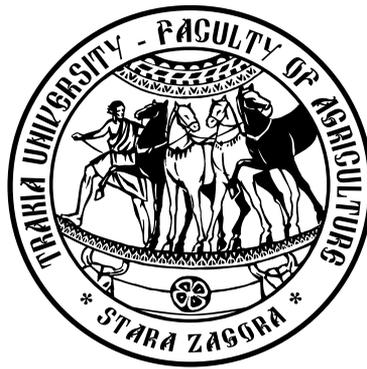


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## Nutrition and Physiology

# Effects of aflatoxin B<sub>1</sub> on histopathological structure of immunocompetent organs in mulard ducks

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**Abstract.** The aim of the present experiment was to investigate the toxic effects of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) on immunocompetent organs (thymus, spleen, bursa of Fabricius) morphology. Also, the possibility for prevention of toxic effects of AFB<sub>1</sub> by feed supplementation of a mycosorbent (Mycotox NB) was studied. The experiments were conducted with 4 groups of 20 10-day-old mulard ducks: group I – control, fed a standard compound feed according to the species and the age; group II – experimental, whose feed was supplemented with 0.5 mg/kg AFB<sub>1</sub>, group III – experimental, supplemented with 0.8 mg/kg AFB<sub>1</sub>, and group IV – experimental, supplemented with 0.5 mg/kg AFB<sub>1</sub> and 2 g/kg Mycotox NG. The duration of the experiments was 42 days. Atrophy and degenerative changes were observed in immunocompetent organs of birds from groups II and III. The supplementation of the feed with 2 g/kg Mycotox NG resulted in partial neutralisation of deleterious effects of AFB<sub>1</sub>, on severity of histological lesions (considerably slighter lymphoid follicle rarefaction).

**Keywords:** aflatoxin B<sub>1</sub>, mallard ducks, histopathological lesion (spleen, bursa of Fabricius and thymus), Mycotox NG

## Introduction

Mycotoxins are bioactive secondary toxic metabolites produced by fungi growing in foodstuffs and induced various toxic effects in vertebrates (Leeson et al., 1995; Bryden, 2007). Animals and people are exposed to the impact of mycotoxins by ingestion of contaminated cereals (corn, wheat, peanuts, sorghum etc.). At a global scale, aflatoxins are responsible for substantial economic losses in the poultry industry as feed concentrations over 75 µg.kg<sup>-1</sup> reduce production traits (Gimeno and Martins, 2011). Fungi belong to the group of storehouse moulds (Hashmi et al., 2006). The main reasons for aflatoxin production during the storage of cereal crops are improper ambient temperature and humidity (Singh and Mandal, 2014). In storehouses, *Aspergillus parasiticus* and *Aspergillus flavus* grow at humidity of 15% and higher, temperature of 6–46°C and aerobic conditions (Lillehoj, 1983). Fungi from the genus *Aspergillus* are widely distributed in the soil, air, organic matter and plants at a global scale. Aflatoxins are secondary toxic metabolites of *Aspergillus parasiticus*, *Aspergillus flavus*, and partly *Aspergillus nomius* (Hassan et al., 2015; Naseer et al., 2016). Aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> are the four primary types found in naturally infected feeds (Cardoso et al., 2016). Aflatoxin B<sub>1</sub> subtype is the most prevalent in contaminated feeds and considered as the most toxic, followed by G<sub>1</sub>, G<sub>2</sub> and B<sub>2</sub> subtypes (Rawal et al., 2010). Aflatoxin B<sub>1</sub> is the most toxic for people, animals, including non-human primates, birds, fish and rodents (Yu, 2012). Oguz et al. (2003) reported that aflatoxins are hepatotoxic, hepatocarcinogenic, mutagenic, teratogenic, cytotoxic and immunotoxic. Aflatoxins inhibit the resistance of birds

to diseases by suppressing postvaccinal immunity (Diekman and Green, 1992). The sensitivity of domestic fowl species to aflatoxin-induced toxicity decreases in the following order: ducklings > turkey poult > goslings > pheasant chicks > chickens (Vekiru et al., 2015). The factors enhancing aflatoxin production in feeds are ambient temperature >27°C, air humidity >62% and feed moisture content >14% (Magdy et al., 2015). The research studies on toxic effects of aflatoxins have demonstrated lower live body weight, alterations in complete blood counts and blood biochemical indices, feed conversion, morphology of internal and haematopoietic organs, altered immune response (Dhanapal et al., 2014; Yalagod, 2014; Kumar et al., 2015; Lakkawar et al., 2015; Lakkawar et al., 2017; Naseem et al., 2017).

