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Original article

Metabolic and tissular effects of artemisinin supplemented diets in broiler chicken

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Abstract

Artemisinin is a powerful antimalarial drug, useful in the treatment of many diseases, including chickens coccidiosis. Its toxic effects have been well studied in humans and experimental animals, but not sufficiently in broiler chickens. Therefore, in the present study, we aimed to assess the side effects of artemisinin in chickens, by measuring the serum level of proteins and enzymes (ALT, AST, GGT, ALP, CK), by histopathological examination and by the evaluation of relative weight of organs (liver, kidney, heart). Artemisinin was administered in the standard feed for chickens in three different concentrations: 5, 50 and 500 ppm.

Each concentration of artemisinin increased the total serum proteins, gamma-globulins and the serum activity of CK and decreased the serum ALP level. The values of ALT and GGT were higher in the chickens treated with 50 and 500 ppm of artemisinin. Multifocal liver necrosis and inflammatory infiltrate were detected in the chickens that received the 50 and 500 ppm dosage of artemisinin. Minimal tubular necrosis, renal tubular epithelium vacuolation, multifocal interstitial nephritis and mild uric nephrosis were detected in chickens treated with the drug. Artemisinin administration produced no significant changes in the organs relative weight.

Artemisinin, at a concentration of 5 mg/kg of feed is well tolerated by broiler chickens, but the concentrations of 50 and 500 mg/ kg feed can produce toxic effects.

Keywords: artemisinin, chickens, toxicity, biochemistry, histology

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Introduction

Artemisinin is a naturally occurring sesquiterpene found in *Artemisia annua* L., first discovered in the 1970's and later introduced as an alternative treatment for malaria caused by the multidrug-resistant protozoan parasite *Plasmodium falciparum* (Miller and Su 2011).

Aside from the antimalarial activities, artemisinin has been found to have additional health benefits including antitumoral, antibacterial, antiviral, antifungal or anti-inflammatory properties (Ho et al. 2014). Antiparasitic effects, including activity on coccidiosis in broiler chickens was previously reported. This parasitic disease produces major economic losses worldwide, despite the use of in-feed anticoccidials, therefore many researchers have begun focusing on alternative treatments as artemisinin (Allen el al. 1997, Arab et al. 2006, Mo et al. 2014).

Besides the beneficial effects in therapy, artemisinin may cause toxic reactions (Efferth and Kaina 2010). Artemisinin administration can produce hormonal imbalance that may adversely affect the ovulation process in rats, or induce reproductive toxicity in male rats, that could result in infertility (Farombi et al. 2014, 2015). Anemia due to artemisinin administration has been reported in various species, including humans (Rehman et al. 2014, Yang et. al. 2015). In general, all of these side effects occur following prolonged administration of artemisinin.

In broiler chickens, there have been few reports of artemisinin toxicity in the liver, kidney, brain or hematological system (Arab el al. 2009, Shahbazfar et al. 2011, Pop et al. 2017), but the information is scarce.

Some proteins and enzymes are useful diagnostic markers of hepatocellular and hepatobiliary lesions in poultry (Subapriya et al. 2007, Kraljevic et al. 2008). Injuries of the hepatobiliary system can lead to the GGT and ALP increase (Subapriya et al. 2007). Serum aIn experimental rats treated with artemisinin and folic acid combination, serum activity of AST increased proportionally with the dose, however, serum ALT concentration was inversely proportional to the concentration of the administrated drug (Udobre et al. 2009).

In this context, the present study aimed to evaluate the side effects of different dosages of artemisinin in chickens by measuring the serum level of proteins (total proteins, gamma globulin and albumin), enzymes (AST, ALT, GGT, ALP, CK), relative weight of internal organs (liver, kidney, heart) and by histopathological evaluation of liver, myocardium and kidneys.

Materials and Methods

Ethics statement

Veterinary conditions regarding the protection of animals used in this research are compiled according to national standards and legislation. All experiments were approved by the Research bioethics Commission of USAMV Cluj-Napoca, Romania (Registration no. of approval of application: 4/19.09.2013).

