immune response[(d)]	Blood[(d)]
R1	weight	serum gamma globulins	-	No additives
R2	weight, histophatology	-	cellular immune response: phagocytosis and lymphocyte proliferation assays	Heparin 50 UI/ml
R3	Weight[(c)]	-	humoral immune response: antibodies against sheep red blood cells	No additives

528 [(a)]10 chickens/replicate; [(b)]after 28 days for bursa of Fabricius, spleen, and thymus; [(c)]+5 days withdrawal; [(d)]after 0, 14 and 28 days.

Table 2

Protocol for phagocytosis assay

	Negative control	Positive control	Experimental control
*Blood+China ink (µl)	83.0	83.0	83.0
Concavalin A (µg)	-	0.5	-
Artemisinin (µg)	-	-	0.5

*250 µl heparinized blood + 0.75 µl supernatant of China ink

Figure legends

Fig.1. Weight ratio and index of lymphoid organs in broiler chickens after consumption of artemisnin for 28 days in feed and after a withdrawal period of 5 days. Control – chickens fed without artemisnin; Art5 - chickens fed with 5 ppm artemisnin; Art50- chickens fed with 50 ppm artemisnin; Art500- chickens fed with 500 ppm artemisnin. Plots filled with grey present the statistic significance: light grey - $p < 0.05$; middle grey - $p < 0.01$; and dark grey - $p < 0.001$.

Fig.2. Histopathological images of the thymus following chronically oral intake of 5, 50 and respectively 500 ppm of atemisin compared with the control group.

A, B, C and D- Low magnification images (obx10*)* of the thymus, assessing the overall morphology and the report between cortex (C) and medulla (M); an important reduction in size of the thymic cortex is observed in images C (artemisnin 50 ppm) and image D (artemisnin 500 ppm) (H&E stain, scale bar=200µm); the demarcated areas represents the zones detailed microscopically in the lower rows of histological images;

E, F, G and H-High power images (obx40 for images E and F and x100 for images G and H) of the thymus medulla and junction between medulla and cortex; Image E presents the normal morphology of the thymic medulla, the arrows indicating a medium-size Hassall's corpuscle (black arrow) and the thymic reticulo-epithelial cell (blue arrow); H&E stain, scale bar=50 µm. In images G (artemisnin 50 ppm) and H (artemisnin 500 ppm) the focal infiltrations with heterophiles (foci containing variable numbers of heterophiles) can be observed in thymic medulla (black arrows); the short arrow present a small-size Hassall's corpuscle *(*H&E stain, scale bar=20µm);

I, J, K and L-High power images (obx40 for images I, J and K and x100 for image L) of the thymic cortex, presenting an normal-sized cortex with typical aspect of densely packed, mature lymphocytes in images I (control) an J (artemisnin 5 ppm), and a narrow cortex consisting in loosely arranged lymphocytes in image K (artemisnin 50 ppm) (H&E stain, scale bar=50 µm). Image L (artemisnin 500 ppm) present multiple foci (indicated by the arrows) of cellular debris and tingible body macrophages *(*H&E stain, scale bar=20µm*).*

Fig.3. Histopathological images presenting bursa of Fabricius following chronically oral intake of 5, 50 and respectively 500 ppm of atemisin compared with the control group.
A, B, C and D- Low magnification images (obx4) of the bursal morphology and the report between the lymphoid component (bursal follicles) and stroma*;* increased amount of stroma is observed in image C (artemisinin 50 ppm) and D (artemisinin 500 ppm) (short arrows); (H&E stain, scale bar=400µm);
E, F, G and H- Detailed images (obx20) of the areas delimited by the rectangles from the upper line of images; Image E presents the normal size and morphology of the bursal follicles (delimited by the accolade); the arrows indicates the bursal interfollicular epithelium (IE); increased amount of interfollicular fibrous stroma (short arrows) is observed in image G (artemisinin 50 ppm) and H (artemisinin 500 ppm); *(*H&E stain, scale bar=100µm);
I, J, K and L-High power images (obx40) of the bursal follicles, presenting an normal morphology of the follicles with their cortex, medulla and corticomedullary epithelium (follicle associated epithelium-FAE), with typical loosely packed lymphocytes in the medulla and more dense disposition in the cortical area. In image J-5ppm (detailed of the area delimited by the small rectangle from image B) a mild intercellular edema (indicated by the arrow) can be observe. In image K (artemisinin 50 ppm), a slight increment of interfollicular fibrous stroma, vacuolization of the cortical zone of the follicles (blue arrow) and focal