The prevention of fungal growth and contamination of feeds with aflatoxins is very important (Ozen et al., 2009). Since the 1990s, the potential of various mycosorbents for detoxication of feed aflatoxins were tested (Kececi et al., 1998; Oguz et al., 2003). The adequate approach to the problem is the use of non-nutritional and inert adsorbents in poultry diets to bind occurring aflatoxins and thus, to reduce their absorption by the digestive tract (Oguz, 2011).

The aim of the present experiment was to investigate the toxic effects of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) on immunocompetent organs (thymus, spleen, bursa of Fabricius) morphology in mulard ducks challenged with different concentrations of aflatoxin B<sub>1</sub> either alone or with the mycosorbent Mycotox NB.

## Material and methods

The experimental design of the study with 80 female mu-

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lard ducks comprised:

- Group I – control. Control birds were fed a standard compound feed according to the age produced by Zoohraninvest feed factory, Stara Zagora and comprised pelleted starter, grower and finisher.
- Group II – experimental, the standard feed was supplemented with 0.5 mg/kg aflatoxin B<sub>1</sub>.
- Group III – experimental, the standard feed was supplemented with 0.8 mg/kg aflatoxin B<sub>1</sub>.
- Group IV – experimental, the standard feed was supplemented with 0.5 mg/kg aflatoxin B<sub>1</sub> and 2 g/kg Mycotox NG (Ceva Sante Animale, France).

The aflatoxin B<sub>1</sub> used in this experiment was produced by *Aspergillus flavus* (99% purity) purchased from Sigma-Aldrich, Germany. The feed of experimental groups of birds was grinded for better homogenisation of aflatoxin B<sub>1</sub>. Microclimatic parameters were optimal and equal for all groups. In the beginning of the trial, air temperature in the living area of ducks was 35°C and until the 15<sup>th</sup> day it decreased at a daily rate of 1°C; by day 28 it was 20°C, and thereafter – +18°C with air humidity of 60–75% (Ordinance No. 44, 2006). The duration of the light day was 24h throughout the experiment. Control and experimental groups were housed in different sections, each with area of 4m<sup>2</sup> in the same premise. The sections were bedded with clean dry wooden shavings with layer depth of 5cm.

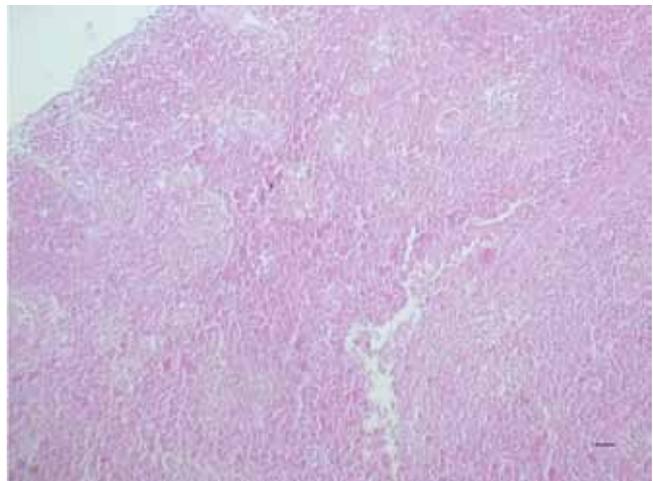
At the end of the experiment (day 42 of life), control and treated ducks were euthanised by cervical dislocation as per Ordinance No. 20 (2012) on the minimum requirements for the protection and welfare of experimental animals and requirements to objects for use, cultivation and/or supply. Histological specimens collected from bursa of Fabricius, thymus and spleen, were fixed in 10% formalin. The samples were embedded in paraffin after dehydration in ascending alcohol series. Blocks were cut on a microtome (Leica model RM 2235) with cross section thickness of 5µm. Cross sections were stained with haematoxylin-eosin.

The experiment was approved by the Ethics Committee of the Faculty of Veterinary Medicine to the Trakia University (Permit No. 42.10.10.2011).

