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Original article

INVESTIGATIONS ON THE LIVER FUNCTION OF BROILER CHICKENS WITH EXPERIMENTAL AFLATOXICOSIS

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Summary

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The present experiment aimed to evaluate the toxic effects of AFB_1 through follow-up of changes in blood activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (γGT), lactate dehydrogenase (LDH), alkaline phosphatase (AP) and liver morphology. Also, the possibility for effective alleviation or prevention of toxic effects of AFB_1 by feed supplementation with the mycosorbent Mycotox NG was evaluated. The experiments were conducted with 50 7-day-old Cobb broiler chickens allotted to one control and 4 experimental groups. The chickens were orally treated with 1 g/kg Mycotox NG, 0.5 mg/kg AFB_1, 0.8 mg/kg AFB_1 и 0.5 mg/kg AFB_1 + 1 g/kg Mycotox NG over 42 days. Blood samples for analysis were collected on days 21 and 42. Blood chemistry revealed that the groups receiving only AFB_1 showed increased activities of studied enzymes and total bilirubin concentrations. Total protein, albumin, cholesterol, triglycerides and blood glucose were lower than respective control values. Histopathological changes consisted in various degree of dystrophy depending on the amount of ingested toxin. The addition of mycosorbent to the feed of group V reduced partially the deleterious impact of AFB_1 as could be seen from blood biochemical changes and the lower frequency and severity of liver lesions.

Key words: aflatoxin B_1, blood biochemical parameters, chickens, Mycotox NG

INTRODUCTION

The group of mycotoxins consists of structurally different secondary metabolites of fungi, which are known to contaminate cereal crops all around the world. Among the huge variety of known mycotoxins, some of them as aflatoxins, ochratoxin A, zearalenone, T-2 toxin and fumonisin are found at greater amount in poultry feeds (Jelinek *et al.,* 1989). According to the World Health Organization, at least one quarter of feedstuffs on a global scale are contaminated with

mycotoxins. The high temperature and humidity in fields, physical and chemical damage by insects, improper storage conditions and the presence of broken grains are favourable factors for mycotoxin accumulation in grains (Binder *et al.,* 2007).

Aflatoxins (AF) are produced by fungi from the genus *Aspergillus* (*A. flavus, A. parasiticus, A. nomius*). Aflatoxin B_1, B_2, G_1 and G_2 are natural contaminants of wheat, corn, soybean, sorghum, rice, cotton seed, sunflower, foodstuffs and animal feeds (Cole & Richard, 1989; Robens & Richard, 1992; Martinez-de-Anda *et al.,* 2010). Among all aflatoxins, AFB_1 is the most toxic for poultry and at the same time, the commonest feed contaminant. It is also a potent hepatotoxin and carcinogen (Girish & Devegowda, 2006).

The toxicity of AF in poultry consists of anorexia, lethargy, poor production traits (live weight and weight gain), lower feed consumption, increased feed conversion and lethality (Kubena *et al.,* 1998; Ledoux *et al.,* 1999; Miazzo *et al*., 2000). In domestic fowl, AF cause anaemia (Oguz *et al*., 2000), decrease the humoral immunity (Oguz *et al*., 2003), exert teratogenic, carcinogenic and mutagenic effects (Sur & Celik, 2003). The liver is the target organ for the toxic effect of aflatoxins (Kubena *et al.*, 1993). Liver metabolism is disturbed by impaired conversion of proteins, vitamins, amino acids, lipids, nucleic acids and enzymes (Ellis *et al.,* 1991). In broiler chickens, the toxic effects of AFB_1 are manifested by lower blood serum total protein, albumin, cholesterol, triglyceride and glucose concentrations (Kubena *et al*., 1993, 1998; Oguz *et al.,* 2000; Zhao *et al.*, 2010). Increased activity of liver enzymes such as ALT, AST, AP, γGT and LDH is used for evaluation of severity of aflatoxicosis in chickens, ducklings and turkey poults (Rao & Joshi, 1993; Leeson *et al.,* 1995; Quist *et al.*, 2000; Cheng *et al.,* 2000; Yildirim *et al.,* 2011). Liver morphology alterations in broiler chickens consist in hepatomegaly, increased fragility, discoloration (yellow taint), impaired liver structure (haemorrhages, dystrophy, parenchymal cell necrosis, fatty infiltration and proliferation of bile duct epithelium) (Ledoux *et al.,* 1999; Ortatatli & Oguz, 2001; Rosa *et al.,* 2001; Sakhare *et al.,* 2007; Hussain *et al.,* 2008; Zhao *et al.,* 2010; Yildirim *et al.,* 2011). Low amounts of AFB_1 in poultry feeds (50 or 100 μg/kg) are reported to be sufficient to disturb the normal liver metabolism (Maurice *et al.,* 1983).

