EFFECTS OF AFLATOXIN B_1 ON THE DEVELOPMENT OF CHICKEN THYMUS AND BLOOD LYMPHOCYTE ALPHA-NAPHTHYL ACETATE ESTERASE ACTIVITY

Effect van aflatoxine B1 op de ontwikkeling van de thymus bij de kip en op de alfa-naftylacetaatesterase activiteit bij bloedlymfocyten

E. Sur, I. Celik

Department of Histology and Embryology, Faculty of Veterinary Medicine,
University of Selçuk, 42031, Kampüs, Konya, Turkey
emrahsur@selcuk.edu.tr

ABSTRACT

In this study, the depressive effects of AFB_1 on the embryonic development of chicken thymus were determined by histological methods and by histochemical demonstration of alpha-naphthyl acetate esterase (ANAE) enzyme. ANAE positivity frequencies of peripheral blood lymphocytes (PBL) were also determined. Embryonic stages were determined according to the Hamburger-Hamilton (H-H) scale. Thymic development was quite similar on day 7 of incubation in all control and AFB_1 groups. In contrast, the development was substantially impaired and retarded on day 10 of incubation in the 20 and 40 ng/egg AFB_1 groups. The ANAE positivity was first detected on the 18th day of incubation in a minority (2%) of PBL. The proportions of PBL and their ANAE positivity frequencies were significantly ($P<0.05$) lower in AFB_1 treated groups than in the controls on the day of hatching.

SAMENVATTING

In deze studie werden de negatieve effecten van AFB_1 op de embryonale ontwikkeling van de thymus bij de kip bepaald door middel van histologische methoden en door middel van de histochemische bepaling van de alfa-naftylesterase (ANAE) activiteit. De frequentie van ANAE-positieve bloedlymfocyten werd eveneens vastgesteld. Het stadium van de embryonale ontwikkeling werd bepaald aan de hand van de Hamburger-Hamilton (H-H) schaal. Op dag 7 van de incubatie was de ontwikkeling van de thymus ongeveer gelijk bij alle groepen, zowel bij de controlegroepen als bij de met AFB1-behandelde groepen. Vanaf dag 10 was de ontwikkeling echter in belangrijke mate vertraagd in de groepen die met 20 en 40 ng AFB_1 per ei behandeld werden. Positieve reacties voor ANAE werden voor het eerst waargenomen op de 18de dag van de incubatie bij een minderheid van de bloedlymfocyten (2%). Op de dag van uitkippen waren de proportie bloedlymfocyten en de frequentie van ANAE-positieve bloedlymfocyten significant lager bij de met AFB_1 behandelde groepen dan bij de controlegroepen.

INTRODUCTION

Aflatoxins (AF) are toxic metabolites produced by certain species of moulds, particularly *Aspergillus flavus* and *Aspergillus parasiticus*. Of the 18 different aflatoxins that have been identified, the most frequently detected AF in food and foodstuffs of domestic animals and poultry are aflatoxin B_1 (AFB_1), AFB_2, AFG_1 and AFG_2. Nevertheless, AFB_1 is classified as the most toxic compound for a variety of animal species (Leeson *et al.,* 1995).

Aflatoxins and their metabolites accumulate in most of the soft tissues and fat depots of the chicken, and they are also found in the egg (Jacobson and Wiseman, 1974; Sudhakar, 1992; Qureshi *et al.,* 1998). Jacobson and Wiseman (1974) found 9 ng/egg AFB_1 on day 10 of feeding in the eggs from animals receiving diets containing 100 ppb dietary AFB_1. Trucksess *et al.* (1983) fed 18 laying hens with a diet containing 8000 ppb AFB_1 for 7 days and found that liver and ova contained the highest levels of AFB_1 and its metabolite Aflatoxin Ro. Besides, the results of the

previous reports (Hamilton, 1982; Trucksess *et al.,* 1983; Qureshi *et al.,* 1998) have revealed that the carry-over of AFB_1 from layer hen's food into eggs has a ratio of 1/2000-1/2500. In poultry food and foodstuffs, this contamination and residue may have little significance for human health, but it can be a serious veterinary problem because of the residues in fertilized eggs (Dietert *et al.,* 1985). This maternally transferred AFB_1 has been reported to affect embryo viability and hatchability (Qureshi *et al.,* 1998) and also to cause various organ malformations, such as spina bifida, anophthalmia, maxillary brachygnatia, distorted legs and evisceration (Cilievici *et al.*, 1980).