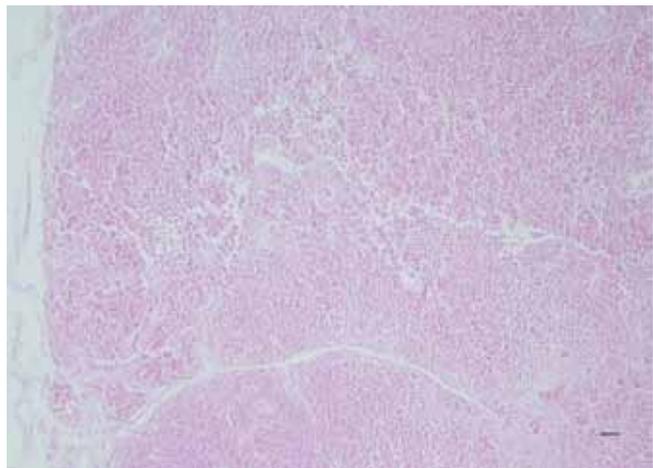
## Results

In birds from group II and III, reduction of lymphatic tissue of the thymus, spleen and bursa of Fabricius was established. In the thymus, there were degenerative changes in rarefaction of lymphatic tissue cells in the cortical layer. The rarefaction was more expressed in birds from group III compared to group II (Figures 1 and 2). In some birds, the rarefaction of cells was so strong that the boundary between cortex and medulla was effaced. Single haemorrhages were encountered. In birds from group IV, the reduction of lymphatic cells was considerably less pronounced (Figure 3).

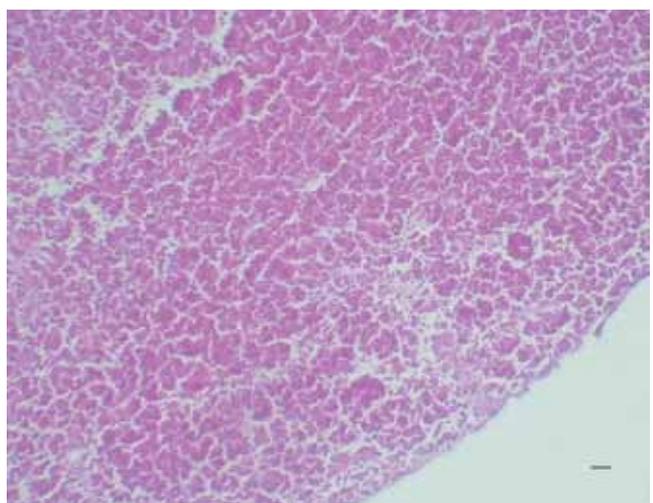
In the bursa of Fabricius, the reduction of lymphoid cell population and their degeneration was moderate to strong. Most commonly, lymphocytes exhibited karyopyknosis and karyorrhexis. Among lymph follicles, oedema was frequently present (Figure 4). In ducklings from group III, the changes



**Figure 1.** Degeneration and rarefaction of lymphatic tissue cells in the thymic cortex in mulard ducks from Group II, whose feed was supplemented with 0.5 mg/kg aflatoxin B<sub>1</sub>. H/E, Bar 15µm.

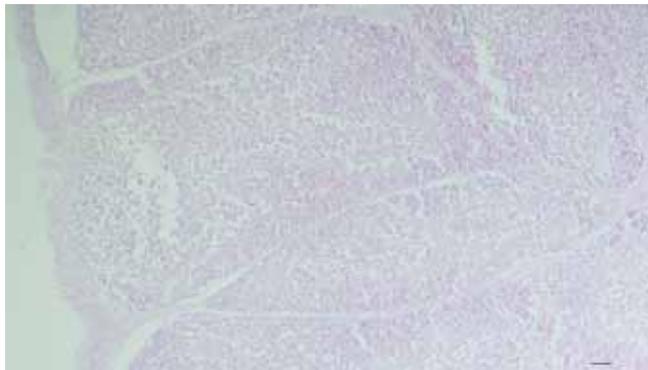


**Figure 2.** Extensive rarefaction of lymphatic tissue cells in the thymic cortex in mulard ducks from Group III, whose feed was supplemented with 0.8 mg/kg aflatoxin B<sub>1</sub>. H/E, Bar 15µm.

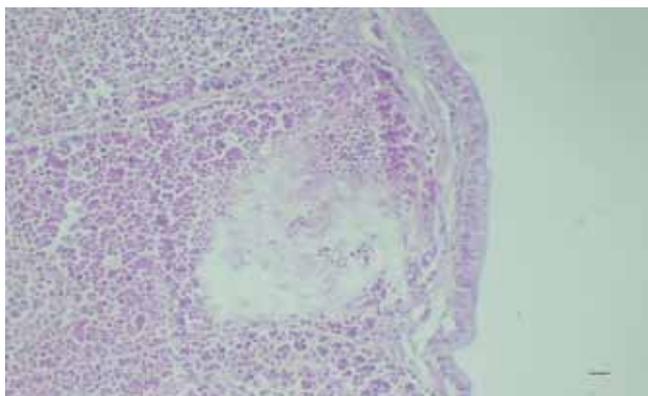


**Figure 3.** Substantially milder degeneration and rarefaction of lymphatic tissue cells in the thymic cortex in mulard ducks from Group IV, whose feed was supplemented with 0.5 mg/kg aflatoxin B<sub>1</sub> and 2 g/kg Mycotox NG, H/E, Bar 15µm.

were the most intensive. In this group, necrotic areas were also encountered (Figure 5). Similar changes were not found out in controls and the other experimental group.