The decontamination of poultry feeds from aflatoxin B_1 is an important problem for poultry industry. Several methods (physical, chemical and biological) are described for removal of aflatoxins from contaminated feeds. Some of these methods however are expensive, labourous and only partly effective (Piva *et al.,* 1995). Since the beginning of the 1990s, different mycosorbents are introduced for that purpose. The natural and synthetic zeolites (Oguz *et al.,* 2000), bentonites (Rosa *et al.,* 2001; Miazzo *et al.,* 2000), clinoptiolites (Oguz *et al.,* 2000; Ortatatli & Oguz, 2001), hydrated calcium aluminosilicate (Kubena *et al.,* 1990a,b; 1998), *Saccharomyces cerevisiae* yeasts (Zhao *et al.,* 2010) are preferred due to their ability to bind to AF and thus, to reduce their absorption from the alimentary tract.

The present experiment aimed to evaluate the hepatotoxic effects of aflatoxin B_1 through follow-up of changes in concentrations of liver-specific blood parameters and liver morphology. The possibility for effective alleviation of toxic effects of AFB_1 by feed supplementation with the mycosorbent Mycotox

NG (Ceva Sante Animale, France) was also evaluated.

MATERIALS AND METHODS

The experiments were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Trakia University (permit No. 49/29.09.2012).

The experiments were carried out with 50 Cobb broiler chickens 7 days of age, from both genders. All birds were housed under optimum microclimatic parameters, identical for all groups, with compliance with Ordinance 44/2006 (Anonymous, 2006). The tested aflatoxin B_1 was produced by *Aspergillus flavus* (99% purity) and purchased by Sigma-Aldrich, Germany. The experimental design included five groups (n=10).

- Group I – control (fed balanced compound feed according to the age produced by Provimi feed plant, Stara Zagora);
- Group II – experimental – the feed of birds was supplemented with 1 g/kg Mycotox NG (Ceva Sante Animale, France);
- Group III – experimental – the feed of birds was supplemented with 0.5 mg/kg aflatoxin B1;
- Group IV – experimental – the feed of birds was supplemented with 0.8 mg/kg aflatoxin B1;
- Group V – experimental – the feed of birds was supplemented with 0.5 mg/kg aflatoxin B1 and 1 g/kg Mycotox NG.

Blood samples were collected from *v. metatarsalis medialis* on days 21 and 42 in sterile heparinised vacutainers (FL medical, Italy) for analysis of ALT, AST, ALP, γGT, LDH, total protein, albumin, blood glucose, total bilirubin, triglycerides and total cholesterol. Within 30 min after blood collection, blood samples were centrifuged at 1,500×g for 10 min. Plasma was harvested and stored at –20 °C until analysis. All biochemical analytes were assayed on an automated biochemical analyser BS–120 (Mindray, China).

After the end of the experiment, liver specimens for histological examination were obtained from control and treated chickens after euthanasia by cervical dislocation, fixed in 10% formalin, dehydrated in an ascending ethanol series, embedded in paraffin and stained with haematoxylin/eosin.

Results were statistically processed by one-way analysis of variance and the Tukey-Kramer *post hoc* test (level of significance $P<0.05$).

RESULTS

Biochemical results

Plasma total protein, albumin and glucose in chickens from groups III and IV (Table 1) were statistically significantly lower than controls on day 21 ($P<0.001$). On the 42nd day, the changes were more pronounced ($P<0.001$). The addition of mycosorbent to the feed reduced partly the deleterious toxin effects ($P<0.05$–$P<0.01$) on studied analytes. Total bilirubin in groups III and IV (Table 2) was substantially higher at both samplings intervals vs control chickens ($P<0.001$). The changes were more distinct on the 21st day. The supplementation of the feed of group V with mycosorbent inhibited considerably the increase in blood total bilirubin ($P<0.05$).

