

Effect of artemisinin on immune system in chickens

Running title: Artemisinin and immune system of chickens

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41 **Summary**

42 The aim of this study was to determine the side effects of artemisinin on immune system in broiler
43 chickens. The lymphoid organs were negatively affected at structural level by chronic consumption
44 of artemisinin. Atherwise, concentration of sera gamma globulins and antibodies titres against sheep
45 red blood cells were higher in chickens exposed orally to artemisinin than in control ones. *In vitro*, T
46 lymphocytes were not stimulated by artemisinin causing a slightly immunosupresion. Spontaneous
47 phagocytic activity was not affected by consumption of artemisinin, it was even significantly
48 stimulated. To our knowledge this is the first study regarding immunotoxicity of artemisinin in
49 poultry.

50 Key words: artemisinin, chickens, immune system.

51

52 **Introduction**

53 Artemisinin is a sesquiterpene lactone isolated from the Chinese herb *Artemisia annua*. This
54 plant has been used for centuries by herbalists to treat fever, in the 70's the scientists proving that
55 it's main bioactive compound, artemisinin, can be used with very good results in treatment of
56 malaria. This compound has also antiviral (Romero *et al.*, 2006; Efferth *et al.*, 2008) anti-tumor
57 effects (Efferth, 2006) and antibacterial properties (Dhingra *et al.*, 2000).

58 The broiler industry is in need of an alternative for coccidiosis control and previous studies
59 have shown promising results for artemisinin (Arab *et al.*, 2006; Naidoo *et al.*, 2008; del Cacho *et*
60 *al.*, 2010).

61 Although artemisinin has been proven to be safe in therapeutic doses, some researchers have
62 shown side effects of this compound. Embryoletality, morphological abnormalities during early
63 pregnancy, hormonal imbalances were induced by treatment with artemisinin in mammals (Boareto
64 *et al.*, 2008). In broiler chickens, artemisinin causes decreases in red blood cells count and
65 hematocrit, anemia, mild degeneration in renal tubules, hepatic degeneration with fatty changes,
66 bile retention in liver, central chromatolysis in brain and neuronal necrosis (Arab *et. al.*, 2009;
67 Shahbazfar *et. al.*, 2011).

68 The effects of *A. annua* extract and leaf powder on broiler chickens immunity was studied in
69 2013 by Gholamrezaie *et al.*, the authors showing that thymus and bursa of Fabricius weights, total
70 anti-SRBC, IgG titer and cellular immunity were increased in chickens treated with *A. annua*.

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

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78 **Materials and methods**

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80 *Ethics statement*

81 Veterinary conditions regarding protection of animals used in this research are compiled
82 according to national standards and legislation. All experiments were approved by the Research
83 bioethics Commission of USAMV Cluj-Napoca (protocol no. 4/19.09.2013).

84

85 *Animals and artemisinin*

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

90 We used artemisinin (purity min. 98%) purchased from Intatrade Chemicals GmbH from Germany,
91 catalog number NN04008. Also, 0.113 mg of artemisinin was diluted in one millilitres of deionised
92 water at 37°C for *in vitro* assays.

93

94 *Experimental design*

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

98 The experimental groups were: a control group (C₁₋₃) that received standard feed and three
99 experimental groups that received 5, 50 and 500 ppm artemisinin in the feed (Art5₁₋₃; Art50₁₋₃ and
100 Art500₁₋₃). Chickens from 1st and 2nd replicates were euthanized by cervical dislocation at the end of
101 the experiment, and those from 3th replicate after 5 days (day 33) of withdrawal period.

102 The effect of artemisinin on immune system in chickens was assessed using the following criteria:
103 (1) morphometric changes in bursa of Fabricius, spleen, and thymus; (2) changes in concentrations

104 of serum gamma globulins; and (3) changes in functional activity of the immune response -
 105 phagocytosis assay, lymphocyte proliferation assay and antibodies titres against sheep red blood
 106 cells (Dohms and Saif, 1984).