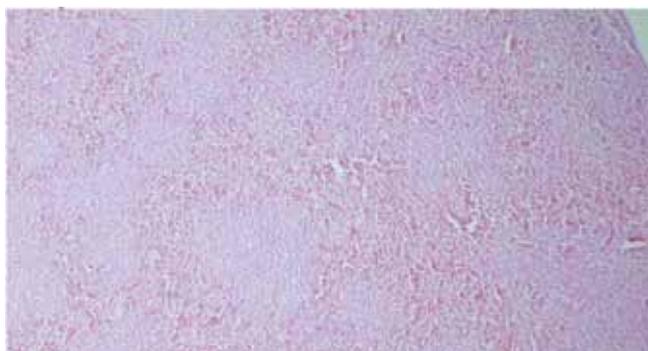


**Figure 4.** Oedema and reduction of lymphoid cell population in the follicles of the bursa of Fabricius in mulard ducks from Group II, whose feed was supplemented with 0.5 mg/kg aflatoxin B1. H/E, Bar 15µm.

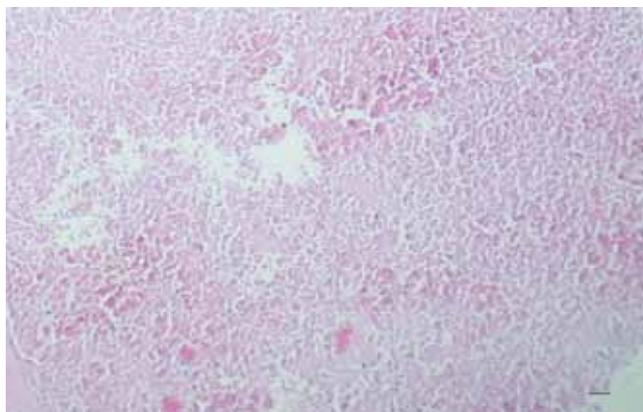


**Figure 5.** Necrosis and degeneration in the follicles of the bursa of Fabricius in mulard ducks from Group III, whose feed was supplemented with 0.8 mg/kg aflatoxin B1. H/E, Bar 15µm.

The main pathomorphological changes in the spleen affected mainly the lymphatic tissue of germinal centres, whose number and size were reduced in birds from group II with karyolysis, karyopyknosis and karyorrhexis of most cells (Figure 6). In the spleens of ducks from group III, the aforementioned observations were further accompanied with haemorrhages, siderocytes, singles necrotic areas (Figure 7). The extent of dystrophy in group IV was less intense.



**Figure 6.** Reduction of lymphatic tissue of splenic germinal centres in mulard ducks from Group II, whose feed was supplemented with 0.5 mg/kg aflatoxin B1. H/E, Bar 15µm.



**Figure 7.** Necrobiotic and necrotic changes in the spleen in mulard ducks from Group III, whose feed was supplemented with 0.8 mg/kg aflatoxin B1. H/E, Bar 15µm.

## Discussion

Mycotoxins, especially aflatoxins, are essential for poultry industry due to the frequent contamination of poultry feeds and incurred economic losses and health problems (Lakkarwar et al., 2017). The thymus and the bursa of Fabricius are the primary immune organs in birds where the proliferation and differentiation of T and B lymphocytes takes place (Riddell, 1987). The immunotoxic effects of aflatoxins are well studied in birds (Celik et al., 2000; Sur and Celik, 2003; Ortatatli et al., 2005). Sur et al. (2011) found out reduction of lymphatic cells in lymphoid organs. The immunotoxicity of aflatoxins is manifested with impaired morphology of the thymus, spleen and bursa of Fabricius (Celik et al., 2000; Ortatatli et al., 2005). It is further associated with generation of intracellular reactive oxygen species (ROS), including superoxide anion, hydroxyl radical and hydrogen peroxide during the conversion of AFB1 by cytochrome P450. High levels of ROS leads to oxidative stress and hence, cellular damage. Oxidative stress could be prevented by both enzyme and non-enzyme antioxidant systems. From the group of the formed, glutathione S-transferase (GST) and glutathione peroxidase (GPx) are the main elements of cell detoxication protecting the cells from ROS (Ren et al., 2009), so they play the role of primary mechanism of defense against oxidative stress (Almar et al., 1998).