accumulation necrotic lymphocytes in the medulla (black arrow) of follicles can be observed. In image L (artemisinin 500 ppm), increased amount of interfollicular fibrous stroma (short arrow), atrophied cortex (black arrow) and evident corticomedullary epithelium (blue arrow) can be observed; *(*H&E stain, scale bar=50µm).

Fig.4. Histopathological images of the spleen following chronically oral intake of 5, 50 and respectively 500 ppm of atemisin compared with the control group.

A, B, C and D- Low magnification (obx4) images of the spleen, assessing the overall morphology and the amount of white and red splenic pulp; for image B (artemisnin 5 ppm), a mild reduction in volume of the red pulp can be observed; important reduction in the overall size of the white pulp is observed in images C (artemisnin 50 ppm) and image D (artemisnin 500 ppm) (H&E stain, scale bar=400µm); the demarcated zones represents the areas detailed microscopically in the lower rows of images;

E, F, G and H- images (ob x20) of the splenic white and red pulp presenting the size and cellularity of the peri-ellipsoid lymphocyte sheath (PELS)(indicatded by blue arrow in image E), peri-arteriolar lymphocyte sheath (PALS, indicatded by blue arrow in Image F) and the cerminal centers (GS, indicated by the black arrows from images E and F). The normal structure of the previously named structures is presented in image E. In image G (artemisnin 50 ppm) a sever vacuolization of the marginal lymphoid tissue can be observed, morphologic change which is also present in a reduced amount in the splenic red pulp. In image H (artemisnin 500 ppm) the severe depletion of the PALS is observed (marked by the black arrows); the vacuolar aspect is present in both white and red pulp; *(*H&E stain, scale bar=100µm).

I, J, K and L- detailed microscopic images (ob x20 for image I and J, obx40 for image K and L) of the splenic white and red pulp presenting, presenting an normal morphology in image I

(control) and a mild depletion of the marginal lymphoid tissue and PALS in image J (artemisnin 5 ppm). Multiple foci of cellular debries (indicated by the black arrows), giving the overall vacuoar aspect of white and red pulp can be obserde in images K (artemisnin 50 ppm) and L (artemisnin 500 ppm). In image L,also the depletion of the PALS and marginal lymphoid tissue can be observed; (H&E stain, for images I and J scale bar=100µm, for images K and L bar=50µm).

Fig.5. Concentration of sera gamma globulins (g/dl) in broiler chickens fed with artemisnin comparing with chickens fed with standard feed. Control – chickens fed without artemisnin; Art5 - chickens fed with 5 ppm artemisnin; Art50- chickens fed with 50 ppm artemisnin; Art500- chickens fed with 500 ppm artemisnin. Plots filled with grey present the statistic significance: light grey - $p < 0.05$; middle grey - $p < 0.01$; and dark grey - $p < 0.001$.

Fig.6. Phagocytic activity of blood cells expressed as the difference between optic densities (absorbance) read at 0 and 30 minutes after incubation of blood samples with China ink. Control – chickens fed without artemisnin; Art5 - chickens fed with 5 ppm artemisnin; Art50- chickens fed with 50 ppm artemisnin; Art500- chickens fed with 500 ppm artemisnin. Plots filled with grey present the statistic significance: light grey - $p < 0.05$; middle grey - $p < 0.01$; and dark grey - $p < 0.001$.