Study design

Two hundred, one-day-old broiler chickens (hybrid Cobb 308) were purchased from a commercial facility (S.C. VISAVIS S.A., Vadul Crisului, Bihor county, Romania) and kept in battery cages (10 chickens/cage) until the age of 17 days. Chickens were fed standard starter with no anticoccidial drugs until the start of the experiment, and water was provided *ad libitum* with a permanent lighting period.

After weighing, eighteen-day-old chickens were randomly divided in 4 experimental groups, each containing 20 chickens, separated into 4 replicates of 5 chickens (2 replicates with females and 2 with males). The experimental groups were divided as follows: C – the control group, fed without anticoccidial drug, and three experimental groups, Art5, Art50 and Art500, fed with artemisinin supplement. The feed intake was measured daily, during the whole experimental period.

Artemisinin administration

Artemisinin (Intatrade Chemicals GmbH, cat. no. NN04008, Germany, purity min. 98%), was administrated continuously in chickens' feed starting from day 18 for 28 days (until the chickens reached 46-days old) in the following dosages: 5 ppm (5 mg artemisinin/kg feed) for Art5 group, 50 ppm for Art50 group and 500 ppm for Art500 group. The diet's composition is described in a previously published article (Pop et al. 2017).

Collection of blood and serum preparation

On days 0, 14 and 28 of the experiment blood (0.5-1.0 ml) was collected aseptically from the ulnar vein of 10 chickens/group (5 females and 5 males) into 1.5 ml Eppendorf tubes without additives using a 2.0 ml syringe and 23G needle,. Serum was obtained after blood coagulation at room temperature for 2 hours and centrifugation at 3000 rpm for 5 minutes. Sera were stored at -20C until further analysis.

Blood biochemistry

The side effect of artemisinin in chickens was assessed by evaluating the level of serum enzymes (ALT, AST, GGT, ALP, CK) and proteins (total proteins, albumins and gamma-globulins) in the blood on day 0, 14 and 28. These biochemical constituents were determined by colorimetric and turbidimetric methods, and by reading the molecular absorption spectrophotometrically on a ScreenMaster Touch® analyzer using commercial kits (Hospitex Diagnostics S.r.l., Italy) and following the manufacturer's instructions. The following methods were used:

- kinetic methods: IFCC, reading at 340 nm, for ALT (Hospitex reference: 4001755L) and AST (ref. 4001785L), Szasz at 405 nm for GGT (ref. 4001703L), DGKC at 405 nm for ALP (ref. 4001702L) and DGKC/IFCC at 340 nm for CK (ref. 4001853L);
- biuret method at 540 nm for total proteins (ref. 4001950L);
- bromocresol green method at 628 nm (580 - 630) for albumins (ref. 4001010L);
- turbidimetric method with 18.5% Na_2SO_4, reading at 546 nm for gamma-globulins.

Relative weight and histopathology of organs

On day 46, chickens were weighed (g) and then euthanized by cervical dislocation. Organs (heart, liver and kidney) were removed, weighed, and their relative weights (organ weight in grams/body weight in grams x 100) were calculated.

Multiple tissues from 10 chickens in each group were trimmed in 5 mm thick samples, immediately fixed in 10% neutral-buffered formalin for 72h and further embedded in paraffin wax following a routine protocol. Tissue sections were automatically stained (Sakura DRS 601 slide strainer) by hematoxylin and eosin (H&E) and examined under a light microscope (Olympus BX41). Images were taken using an Olympus UC30 camera and finally processed by Olympus Stream Basic image analysis software.

Statistical analysis

The data obtained from blood biochemistry and organs weight were expressed as mean±SD. First, data were checked for normality using D'Agostino-Pearson test. Then, analysis of variance (ANOVA) was done by one-way and repeated measures ANOVA followed by Tukey-Kramer multiple comparison test. If the data were not normally distributed, Kruskal-Wallis test was applied followed by Dunn's multiple comparison test. $P \leq 0.05$ was taken to be statistically significant. All statistical analysis was performed with Medcalc software version 17.9.7.

Results

Feed intake

The chickens treated with 5 ppm of artemisinin consumed a higher amount of feed than the control group, but the chickens from Art500 group had a lower feed intake compared with the unmedicated group (Pop et al. 2017).