The nuclear debris in the thymic cortex found out in this study could be associated with lymphocytopenia (Hussain et al., 2008). Decreased lymphocytic population and increased nuclear debris and reticulocytes in lymph follicles of the bursa of Fabricius indicated reduced proliferation. The same events in the splenic pulp along with lower lymphocyte count in lymphatic follicles were a sign of impaired humoral and cellular immune functions of the organ.

Broiler chickens fed rations containing 4 mg/kg total aflatoxin B exhibited reduction of lymphoid cells, atrophy, necrosis of lymphatic follicles and haemorrhages in lymphoid organs (thymus, spleen, bursa of Fabricius) (Ortatali and Oguz, 2001; Ortatali et al., 2005; Sakhare et al., 2007; Mohamed and Mohamed, 2009). Broiler chickens challenged with 0.2 mg/kg aflatoxin B1 (Sakhare et al., 2007) had reduced lympho-

cyte population in bursal follicles, necrotic lymphatic follicles, proliferation of fibrous tissue in interfollicular spaces. At the same time, the authors found no substantial changes in the histological structure of the bursa after supplementation of the feed with mycosorbent.

Reduced density of lymphoid cells and immunocompetent organs (bursa of Fabricius, thymus, spleen, caecal tonsils, Harderian glands) were present in broiler chickens treated with citrinin and aflatoxin, either independently or in combination (Anandkumar et al., 2014). Balachandran (1998) and Mohamed and Mohamed (2009) reported lower density of lymphoid cells and necrosis of the bursa of Fabricius and the spleen, increased number of germinal centres and reticular hyperplasia of splenic cells in broiler chickens supplemented with 1 mg/kg aflatoxin throughout 28 days. Observed splenic lesions also supported immunotoxic and haemotoxic effects of aflatoxins (Gabal and Azzam, 1998; Ibrahim et al., 2000). The immunotoxicity induced by aflatoxins is well studied in domestic fowl species (Celik et al., 2000; Ortatli et al., 2005; Sur and Celik, 2005; Sur et al., 2011). In a previous experiment of ours (Valchev et al., 2014) with broiler chickens that received 0.5 mg/kg AFB<sub>1</sub> or 0.8 mg/kg AFB<sub>1</sub>, lymphoid cell dystrophy and rarefaction of lymphoid cells (reduction of lymphatic tissue) were established. In rats treated with 1 mg/kg AFB<sub>1</sub> in the feed for six weeks Omar (2012) demonstrated lymphocytic degeneration, haemorrhages, degenerated blood cells, congestion in blood sinuses, necrotic foci, atrophy of the white pulp of the spleen and irregular arrangement of leucocytes in the white pulp.

Intravasal haemolysis and lymphatic follicular haemorrhages present in this study could be associated with increased fragility of blood vessels due to increased concentrations of hydrolases in vascular walls (Tung et al., 1970) and impaired extrinsic coagulation system with subsequent reduction of thromboplastin, prothrombin, fibrinogen, factors V, VII and X (Doerr et al., 1974), as well as reduced factors VII and IX from the intrinsic haemocoagulation system (Doerr and Hamilton, 1981).

Aflatoxins impair the protein synthesis by forming adducts with DNA, RNA and proteins (Busby and Wogan, 1984), RNA synthesis inhibition, decreased activity of DNA-dependent RNA polymerase and degranulation of granular endoplasmic reticulum (Verma and Nair, 2001), mechanisms of structural changes occurring in different tissues (liver, kidneys, skeletal muscles, heart, pancreas, immunocompetent organs) (Wangikar et al., 2005; Mohammed and Metwally, 2009; Sharma et al., 2011). ROS formation secondary to the toxic impact of AFB<sub>1</sub> occurs mainly in the mitochondria of hepatocytes and renal epithelial cells, damaging important biomolecules as DNA, proteins and lipids (Hwang and Kim, 2007). It is acknowledged that aflatoxins enhance lipid peroxidation and induce cellular damage with impairment of the normal morphology of parenchymal organs (Verma and Chakraborty, 2008; Darwish et al., 2011).

## Conclusion

The results demonstrated that the presence of 0.5 mg/kg or 0.8 mg/kg AFB<sub>1</sub> in the compound feed of mulard ducks had a deleterious effect on the morphology of immunocompetent organs (lymph follicle rarefaction, degenerative changes, oedema and hemorrhages). The supplementation of the feed with 2 g/kg Mycotox NG resulted in partial neutralisation of deleterious effects of AFB<sub>1</sub> on severity of histological lesions (considerably slighter lymphoid follicle rarefaction and degenerative changes).

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