Among the most important problems caused by maternally transferred AFB_1 are functional defects in the immune system of the developing embryo (Çelik *et al.*, 2000a). There may be a functional impairment of the immune system, resulting in increased susceptibility to pathogenic micro-organisms during growth and maturation of compromised chicks. Such animals develop a deficient resistance to infectious diseases and a reduced immunity after vaccination (Batra *et al.,*1991; Azzam and Gabal, 1997; Gabal and Azzam, 1998; Qureshi *et al.,* 1998).

The thymus is a central lymphoid organ of both avian and mammalian species in which T-lymphocytes gain enzymatic maturation and immunocompetence. Thus the organ plays a crucial role in the cellular immune system. Disturbances in the embryonic development of the thymus might give significant clues as to the immune status of the affected animals. Enzyme histochemical studies can be used to evaluate the functional development and maturation of the immune system (Coskun *et al.,* 1998; Çelik *et al.,* 2000a; Sur and Çelik, 2003). Enzymatic positivity profiles of thymocytes have been assumed to be a useful tool for the assessment of T cell maturation (Basso *et al.,* 1980). A lymphocyte lysosomal enzyme (Knowles *et al.,* 1978), alpha-naphthyl acetate esterase (ANAE), has been demonstrated in mature, immunocompetent circulating T-lymphocytes of many animal species. ANAE positivity has widely been used to differentiate T and B lymphocytes and monocytes in various species, including humans (Zicca *et al.,* 1981; Çelik *et al.,* 1991), chicken (Pruthi *et al.,* 1987; Maiti *et al.,*1990), cattle (Kajikawa *et al.,*1983; Çelik *et al.,* 1994), dog (Wulff *et al.,* 1981) and mouse (Mueller *et al.,* 1975). The enzyme is assumed to be responsible for the cytotoxic effects of T-lymphocytes and the phagocytic activity of monocytes (Mueller *et al.,* 1975).

Knowles *et al.* (1978) have reported that the ANAE positivity of peripheral blood T-lymphocytes is represented by 3 to 5 reddish-brown granules that are localized adjacent to the cell membrane. In contrast, lymphocytes lacking the granules are considered to be ANAE-negative B-lymphocytes. Monocytes display a fine, diffuse granular positivity.

The proportion of ANAE-positive peripheral blood lymphocytes (PBL) differs largely between animal species, varying from 56% to 78% of PBL in the dog (Wulff *et al.,* 1981), 47.7% in cattle (Kajikawa *et al.,* 1983), 69% in humans (Çelik *et al.,* 1991) and 35% in chickens (Pruthi *et al.,* 1987).

In this study, the embryotoxicity and depressive effects of AFB_1 on the embryonic development of chicken thymus were tested in fertilized chicken eggs by histological methods and enzyme histochemical demonstration of alpha-naphtyl acetate esterase (ANAE).

MATERIAL AND METHODS

Preparation of AFB_1 solutions

Pure AFB_1 was obtained from Makor Chemical Co., (Jerusalem, Israel). The AFB_1 was diluted in benzene to prepare a stock solution containing 20 µl/ml AFB_1. This solution was then transferred into the vials containing the desired concentrations of AFB_1 for each dose group and left overnight to evaporate the benzene. The AFB_1 residue was dissolved in absolute ethanol (99.9%) and then the ethanol concentration was reduced to 30% by adding sterilized bidistilled water. The AFB_1 concentration of these solutions was measured in duplicate by a Thin Layer Chromatography (TLC) densitometer equipped with a fluorescence detector (Perkin Elmer MPF 43A) at 365 nm excitation and 425 nm emission wave lengths, and by a UV-VIS Recording Spectrophotometer (Shimadzu-UV 2100) using standards.

Treatment groups and embryonic exposure

For the experiments, 651 fertilized eggs of laying hens (Babcock B-380) were used. The eggs were fumigated (80 g potassium permanganate in 130 ml 40% formaldehyde solution per m^3 for 20 min) and divided into 7 groups as follows: non-treated controls (86 eggs), drilled-sealed group (80 eggs), solvent 30% ethanol-injected group (83 eggs), 5 ng/egg AFB_1 group (88 eggs), 10 ng/egg AFB_1 group (97 eggs), 20 ng/egg AFB_1 group (97 eggs) and 40 ng/egg AFB_1 group (120 eggs).