Blood biochemistry

The activity of ALT differed significantly only in the group that received 50 ppm of artemisinin after two weeks of drug administration, but in the end of the experiment ALT activity was similar to that of the control group. In dynamics, the groups treated with 5 and 50 ppm of artemisinin as well as the control group had significantly increased AST activity in the final day of the experiment compared with the other 2 samplings. The GGT activity was increased in all artemisinin treated groups compared with the control group in the initial day of the experiment, but after 14 and 28 days of drug administration the activity of GGT was similar to that found in the control group. Inall the groups, the GGT activity increased gradually from the first day of the experiment until the last. For the group treated with 50 ppm of artemisinin, the ALP activity was significantly decreased in the final determination compared with the control group. However, in all the groups, the activity of ALP significantly decreased from the first determination until the last. The groups treated with 5 and 50 ppm of artemisinin registered lower levels of creatine kinase compared with the control group in the initial determination, but in the second determination all the groups had significantly increased levels of CK compared with the other two blood samplings. The concentration of total proteins was similar in all groups, but in dynamics the artemisinin treated groups recorded a higher concentration in the final determination compared with the other two samplings ($P < 0.05 – 0.01$). The albumin concentration did not differ significantly between groups, but in the second determination a higher concentration of albumins was recorded in all the groups compared with the first and third determination. The gamma globulin concentration was similar in all the groups in the initial determination. For the group treated with 50 ppm of artemisinin in the second and also the third blood sampling the concentration of gamma globulins was higher compared with the control group. This was also

Table 1. The effects of artemisinin on the biochemical constituents of broiler chickens.

	Control			Art5			Art50			Art500		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
ALT (U/l)	23.34 ±3.31 ^{a,d}	19.05 ±1.58 ^{a,de}	14.98 ±1.52 ^{a,e}	20.19 ±4.99^{a,d}	19.07 ±1.05^{a,d}	16.68 ±2.24^{a,d}	23.51 ±2.77^{a,d}	36.62 ±3.65	15.34 ±1.88^{a,d}	19.39 ±2.29^{a}	13.69 ±0.99^{a}	27.43 ±11.57^{a}
AST (U/l)	197.76 ±11.61 ^{ab,d}	167.75 ±3.62^{a,d}	279.43 ±20.74^{a}	170.67 ±10.24^{a,d}	179.2 ±13.1^{ab,d}	271.09 ±17.44^{a}	188.14 ±10.9^{ab,d}	215.35 ±11.79^{b,d}	265.51 ±16.46^{a}	231.27 ±19.8^{b}	183.02 ±37.65^{ab}	220.99 ±16.57^{a}
GGT (U/l)	15.53 ±1.02	22.79 ±1.41^{a}	30.03 ±2.66^{ab}	19.42 ±0.83^{a,d}	25.55 ±2.25^{a,de}	27.64 ±2.38^{a,e}	19.2 ±1.21^{a}	29.33 ±2.48^{a,d}	27.46 ±2.21^{a,d}	22.33 ±1.4^{a,d}	30 ±2.69^{a,de}	38.54 ±3.72^{b,e}
ALP (U/l)	15541.5 ±723.94^{a,d}	11772.25 ±1157.8^{a,de}	7762.28 ±1008.8^{a,e}	16186.6 ±768.88^{a}	10342.4 ±1033.1^{a}	5469.1 ±766.89^{ab}	14646 ±875.03^{a}	10985.7 ±1537.4^{a}	3796.9 ±460.72^{b}	15371 ±504.81^{a}	10510.3 ±1172.4^{a}	4907.2 ±857.39^{ab}
CK (U/l)	100.7 ±11.77^{a}	257.51 ±26.92^{a}	34.53 ±4.21^{a}	53.94 ±9.13^{b,d}	313.4 ±20.52^{a}	36.81 ±5.86^{a,d}	45.66 ±3.6^{b,d}	433.2 ±135.37^{a}	46.88 ±9.12^{a,d}	75.41 ±6.41^{ab,d}	296.98 ±36.28^{a}	40.04 ±5.87^{a,d}
PROT (g/dl)	3.53 ±0.12^{a,d}	3.32 ±0.13^{a,d}	3.76 ±0.24^{a,d}	3.04 ±0.12^{a,d}	3.47 ±0.19^{a,de}	4.06 ±0.23^{a,e}	3.27 ±0.15^{a,d}	3.52 ±0.12^{a,de}	4.16 ±024^{a,e}	3.36 ±0.17^{a,d}	3.53 ±0.1^{a,de}	4.08 ±0.18^{a,e}
ALBUM (g/dl)	1.15 ±0.08^{a,,d}	1.45 ±0.03^{a}	1.01 ±0.05^{a,d}	0.92 ±0.05^{a}	1.39 ±0.05^{a,d}	1.26 ±0.05^{a,d}	1.09 ±0.04^{a,d}	1.42 ±0.06^{a}	1.19 ±0.08^{a,d}	1.11 ±0.06^{a,d}	1.46 ±0.02^{a}	1.2 ±0.09^{a,d}
GammaG (g/dl)	0.59 ±0.03^{a,d}	0.46 ±0.02^{a,e}	0.55 ±0.04^{a,de}	0.5 ±0.03^{a,d}	0.56 ±0.04^{b,d}	0.63 ±0.06^{ab,d}	0.43 ±0.02^{a,d}	0.62 ±0.04^{b,d}	0.82 ±0.06^{bc}	0.56 ±0.05^{a,d}	0.53 ±0.02^{ab,d}	0.85 ±0.06^{c}