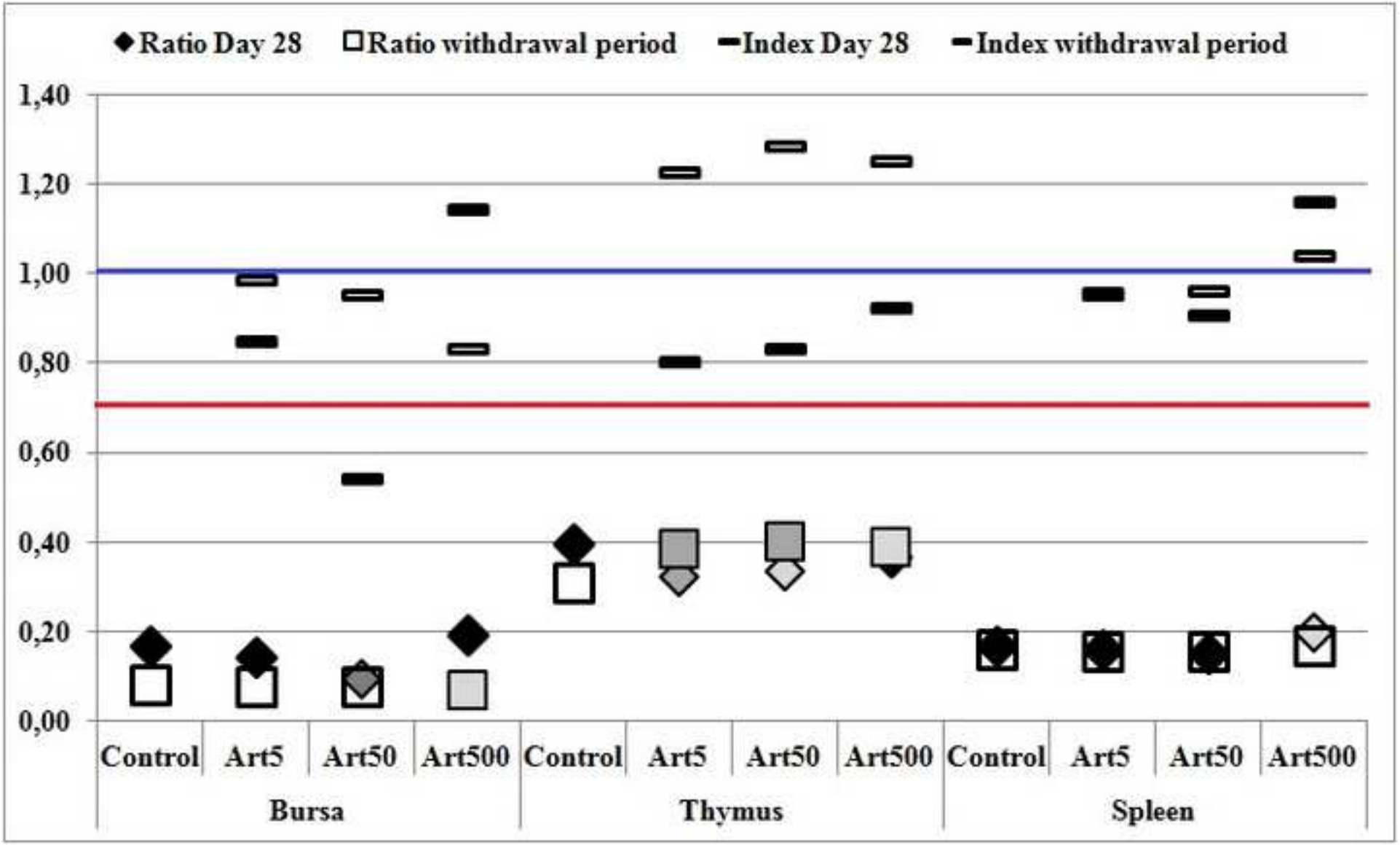
Fig.7. Stimulation index of lymphocyte in broiler chickens fed with artemisinin. Control – chickens fed without artemisnin; Art5 - chickens fed with 5 ppm artemisnin; Art50- chickens fed with 50 ppm artemisnin; Art500- chickens fed with 500 ppm artemisnin. Plots filled with grey present the statistic significance: light grey - $p < 0.05$; middle grey - $p < 0.01$; and dark grey - $p < 0.001$.