The egg shells in drilled-sealed group were drilled and immediately sealed with melted paraffin in a sterile cabinet. Injections of the test solutions were performed just prior to placing the eggs in the incubator. After the shell was drilled at the blunt ends of the eggs, 20 µl of test solution was injected into the air sac (Prelusky *et al.,* 1987; Çelik *et al.,* 2000a). For injections, micropipettes (Sealpette, Jencons, Finland) with sterile tips were used. After the injections, the holes were immediately sealed with melted paraffin and the eggs were then placed in an incubator (Söktav, Turkey) at 37°C, 65% RH. The eggs were turned every 2 hours.

Staging of embryonic development

The eggs were opened on days 7, 10, 12, 13, 15 and 18 of incubation. Embryonic stages were determined according to the Hamburger-Hamilton (H-H) scale (Hamburger and Hamilton, 1951).

Histological investigations

On the 7th and 10th days of incubation, 6 randomly selected whole embryos from each group were fixed in buffered formaldehyde-saline solution (pH 7.4), dehydrated and embedded in paraffin blocks (Culling *et al.,* 1985). On days 12, 13, 15 18 and 21 of incubation, 6 embryos were sampled from each group. Half of each embryo and its thymic lobes were fixed in a phosphate buffered formaldehyde-sucrose solution (+4°C, pH 6.8) for 24 h and then kept in Holt's solution (+4ºC) for an additional 24 h for the histochemical demonstration of ANAE (Knowles and Holck, 1978; Pruthi *et al.,* 1987; Maiti *et al.,* 1990). For routine histological examinations, 6 µm thick tissue sections taken from paraffin blocks were stained with Crossman's trichrome staining (Culling *et al.,* 1985) and Pappenheim's panoptic stain (Konuk, 1981). Histological changes were investigated with the light microscope.

ANAE demonstration and May Grünwald-Giemsa staining on the blood smears

Cardiac blood samples were taken into heparinized tubes (10 IU ml/blood) on days 12, 13, 15, 18 and 21 of incubation. From each sample, 4 smears were prepared and air dried. The smears were fixed in phosphate buffered glutaraldehyde-acetone solution (pH 4.8) at –10°C for 3 minutes. Two of the smears were used for ANAE demonstration (Coskun *et al.,* 1998), the remaining ones were stained with May Grünwald-Giemsa (Konuk, 1981) for the determination of PBL percentages.

ANAE histochemistry

ANAE was demonstrated on both blood smears and in 12 µm thick cryostat (Slee London) sections, according to the method of Maiti *et al.* (1990). The incubation solution was prepared by mixing 80 ml of 0.067 M phosphate buffer (pH 5.0), 4.8 ml of hex azotized pararosaniline [2.4 ml of pararosaniline (Sigma, C.I.N. 42500) and 2.4 ml of 4% sodium nitrite in distilled water] and 20 mg of alpha naphthyl acetate (Sigma, N-8505) in 0.8 ml of acetone. The final pH of the incubation solution was adjusted to 5.8 with 1 N NaOH. Tissue sections were incubated for controlled periods, whereas the blood smears were incubated for 2 h at 37°C. After incubation, the smears and sections were rinsed in distilled water and counter-stained for 10 minutes with 1% methyl green (Merck, C.I.N., 2585) prepared in 0.1 M acetate buffer (pH 4.2). The slides were dehydrated and mounted with synthetic mounting medium (Entellan, Merck).

Evaluation of histochemical staining

In both tissue sections and blood smears, the cells having lymphocyte morphology and 1 to 3 large, reddish-brown granules were classified as ANAE-positive T lymphocytes. Similarly, PBL percentages were also determined by counting 200 leukocytes on the May Grünwald-Giemsa stained smears. The positivity rates were expressed as percentages of the lymphocytes counted. The localization patterns of both ANAE-positive and negative cells were determined microscopically in the sections.

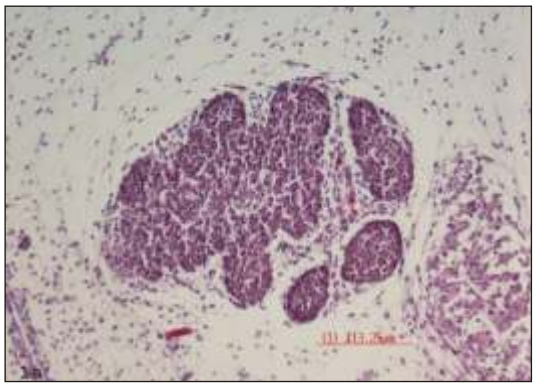
Statistical analyses

Statistical analyses were performed with a standard computer program (Minitab for Windows, Release, 9.2, 1993). Differences between the arcsine transformed ANAE positivity values of PBL were analyzed by one-way ANOVA (Yıldız and Bircan, 1991).