Values with no common superscript differ significantly (a, b, c: between groups, for each day; d,e,f: inside group, in dynamics). Results are expressed as means ± SEM.
Control – group fed without anticoccidial drug; Art5, Art50, Art500 – experimental groups supplemented with artemisinin in concentrations of 5, 50 and 500 ppm;
ALT – alanine transaminase, AST – aspartate transaminase, GGT – gamma-glutamyl transferase, ALP – alkaline phosphatase, CK – Creatine kinase; PROT – total proteins; ALBUM – albumins; Gamma G – gamma-globulins

the case for the group treated with 5 ppm in the second determination and the group Art500 in the third determination (Table 1).

Histopathology and relative weight of organs

The liver histopathology revealed different degrees of hepatocyte steatosis in all groups treated with artemisinin as well as in the control group. However, these lesions were encountered only in 3 chickens from the Art50 group compared to 7 chickens in the control group, 8 chickens in the Art5 group and 6 chickens in the Art500 group. In the absence of a consistent difference between the groups, this observation could mostly be interpreted as individual changes due to different food intake or energy consumption and not related to the tested compound. However, in one chicken from each of the groups treated with 50 and 500 ppm of artemisinin, multifocal liver necrosis with inflammatory cell infiltration was also observed (Fig. 1). Extramedullary hematopoiesis was also found in all the groups, as an incidental finding, in 2 chickens from the Art500 group, and 3 chickens from the other groups.

There were no lesions in the heart in the control group or the groups treated with 50 and 500 ppm of artemisinin. Minimal multifocal and perivascular heterophilic infiltration, and infiltrate between myocytes were observed in two chickens from the Art5 group (Fig. 2).

Minimal acute tubular necrosis/renal cell apoptosis were observed in 3 chickens from the group Art500 and control group, and 4 chickens from the group Art5. Renal tubular epithelial cells vacuolation was observed in 2 chickens from the control and Art5 groups, a discreet proteinaceous material was detected in 1 chicken from the control group and 2 chickens from Art50 group, and extramedullary hematopoiesis was observed in 1 chicken treated with 500 ppm artemisinin. Besides this, mononuclear interstitial nephritis was observed in 1 chicken from the groups Art5 and Art50. One chicken treated with 5 ppm of artemisinin and 3 chickens treated with 50 ppm of artemisinin also had multifocal uric nephrosis (Fig. 3).

There were no significant differences in organ relative weight between the groups treated with artemisinin and the control group for none of the organs evaluated (Table 2).