107

108 *Blood and serum samples*

109 Blood was collected aseptically from ulnar vein on days 0, 14 and 28 of the experiment. We
 110 collected 0.5-1.0 ml of blood without additives from 1st and 3th replicates, in order to obtain serum,
 111 and 0.7 ml blood with heparin (50 UI/ml) from 2nd replicate.

112

113 *Weight ratio and index of lymphoid organs*

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

118

119 *Histopathology of lymphoid organs*

120 After euthanasia complete necropsy was provided and multiple tissue sample including bursa of
 121 Fabricius, thymus, spleen were collected, trimmed in 5 mm thick samples and immediately fixed in
 122 10% neutral-buffered formalin. After 72h of aldehydic fixation the samples were included in
 123 paraffin wax following a standard procedure (Prophet *et al.*, 1992), cut at 2-3 μ m and automatically
 124 stained with hematoxylin and eosin (H&E). Finally bright field images were collected with an
 125 Olympus UC30 camera monted on a Olympus BX41 microscope and processed using Olympus
 126 Stream Basic image analysis software.

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130 *Serum gamma globulins*

131 We analysed sera gamma globulins (SGGs) on days 0, 14 and 28 of the experiment, in 1st replicate.

132 We used turbidimetric method with 18.5% Na₂SO₄. The concentration of SGGs is proportional with
133 the intensity of turbidity, and it was expressed as g/dl.

134

135 *Phagocytosis assay*

136 The activity of phagocytic cells was measured by a modified carbon particle inclusion test on days
137 0, 14 and 28 of the experiment. Phagocytic cells engulf inert particles such as carbon due to the
138 defensive capacity of these cells (Spînu *et al.*, 1993). First, each heparinized blood sample was
139 mixed with China ink, and then divided in 3 equally controls: negative (NC), positive (PC) and
140 experimental (EC) controls. The controls were incubated at 41°C for 0 and 30 minutes. After each
141 incubation time, 20 µl of controls were transferred in 266 µl of physiologic salt solution (PSS) and
142 centrifuged 5 minutes at 800 rpm. The supernatants (200 µl) were read spectrophotometrically
143 (Biorad 1100 reader plate) in a 96 well flat bottom plates at 540 nm. The results were interpreted as
144 the difference between optic densities (OD) read at 0 and 30 minutes after incubation (OD_{0'} –
145 OD_{30'}).

146

147 *Lymphocyte proliferation assay (LPA)*

148 Three hundred forty microlitres of heparinized blood were mixed with 1.360.00 µl RPMI 1640
149 suplemeneted with peniciline/streptomycine (1000UI/1000µg/ml) and fetal bovine serum (5%) and
150 with a pH adjusted at 7.2-7.4%. Then, 200 µl of mixture were distributed in 8 wells of a 96-sterile
151 flat bottom well plate. For each sample, we used 4 experimental variants (2 wells/variant): (1)
152 control culture, (2) concavalin A culture, (3) lipopolysaccharides culture and (4) artemisinin culture.
153 According to working variants, we added the mitogens for leukocytes (concavalin A,
154 lipopolysaccharides from *Escherichia coli* and artemisinin) in a concentration of 0.5 µg/well. The
155 cultures were incubated for 48·h at 41.0°C and 5% CO₂. After incubation, 30 µl of culture

156 supernatant/well was collected and mixed with its duplicate. Cell growth was quantified by means
157 of the glucose consumption technique. Concentration of residual glucose (GC) in culture
158 supernatant was measured spectrophotometrically using an enzymatic colorimetric CHOD-PAP
159 method (GLUCOSE 4x250 ml kit, ref 4001325L, Hospitex Diagnostics, Italy) following
160 manufacturer's instructions. The stimulation index (SI) was calculated as follows: GC of
161 unstimulated cells (control culture)/GC of stimulated cells (concanavalin A, lypopolizaharide and
162 artemisinin cultures).