633 Fig.8. Antibodies titres (geometric mean) against sheep red blood cells in broiler chickens fed
634 with artemisinin. Control – chickens fed without artemisnin; Art5 - chickens fed with 5 ppm
635 artemisnin; Art50- chickens fed with 50 ppm artemisnin; Art500- chickens fed with 500 ppm
636 artemisnin. Plots filled with grey present the statistic significance: light grey - $p < 0.05$;
637 middle grey - $p < 0.01$; and dark grey - $p < 0.001$.

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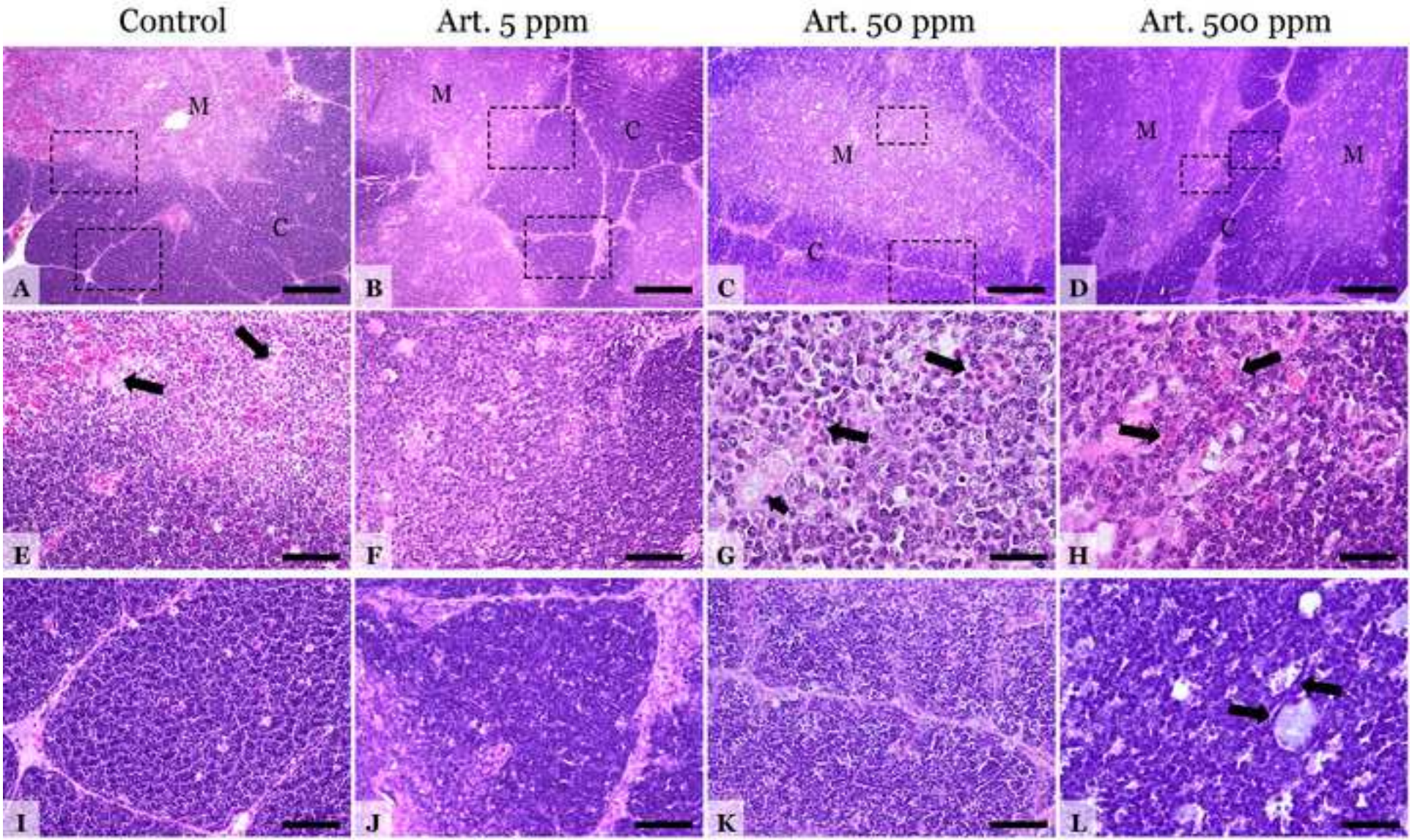
Weight ratio and index of lymphoid organs