Discussion

Previous research studies showed that the intense metabolism associated with the rapid growth rate of broiler chickens requires an increased hepatic protein synthesis as source of amino acids, especially necessary

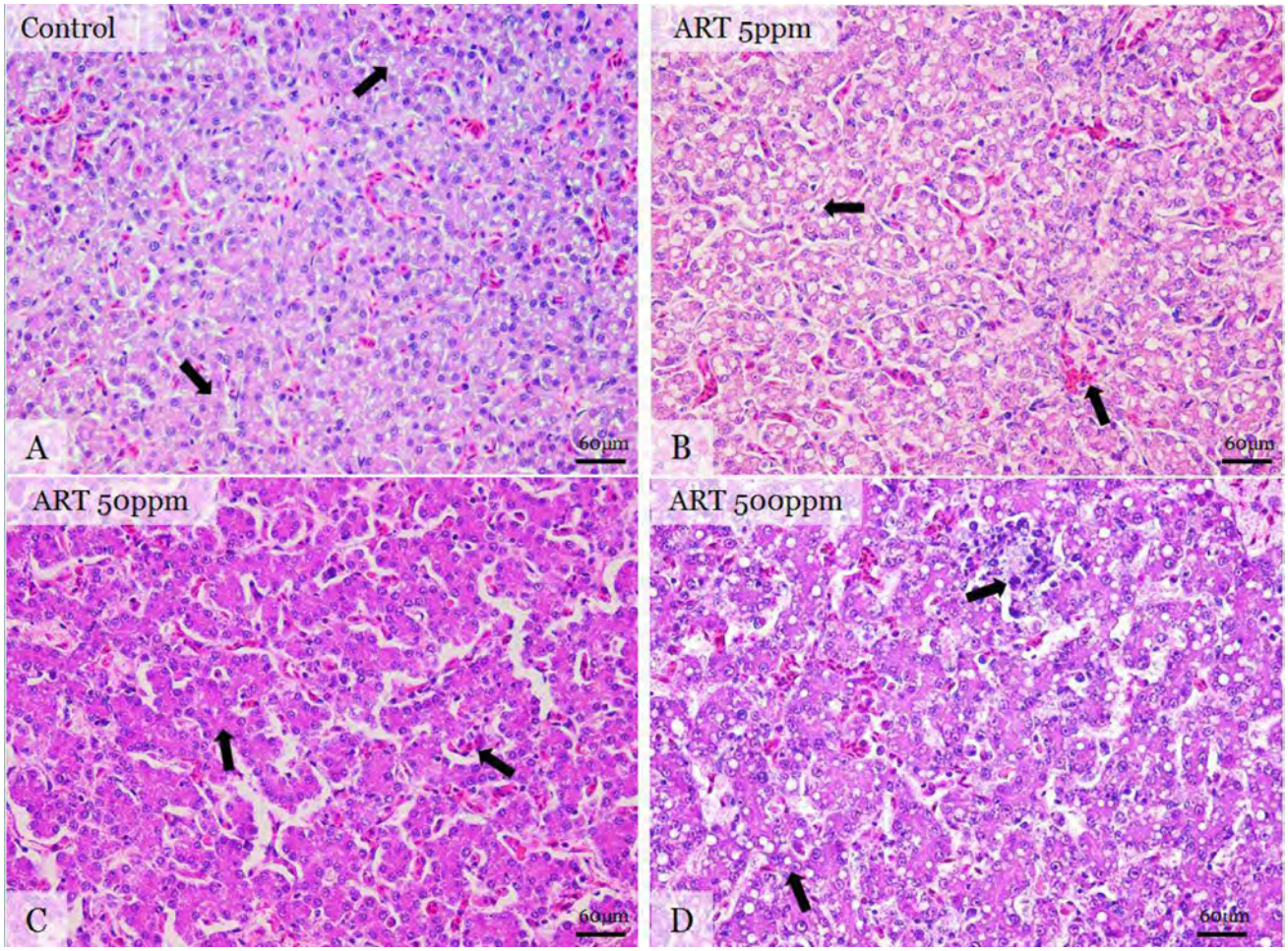

Fig. 1. Histopathology of the liver collected from the three groups compared to the controls. In images A and C the arrows indicate the normal appearance of hepatic chords and sinusoids. For the groups who received 5 ppm and 500ppm respectively, arrows indicate the mild hepatocyte steatosis and a minute focus of hepatocyte necrosis and leukocyte infiltration (the upper arrow from image D). Hematoxylin and eosin, x40.