163

164 *Antibodies titres against sheep red blood cells*

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

167 Chickens from 3rd replicate (10/group) were subcutaneously stimulated with 0.5 ml of sheep red
168 blood cells (SRBC) suspension, two times, on days 0 and 14. Sheep red blood cells were prepared
169 from whole sheep blood obtained aseptically. Heparinized blood (50 UI/ml) was centrifuged 10 min
170 at 2500 rpm and then the sediment with red cells washed 3 times with PSS. Finally, red cells were
171 resuspended in PSS to a concentration of 5% (vol/vol). Blood from chickens to evaluate the titre of
172 antibodies against sheep red blood cells (AbSRBC) was collected on days 0, 14 and 28 of the
173 experiment.

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

180

181

182 *Statistical analysis*

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

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

193

194 **Results**

195 *Weight ratio and index of lymphoid organs*

196 Globally, the lymphoid organs to body weight ratio (WR) was lower in treated groups than
 197 in control group, either at day 28 of experiment or even after withdrawal period of 5 days; the WR
 198 was significantly higher for all experimental groups ($p = 0.01$ Art5, Art 50; $= 0.05$ Art500) after
 199 withdrawal period for thymus (Fig. 1). This decrease of WR was statistically significant for all
 200 lymphoid organs but for different experimental groups: in group Art5 only thymus ($p = 0.01$); in
 201 group Art50 bursa ($p < 0.0001$) and thymus ($p = 0.04$). Interestingly, on day 28, in Art500 group the
 202 WR was higher in case of bursa and spleen ($p = 0.05$) than in control group; the picture remains
 203 during withdrawal period only for spleen, the WR for bursa turned significantly lower ($p = 0.03$)
 204 comparing with control group (Fig. 1).

205 The weight index (WI) followed the same pattern as WR, but it was lower (0.5) than 0.7 in case of
 206 chickens treated with 50 ppm artemisinin 28 days for bursa, suggesting bursal atrophy; after 5 days
 207 of withdrawal period WI increased at 0.954 (Fig. 1).

208 *Histopathology of lymphoid organs*

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

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

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

223 *Sera gamma globulins*

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

228 *Phagocytosis assay*

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

234 artemisinin we noticed a very low immunosupresion of phagocytic cells; the immunosupresion was
 235 statistically significant in chickens treated with 50 ppm artemisinin, after 28 days of consupcion ($p =$
 236 $0.04 - 0.05$) (Fig. 6).

237 *Lymphocyte proliferation assay*

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

246 *Antibodies titres against sheep red blood cells*

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

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

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259

260 Discussion

261 Toxicity of artemisinin and its derivatives (artesunate, artemether, arteminol, arteether) was
 262 well documented in mammals and revealed neurotoxicity (Erickson *et al.*, 2011), hormonal
 263 imbalance in the absence of malarial parasite infection (Farombi *et al.*, 2015), embryotoxicity
 264 (Clark, 2009) and immunosuppressive activity (Li *et al.*, 2013).

265 In birds there are few studies regarding the side effects of artemisinin. It is hepatotoxic for
 266 turkeys in dose of 2600 mg/kg (Thøfner *et al.*, 2013) and in poultry following single oral dose
 267 (1250 and 2500 mg/kg) or chronic oral intake (for 36 days 17, 34, 68, and 136 ppm artemisinin)
 268 causes microscopical lesions in brain, liver and kidney (Arab *et al.*, 2009; Shahbazfar *et al.*, 2011).

269 To our knowledge this is the first study regarding immunotoxicity of artemisinin in poultry.
 270 We used the artemisinin in feed, for 28 days in three concentrations: 5, 50 and 500 ppm. The side
 271 effects on immune system were assessed following morphometric changes in lymphoid tissues
 272 (thymus, bursa and spleen), changes in concentrations of immunoglobulins (serum gamma
 273 globulins) and changes in functional activity of the immune response (*in vitro* assays for cellular
 274 response and *in vivo* assays for humoral response) (Dohms and Saif, 1984).

275 Based on lymphoid organs to body weight ratio, all three lymphoid organs (thymus, bursa
 276 and spleen) were negatively effected by chronic consumption of artemisinin; this was reversibilly for
 277 thymus after a withdrawal period of 5 days. The most affected lymphoid organ was bursa at 50
 278 ppm dose, weight index (0.5) suggesting bursal atrophy. This finding was suported by
 279 histopathology that revealed increment of interfollicular fibrous stroma, vacuolization of the cortical
 280 zone of the follicles and focal accumulation of necrotic lymphocytes in the medulla.