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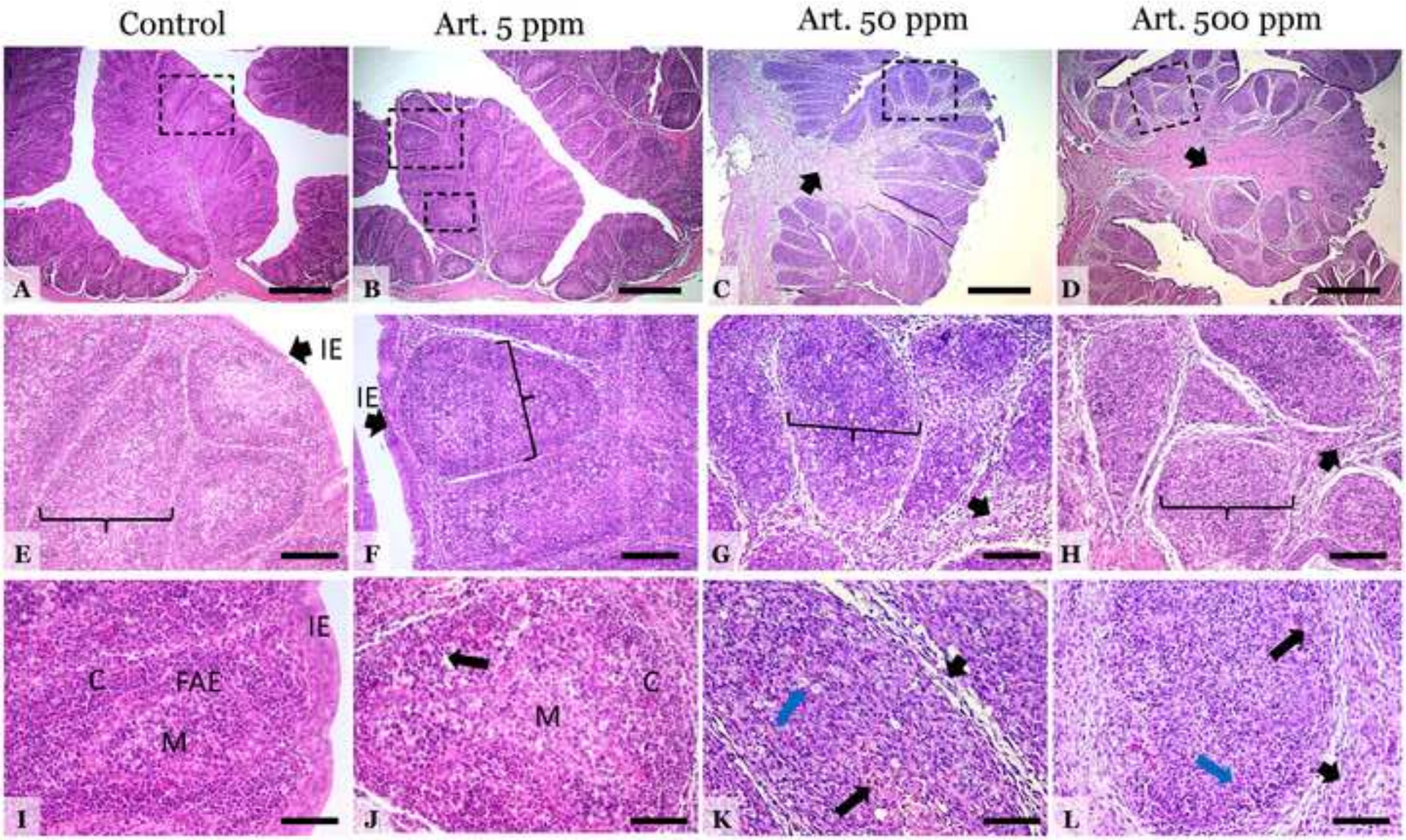
Thymus