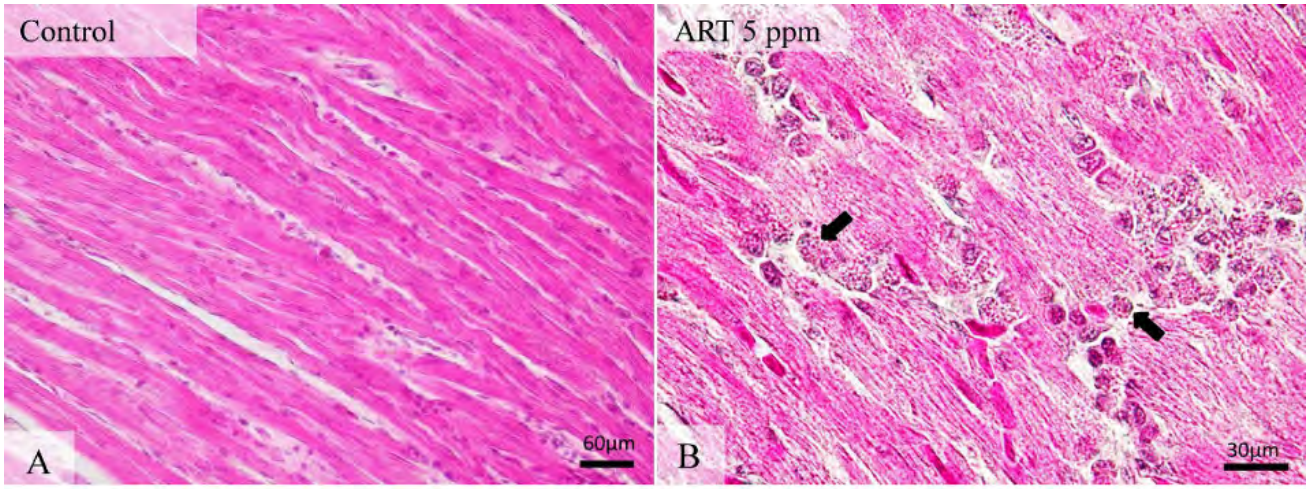

Fig. 2. Histopathology of the myocardium, showing a minimal, focal, myocardial infiltration with heterophils (indicated by arrows) in the ART 5 group. HE, x40 for image A and, respectively, x100 for image B.

for skeletal muscle development (Filipovic et. al. 2007). Consequently, the impairment of the metabolic function of the liver would be timely reported by the decrease in serum level of total proteins. The present results would indicate that the given dosages of artemisinin do not inhibit the hepatic synthesis of total proteins.