As seen from Table 2, triglyceride and cholesterol concentrations in groups III and IV were reduced 21 and 42 days after treatment ($P<0.001$) as compared to controls. The addition of mycosorbent to

Table 1. Effect of aflatoxin B_1 (AFB_1) only or co-administered with Mycotox NG on blood plasma total protein, albumin and glucose in broiler chickens. Group I – control; group II – Mycotox NG; group III – 0.5 mg/kg AFB_1; group IV – 0.8 mg/kg AFB_1; group V – 0.5 mg/kg AFB_1 + Mycotox NG. Data are presented as mean ± SEM; n=10

Groups	Total protein, g/L		Albumin, g/L		Glucose, mmol/L	
	Days of age					
	21	42	21	42	21	42
I	39.7±1.33	38.7±0.76	20.0±0.63	20.6±0.80	18.59±0.48	17.86±0.28
II	40.0±0.95	38.8±1.06	20.1±0.54	20.2±0.59	18.71±0.48	17.80±0.32
III	30.4±0.79 1c,2c	23.2±0.82 1c,2c	15.0±0.51 1c,2c	13.4±0.85 1c,2c	14.70±0.40 1c,2c	13.33±0.38 1c,2c
IV	25.4±1.01 1c,2c,,3a	19.7±0.51 1c,2c,3a	12.0±0.57 1c,2c,3b	11.4±0.71 1c,2c	14.03±0.45 1c,2c	10.82±0.68 1c,2c,3b
V	35.1±1.22 1a,2a,3a,4c	34.4±0.93 1b,2b,3c,4c	17.4±0.58 1a,2a,3a,4c	16.5±0.47 1b,2b,3a,4c	16.69±041 1a,2a,3a,,4c	16.00±0.40 1a,2a,3c,4c

* Level of significance: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; 1 – vs control group I; 2 –vs group II; 3 – vs group III; 4 – vs group IV.

Table 2. Effect of aflatoxin B_1 (AFB_1) only or co-administered with Mycotox NG on blood plasma bilirubin, triglycerides and total cholesterol in broiler chickens. Group I – control; group II – Mycotox NG; group III – 0.5 mg/kg AFB_1; group IV – 0.8 mg/kg AFB_1; group V – 0.5 mg/kg AFB_1 + Mycotox NG. Data are presented as mean ± SEM; n=10

Groups	Bilirubin, µmol/L		Triglycerides, mmol/L		Total cholesterol, mmol/L	
	Days of age					
	21	42	21	42	21	42
I	6.98±0.23	7.04±0.12	1.42±0.055	1.38±0.039	4.19±0.10	4.17±0.11
II	6.87±0.185	7.07±0.13	1.45±0.043	1.36±0.047	4.14±0.13	4.19±0.11
III	9.01±0.19 1c,2c	8.60±0.12 1c,2c	1.05±0.035 1c,2c	0.65±0.053 1c,2c	3.24±0.12 1c,2c	2.82±0.12 1c,2c
IV	9.43±0.29 1c,2c	9.0±0.17 1c,2c	0.94±0.047 1c,2c	0.49±0.036 1c,2c	3.01±0.10 1c,2c	2.51±0.12 1c,2c
V	8.03±0.18 1a,2b,3a,4c	7.72±0.15 1a,2a,3c,4c	1.24±0.038 1a,2a,3a,4c	1.16±0.036 1b,2a,3c,4c	3.72±0.34 1a,2a,3a,4c	3.59±0.98 1b,2b,3c,4c

* Level of significance: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; 1 – vs control group I; 2 –vs group II; 3 – vs group III; 4 – vs group IV.

the feed of group V increased the concentrations of these two parameters ($P<0.05$–$P<0.01$).