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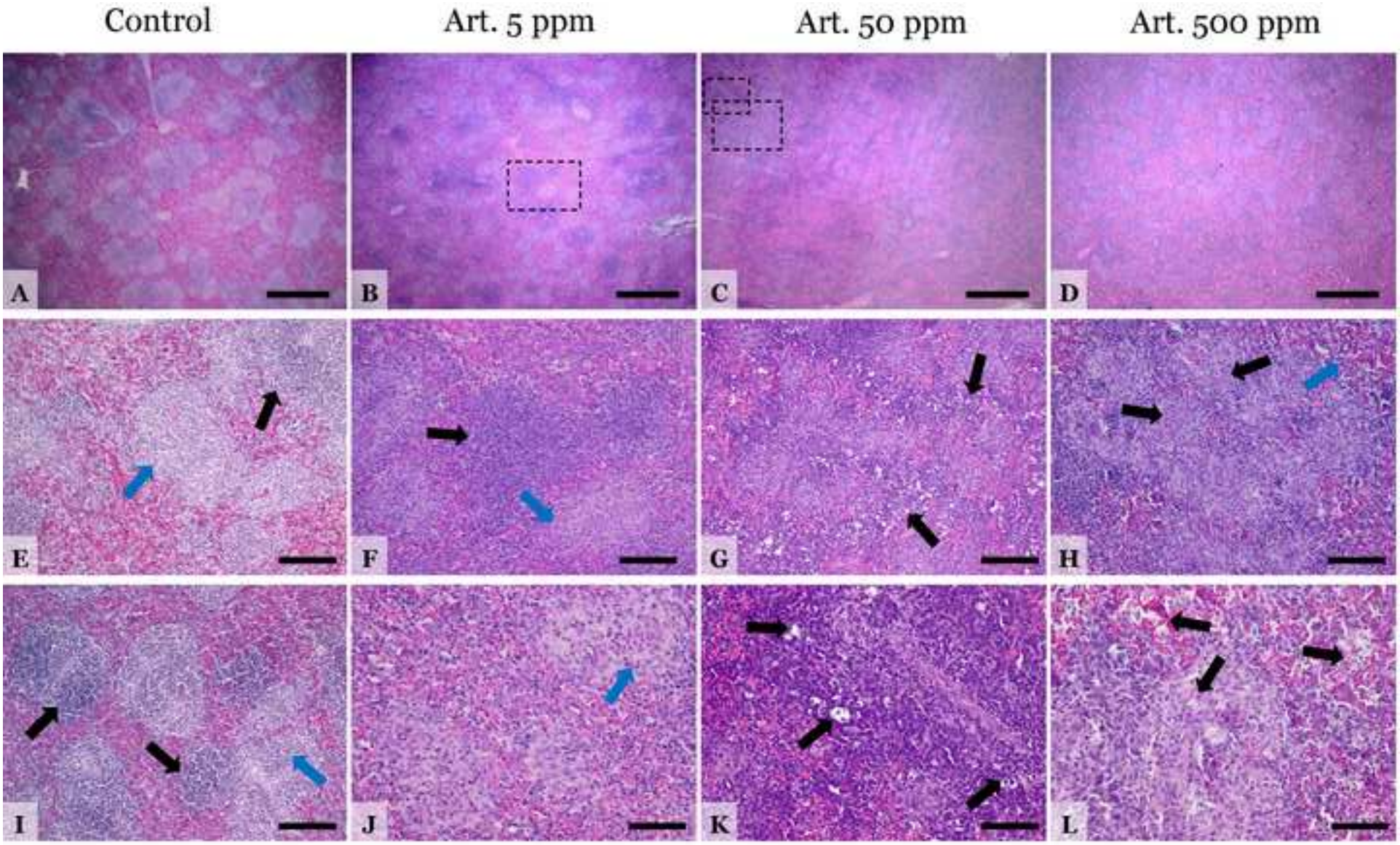
Bursa