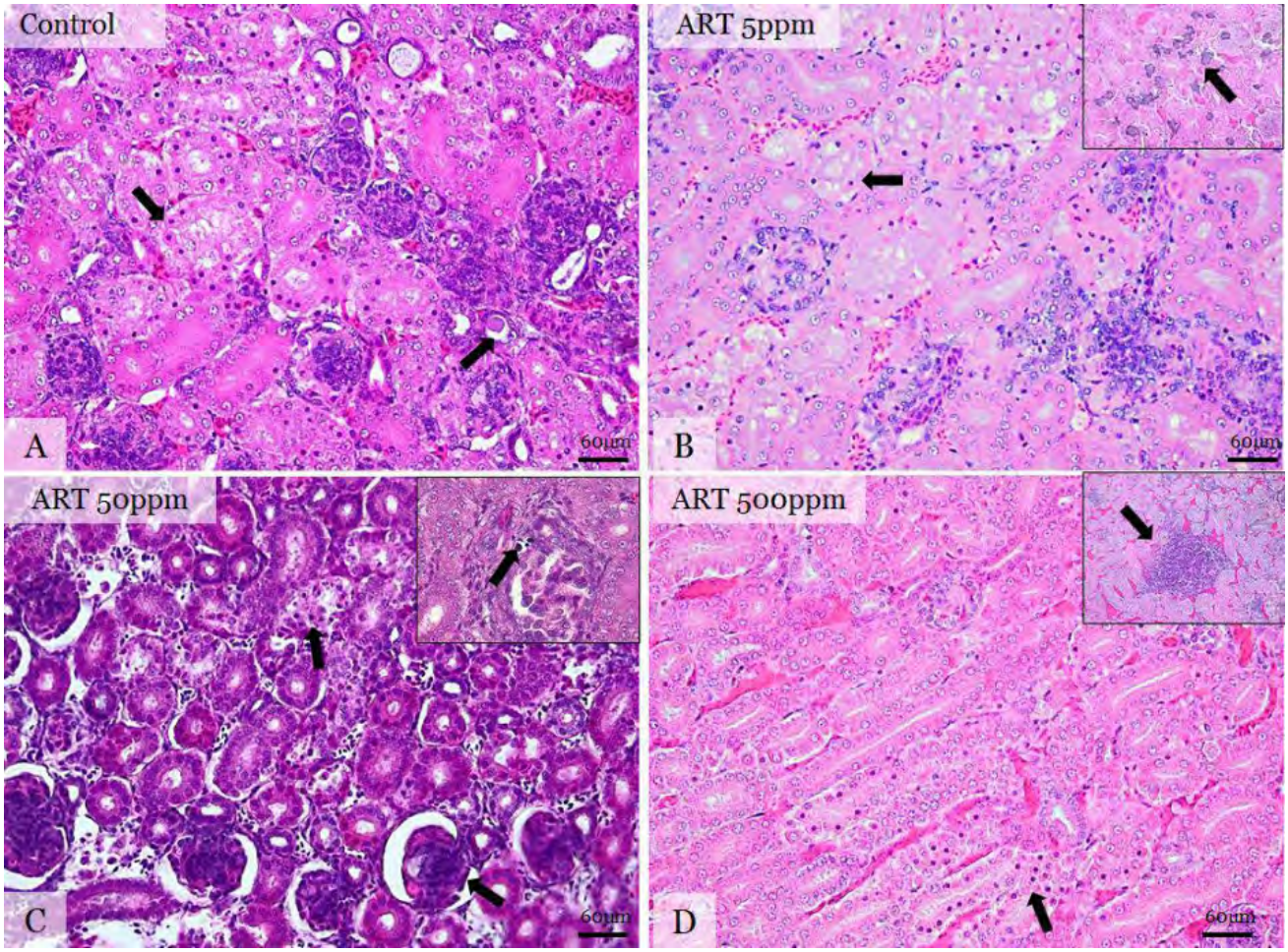

Fig. 3. Histopathology of the renal cortex in the three experimental groups and control group, showing epithelial cell vacuolation and necrosis in the proximal tubules, with occasional desquamation (image C). Arrows from the insets indicate focal mineralization of the epithelium and interstitium (minimal finding)(image B and C) and a focus of extramedullary hematopoiesis (image D). HE, x 40.

Table 2. Weight of broiler chickens organs following treatment with artemisinin compared with the control group.

	Body weight (g)	Heart		Liver		Kidney	
		Weight (g)	Relative weight (%)	Weight (g)	Relative weight (%)	Weight (g)	Relative weight (%)
Control	1659.1±48.34 ^{a}	7.59±0.35 ^{a}	0.46±0.02 ^{a}	42.80±1.51 ^{ab}	2.61±0.11 ^{a}	11.11±0.45 ^{a}	0.67±0.03 ^{a}
Art5	1717.0±52.91 ^{a}	7.37±0.26 ^{a}	0.43±0.01 ^{a}	45.11±1.44 ^{a}	2.65±0.09 ^{a}	10.49±0.42 ^{a}	0.61±0.02 ^{a}
Art50	1507.5±56.90 ^{ab}	6.57±0.30 ^{a}	0.44±0.02 ^{a}	41.44±2.07 ^{ab}	2.77±0.12 ^{a}	10.40±0.46 ^{a}	0.70±0.04 ^{a}
Art500	1314.8±42.77 ^{b}	6.45±0.29 ^{a}	0.49±0.02 ^{a}	35.19±1.53 ^{b}	2.68±0.09 ^{a}	9.27±0.32 ^{a}	0.71±0.03 ^{a}

Different superscripts in one row indicate statistical difference ($p<0.05$); Results are expressed as means±SEM

However, the albumin synthesis appears to be negatively influenced after 28 days of drug administration. This appears to be compensated by an increase in gamma globulins , thus the serum level of total proteins maintains the upward trend described. However, the gamma globulin levels in group Art50 and Art500 exceeded the reference values [0.416±0.072 (Filipovic et. al. 2007)], which suggests a reaction of hepatic parenchyma in groups treated with a higher dose of artemisinin. Besides this, the identified hepatic lesions were more severe in the groups medicated with 50 and 500 ppm of artemisinin. Fatty appearing pale liver, distended gallbladder, enlarged kidneys, urate deposits in the ureters and increased mortality were observed in turkeys supplemented with artemisinin (Thøfner et al. 2012), but the dosage used was higher (2600 mg/kg feed) compared to that found in our study.