The toxic effects of AFB_1 on liver function was demonstrated by elevated enzyme activity of AST, ALT, γGT, LDH and AP ($P<0.001$) (Table 3). The changes

Table 3. Effect of aflatoxin B_1 (AFB_1) only or co-administered with Mycotox NG on blood plasma activities of aspartate aminotransferase (AST) alanine aminotransferase (ALT), gamma glutamyltransferase (γGT), lactate dehydrogenase (LDH) and alkaline phosphatase (AP) in broiler chickens. Group I – control; group II – Mycotox NG; group III – 0.5 mg/kg AFB_1; group IV – 0.8 mg/kg AFB_1; group V –0.5 mg/kg AFB_1 + Mycotox NG. Data are presented as mean ± SEM; n=10

	AST, U/L		ALT, U/L		γGT, U/L		LDH, U/L		AP, U/L	
	Days of age									
	21	42	21	42	21	42	21	42	21	42
I	116.0±5.41	131.0±5.26	12.2±0.62	14.3±0.68	9.9±0.90	11.8±0.59	349.5±7.6	343.4±8.4	34.7±1.1	40.0±1.5
II	115.3±5.04	138.0±5.53	11.2±0.57	13.8±0.74	9.6±0.33	11.5±0.47	352.8±7.4	349.6±9.2	31.5±1.9	36.8±1.8
III	172.9±5.52 ^{1c,2c}	200.5±6.07 ^{1c,2c}	26.0±1.66 ^{1c,2c}	31.1±1.71 ^{1c,2c}	17.7±0.65 ^{1c,2c}	25.4±1.90 ^{1c,2c}	412.1±6.3 ^{1c,2c}	524.0±15.1 ^{1c,2c}	57.1±2.6 ^{1c,2c}	82.9±3.6 ^{1c,2c}
IV	198.2±7.93 ^{1c,2c,3a}	252.8±6.77 ^{1c,2c,3c}	29.1±1.74 ^{1c,2c}	34.7±1.55 ^{1c,2c}	23.7±1.35 ^{1c,2c,3c}	32.5±1.82 ^{1c,2c,3b}	461.1±6.6 ^{1c,2c,3c}	541.9±13.6 ^{1c,2c}	76.8±4.5 ^{1c,2c,3c}	104.2±6.0 ^{1c,2c,3c}
V	143.0±5.78 ^{1a,2a,3a,4c}	167.4±6.83 ^{1a,2a,3a,4c}	17.9±1.45 ^{1a,2b,3c,4c}	19.2±0.44 ^{1a,2a,3c,4c}	13.6±0.92 ^{1a,2a,3b,4c}	17.7±0.68 ^{1a,2a,3c,4c}	383.9±4.7 ^{1b,2a,3a,4c}	399.6±14.3 ^{1a,2a,3c,4c}	46.2±2.2 ^{1a,2b,3a,4c}	54±2.8 ^{1a,2b,3c,4c}

* Level of significance: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; 1 – vs control group I; 2 –vs group II; 3 – vs group III; 4 – vs group IV.

in the activity of studied enzymes were dependent on the dietary AFB_1 amount and exposure time. The activities in the group that received aflatoxin and mycosorbent was lower than those in the groups treated only with AFB_1 ($P<0.05$–$P<0.01$). There were no statistically significant changes in studied blood biochemical analytes between the group supplemented with mycosorbent (Group II) and untreated chickens.

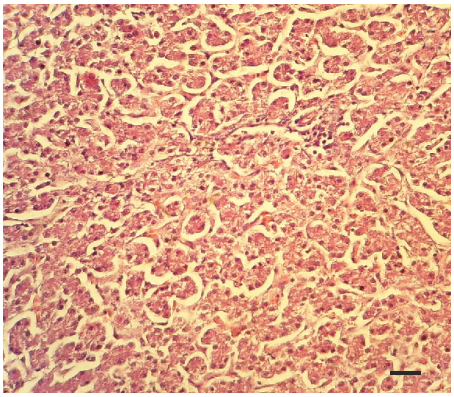

Fig. 1. Liver of a chicken treated with 0.5 mg/kg AFB_1. Granular and fatty dystrophy of the cytoplasm of hepatocytes. H/E, bar=20 µm.

Morphological studies

The livers of chickens treated with 0.5 mg/kg AFB_1 exhibited strong dilation of capillaries, with activation of their endothelium, granular and fatty dystrophy of hepatocytes and weak perivascular mononuclear and connective tissue proliferation (Fig. 1).

In chickens treated with 0.8 mg/kg AFB_1, the normal liver structure was severely impaired. Multiple haemorrhages, hepatocellular necroses, extensive mononuclear and connective tissue proliferations and bile duct hyperplasia could be observed (Fig. 2 and 3).