281 In previous studies the authors didn't find any lesions in spleen even after single oral dose or
 282 chronic oral intake (Arab *et al.*, 2009; Shahbazfar *et al.*, 2011). The doses used by these authors
 283 were 10, 50, 250, 1250 and 2500 mg/kg for single oral dose study and 17, 34, 68, and 136 ppm for
 284 36 days in case of chronic oral intake study. We used 5, 50 and 500 ppm for 28 days and moderate
 285 and severe lesions in spleen were noticed in chickens treated with 50 and 500 ppm artemisinin.

Even morphometric data and histopathology 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 orally to artemisinin comparing with unexposed chickens. To notice that the highest antibodies titres and statistically significant were registered in chickens treated with 5 ppm artemisinin 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 immunotolerant, 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 immunosuppression *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 immunosuppression mainly for T lymphocytes. Histopathology of thymus revealed changes in chickens exposed to 50 and 500 ppm artemisinin: 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 artemisinin and its derivatives against T lymphocytes both *in vitro* and *in vivo* (Wang *et al.*, 2007; Li *et al.*, 2013).

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

In vitro assays (phagocytosis and lymphocyte proliferation assays) revealed a slight immunosuppression, mainly for T lymphocytes, that was not supported *in vivo* (serum gamma

312 globulins concentration and antibodies titres against SRBCs), even lymphoid organs were affected
313 at structural level by artemisinin in high doses (50 and 500 ppm for 28 days in feed). We added the
314 artemisinin when chickens were 18 days-old; in control of coccidiosis drugs (coccidiostats) are
315 added to day-old chickens and it will be important to find the changes in this case.

316

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324

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394 **Figure legends**

395

396 **Fig.1.** Weight ratio and index of lymphoid organs in broiler chickens after consumption
397 of artemisinin for 28 days in feed and after a withdrawal period of 5 days.

398

399 **Fig.2.** Histopathological images of the lymphoid organs following chronically oral
400 intake of 5, 50 and respectively 500 ppm of atemisin compared with the control group.

401 A-D: High power images (obx40) of the bursal follicles. A - control presenting an
402 normal morphology. B – a mild intercellular edema (indicated by the arrow). C - a slight
403 increment of interfollicular fibrous stroma, vacuolization of the cortical zone of the
404 follicles (blue arrow) and focal accumulation necrotic lymphocytes in the medulla
405 (black arrow) of follicles. D - increased amount of interfollicular fibrous stroma (short
406 arrow), atrophied cortex (black arrow) and evident corticomedullary epithelium (blue
407 arrow) (H&E stain, scale bar=50µm).

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

412 I-L: Images (ob x20) of the splenic white and red pulp presenting the size and cellularity
413 of the peri-ellipsoid lymphocyte sheath (PELS)(indicatded by blue arrow in image I),
414 peri-arteriolar lymphocyte sheath (PALS, indicatded by blue arrow in Image J) and the
415 cerminal centers (GS, indicated by the black arrows from images I and J). The normal
416 structure of the previously named structures is presented in image I. In image K a sever
417 vacuolization of the marginal lymphoid tissue can be observed, morphologic change
418 which is also present in a reduced amount in the splenic red pulp. In image L the severe

419 depletion of the PALS is observed (marked by the black arrows); the vacuolar aspect is
420 present in both white and red pulp; (H&E stain, scale bar=100µm).

421

422 **Fig.3.** Concentration of sera gamma globulins (g/dl) in broiler chickens fed with
423 artemisinin comparing with chickens fed with standard feed.

424

425 **Fig.4.** Phagocytic activity of blood cells expressed as the difference between optic
426 densities (absorbance) read at 0 and 30 minutes after incubation of blood samples with
427 China ink.

428

429 **Fig.5.** Stimulation index of lymphocyte in broiler chickens fed with artemisinin.

430

431 **Fig.6.** Antibodies titres (geometric mean) against sheep red blood cells in broiler
432 chickens fed with artemisinin.