The recorded values of serum enzymes in group Art5 indicate that artemisinin did not induce disruptions at hepatocellular and hepatobiliary level at this lower

dose. These findings are supported by the histopathological findings. However, the increased level of AST and GGT in the control group after 28 days of medication shows the existence of liver dysfunction which is unrelated to the administration of artemisinin. The possible explanation could be the occurrence of enzymatic induction under the effect of trace amount of mycotoxins, considering that mycotoxicological analysis of the basic feed has not been performed. This possibility has also been reported in previous research studies on the biochemical blood constituents in broiler chickens (Silva et al. 2007). The mycotoxins could also be the cause of the mild hepatic lesions observed even in the group fed with the standard diet. The renal lesions observed regardless of the artemisinin administration, set the presence of a concomitant acting toxic substance. However, weights of the liver and kidney were similar in the untreated and treated chickens.Arab et al. (2009) showed that the administration of a single oral dose of artemisinin in broiler chickens produces hepatic and kidney degeneration and bile retention which are independent of the used drug dose. However, the authors used an alcoholic solution given directly by a crop tube, consequently most of the doses were higher than those employed in our study. Shahbazfar et al. (2011) observed the same pathological changes in the liver and kidney of broiler chickens as those found in the previous mentioned study, and they used similar dosages as used in the present study. These findings are in accordance with our results for the chickens treated with 50 and 500 ppm of artemisinin.

In the present study, a significant increase in ALT was recorded after 14 days of drug administration in the chickens supplemented with 50 ppm or artemisinin, but not in those who have received the 500 ppm dose of the drug. Seemingly paradoxical, the hepatocytes respond to a higher dose of artemisinin through a diminution of ALT release from cytosol. This behavior cannot be interpreted as an absence of dietary toxicity of the 500 ppm dose, but rather a reduction of enzyme synthesis or its inactivation might be suspected, as an effect of the increasing artemisinin concentration. Similar effects were recorded in radiation-induced hepatic lesions in broilers (Kraljević et al. 2008). Subsequently, ALT peaked after 28 days of 500 ppm artemisinin administration. This phenomenon was associated with the hepatocyte necrosis identified at the necropsy. These facts were supported by the elevation of GGT enzyme levels. In this context, ALT and GGT enzymes showed the hepatoxicity of higher dosages of artemisinin. In a study on the administration of artemisinin in dogs with tumors, no biochemical toxicity was observed, only the level of ALT had increased and just in two specimens, and therefore, was considered clinically irrelevant (Hosoya et al. 2014). Alkaline phosphatase has not been influenced by the artemisinin administration. One possible explanation could be its localization in the biliary pole of hepatocytes and in the biliary duct epithelium that facilitates its release with predominance in bile and less toward sinusoidal capillaries. However, statistically significant decrease inalkaline phosphatase was recorded for all the groups after 14 and 28 days of drug administration. This trend was associated with the reduction in phosphocalcic bone metabolism as the chickens get older and bone growth decreases(Silva et al. 2009). Therefore, the reduction in the serum activity of ALP in all artemisinin treated chickens occurred more likely from the diminishing of the bone ALP isoenzyme serum activity rather than of hepatobiliary enzyme. Significant changes in the CK levels occurred after 14 days of artemisinin administration. In this period, skeletal muscles of broilers reach the maximum development and the increased values of CK and partially AST are either results of abnormal permeability of sarcolemma or even of own sarcolemma lesions (Szabo and Milisits 2007). Therefore, it is unlikely that artemisinin is responsible for the lesions of striated muscles since, after 28 days of drug administration, the recorded values of creatine kinase decreased up to initial determinations without any therapeutic interventions.

changes withinmajour If we correlate the results from histopathology and biochemistry, we can state that artemisinin, in the concentrations of 50 and 500 ppm, can produce harmful effects in broiler chickens organism.

Our results showed that artemisinin, in concentrations of 5 mg/kg of feed, is well tolerated by broiler chickens, and can be used as a feed additive.

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