Chickens treated with both 0.5 mg/kg AFB_1 and Mycotox NG exhibited less severe dystrophy of hepatocytes in comparison with groups treated with both doses aflatoxin. A generalised hyperaemia of capillaries and multiple necrobiotic areas (Fig. 4) were observed.

In chickens from group I (control) and group II (Mycotox NG) showed no histopathological evidence of liver parenchyma lesions.

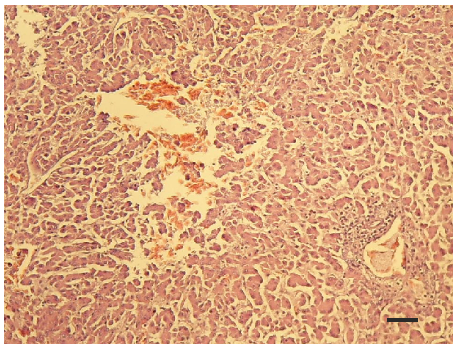

Fig. 2. Liver of a chicken treated with 0.8 mg/kg AFB_1. Hepatocellular necroses and pericapillary proliferations in the parenchyma of the organ. H/E, bar=20 µm.

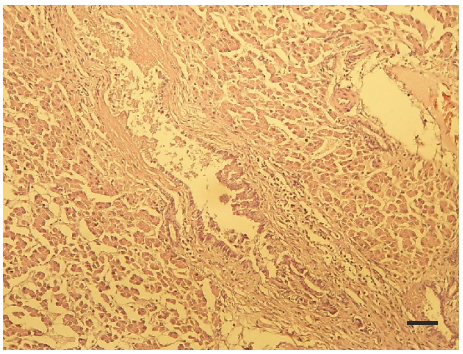

Fig. 3. Liver of a chicken treated with 0.8 mg/kg AFB_1. Bile duct hyperplasia. H/E, bar=20 µm.

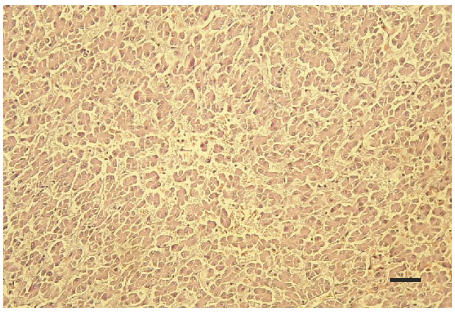

Fig. 4. Liver of a chicken treated with 0.5 mg/kg AFB_1 and Mycotox NG. Hyperaemia and activation of the capillary endothelium. H/E, bar=20 µm.

DISCUSSION

Aflatoxin B_1 is among the most commonly encountered poultry feed contaminants, incurring economic losses to poultry industry (Rizzi *et al.,* 1998). Aflatoxins are hepatotoxic for all vertebrates, causing fatty infiltration, hepatocytic degeneration and necrosis, which impair the normal liver function (Riley & Pestka, 2005). It is established that changes in blood biochemical analytes occur when dietary aflatoxin B_1 levels are equal to or higher than 300 µg/kg (Raju & Devegowda 2000). The present results showed statistically significant elevation in activities of amino transferases (AST, ALT and γGT), LDH and ALP. The high activities of AST, ALP, γGT, LDH and ALT in blood are bioindicators of liver damage (Kubena *et al.*, 1991, 1993; Abdel-Wahhab *et al.,* 1999; Oguz *et al.,* 2000; Ortatatli & Oguz, 2001; Rosa *et al.,* 2001; Miazzo *et al*., 2000; Safameher *et al.,* 2008; Mohamed & Mohamed, 2009; Zhao *et al.,* 2010; Yildirim *et al.,* 2011). These changes are observed in birds and rats with signs of liver parenchyma and bile system damage. These enzymes are located into the cytoplasm and mitochondria of hepatocytes and when the stuctural integrity of the liver is damaged, they pass into the blood plasma (El-Nekeety *et al.,* 2011). Increased values of these enzymes result from enhanced cell membrane permeability or hepatocyte necrosis with consequent transfer from the cytosol in blood serum (Saad & Abdel-Fattah, 2008). The increased activity of LDH is interpreted as sign of liver and heart damage (Cardinet, 1989). The increased γGT, apart being a marker of hepatocytic degeneration (Afzali & Devegowda, 1999) indicates also bile duct hyperplasia in birds with aflatoxicosis (Mohamed & Mohamed, 2009; Zhao *et al.,* 2010; Yildirim *et al.,* 2011).