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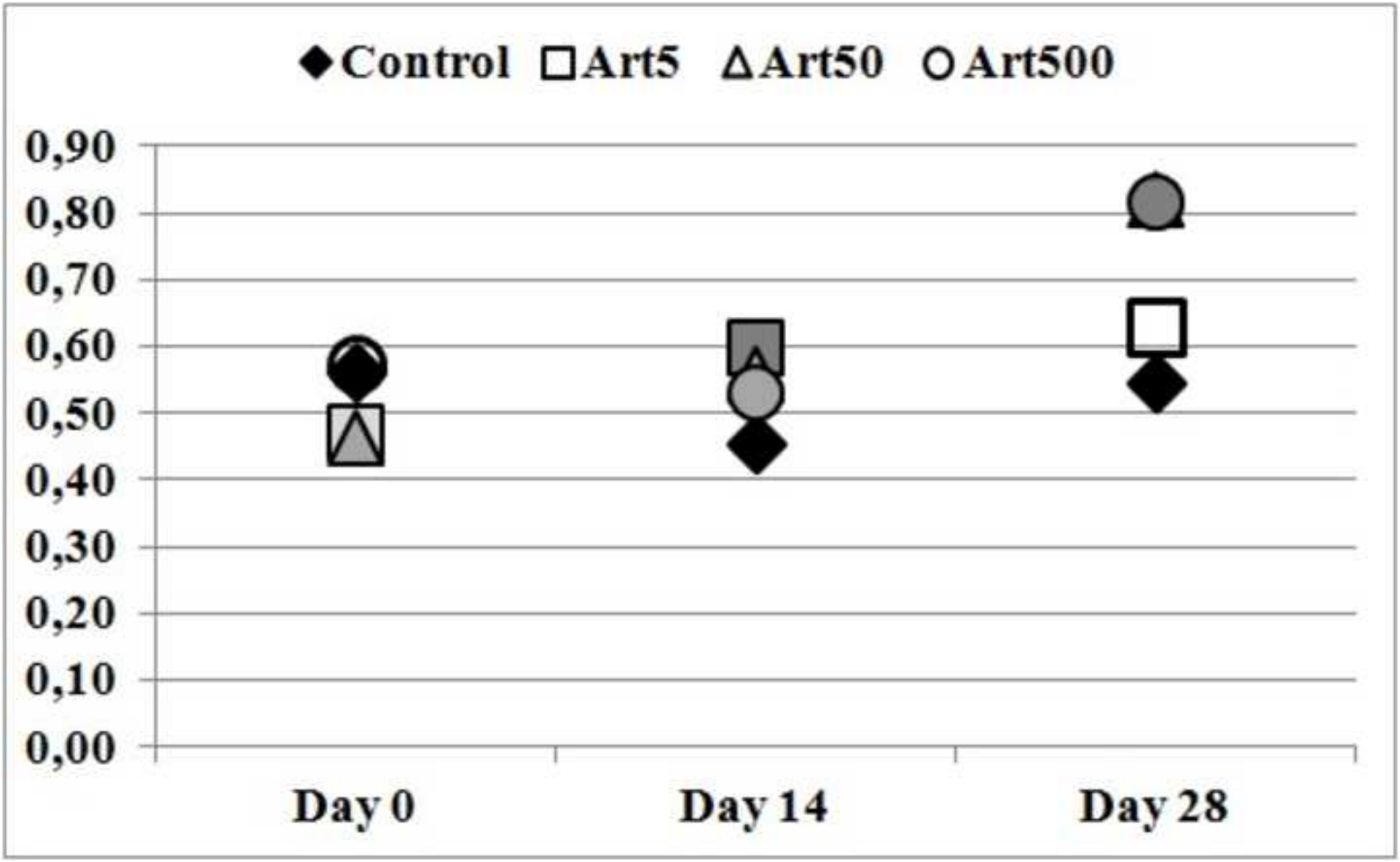
Spleen