Metabolic disturbances in birds affected by aflatoxicosis are characterised with protein synthesis inhibition and secondary reduction of plasma protein and albumin concentrations (Kubena *et al.,* 1991; Ledoux *et al.,* 1999; Rosa *et al.,* 2001; Sakhare *et al.,* 2007; Zhao *et al.,* 2010; Yildirim *et al.,* 2011), in agreement with our results. Aflatoxins interfere with protein synthesis through binding to DNA, RNA and proteins, inhibition of DNA synthesis and activity of DNA-dependent RNA polymerase (Cullen & Newberne, 1994).

Pathomorphological liver changes consist in dystrophic and necrotic changes in hepatocytes and bile duct epithelial hyperplasia (Ledoux *et al.,* 1999; Miazzo *et al.,* 2000; Rosa *et al.,* 2001; Mohamed & Mohamed, 2009; Zao *et al.,* 2010; Yildirim *et al.,* 2011). The observed dystrophic and necrotic changes in the liver result from altered primary macromolecules (lipids, proteins and DNA) provoked by oxidative stress-induced damage of DNA and lipid peroxidation (Mohamed & Mohamed, 2009). On the other side, the accumulation of calcium in

hepatocytes provokes mitochondrial dysfunction and reduced adenosine triphosphate synthesis and hence, changes in liver morphology (Quezada *et al.,* 2002; Fatemi *et al.,* 2006). The liver is a target organ for AFB_1 toxicity as it is the site where aflatoxins undergo bioactivation to reactive 8,9-epoxide, which then binds to DNA and proteins (Miazzo *et al.,* 2005; Bailey *et al.,* 2006; Pasha *et al.,* 2007). Aflatoxin B_1 is cytotoxic for hepatocytes and inhibits their proliferation (Abdel-Wahhab *et al.,* 2002; 2007). The hyperplasia of bile duct epithelium occurs consequently to the direct toxic effect of AFB_1 on bile epithelium or excessive production of prostaglandins due to AFB_1-induced lipid peroxidation (Quist *et al.,* 2000; Saif *et al.,* 2003; Mohamed & Mohamed, 2009).

The statistically significant lower blood cholesterol and triglyceride concentrations result from impaired liver metabolism following hepatocellular damage. The altered lipid metabolism in aflatoxicosis is suggested to cause lower cholesterol and triglyceride release by the liver and correspondly lower blood levels (Kubena *et al.,* 1993, 1998; McKenzie *et al.,* 1998; Ledoux *et al.,* 1999; Sakhare *et al.,* 2007).

Increased blood total bilirubin concentrations observed in this study are compatible to other reported results (Rizvi & Shakoori, 2000; Soliman *et al.,* 2008) and also a sequel to impaired liver function.

Reduced blood glucose concentrations in treated birds could be attributed to lower feed intake and/or lower activity of enzymes involved in carbohydrate catabolism, and liver dystrophy associated with glycogenolysis and glyconeogenesis (Simon, 1989; Ledoux *et al.,* 1999; Soliman *et al.,* 2008; Zhao *et al.,* 2010).

The results from the present study confirm previous investigations by showing that the use of specific adsorbents could reduce at a significant extent the toxic effects of aflatoxins in growing birds (Ledoux *et al.*, 1999; Miazzo *et al.,* 2000; Sakhare *et al.,* 2007; Mohamed & Mohamed, 2009; Zhao *et al.,* 2010; Yildirim *et al.,* 2001).

In conclusion, the tested concentrations of AFB_1, added to the compound feed, had a negative impact on the liver function of broiler chickens, manifested by increased blood total bilirubin, AST, ALT, LDH, γ GT and AP concentrations. At the same time, the tested aflatoxin B_1 doses provoked a reduction in blood total protein albumin, glucose, triglycerides, cholesterol and impaired the normal morphology of the liver. The addition of 1 g/kg Mycotox NG to the ration containing 0.5 mg/kg AFB_1 could reliably alleviate the severity of changes in monitored blood parameters and histological lesions resulting from aflatoxicosis.

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