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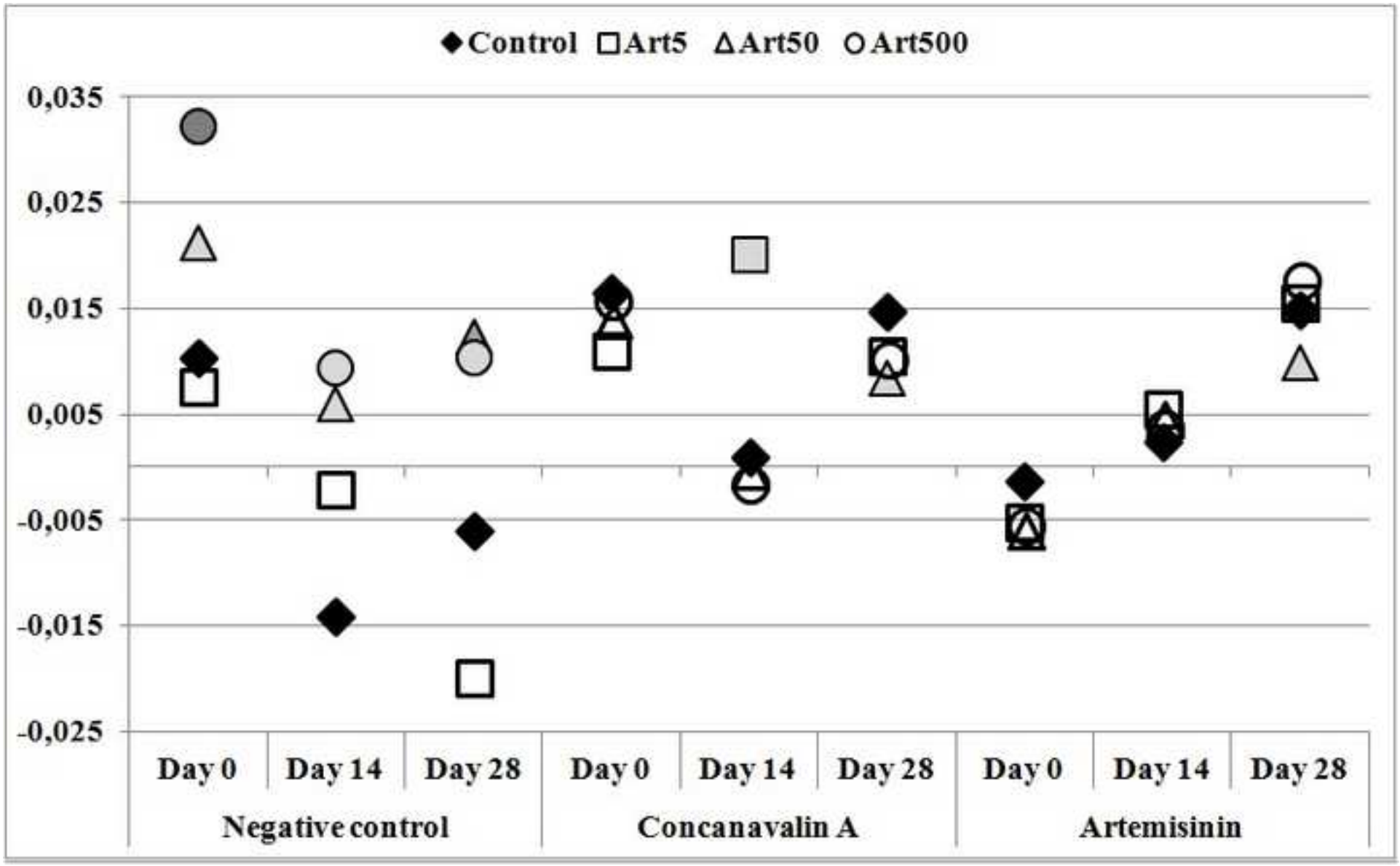
Sera gamma globulins (g/dl)

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Phagocytic activity of blood cells

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Stimulation index of lymphocyte

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