RESULTS

Histology of the thymus

Histology of the presumed thymic lobules displayed a similar morphology in all control and experimental groups at day 7 of incubation. However, at d 10, the development of the organ was retarded in the 10, 20



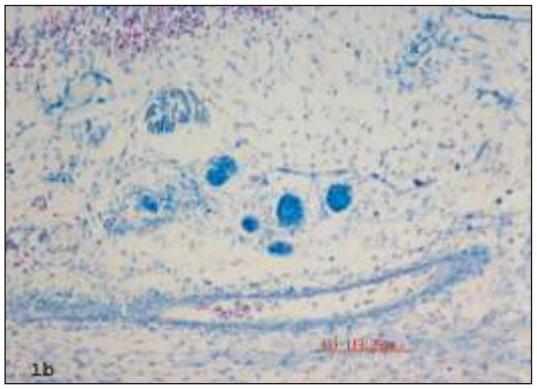
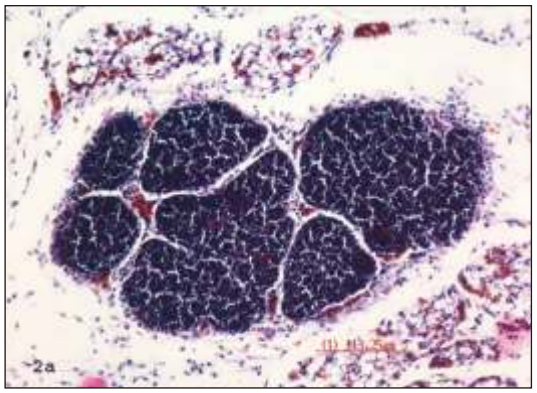


Figure 1 (a, b). Histologic sections of developing thymic primordium from control chicken embryo (a, Trichrome stain) and 20 ng AFB_1 group (b, Pappenheim's panoptic stain) on d 10 of incubation. Retarded development of thymic lobes of the thymic primordium is clearly seen in the animal treated with AFB_1.



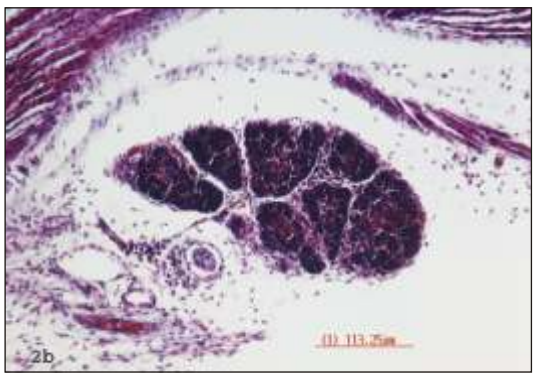


Figure 2 (a, b). Histologic sections of thymuses from control (a) and 20 ng AFB_1 group (b) on d 13 of incubation (Trichrome stain). Retarded development of thymic lobules is clearly seen in the animal treated with AFB_1.

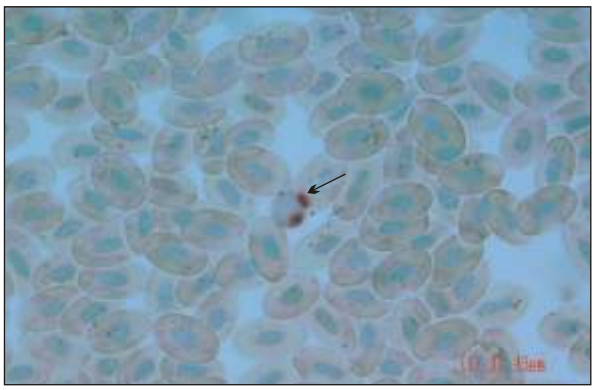


Figure 3. ANAE-positive peripheral blood lymphocyte (arrow) is observed on d 18 of incubation (ANAE demonstration, according to Coşkun *et al*., 1998).

and 40 ng/egg AFB_1 groups. The development and histological organization of the lobules were substantially impaired and suppressed in the 10, 20 and 40 ng/egg AFB_1 groups between days 10 and 18 of incubation (Figures 1, 2). The animals in the 5 and 10 ng AFB_1-treated groups hatched with the thymuses displaying a similar histology to those of the controls. By contrast, no chicken hatched in the 20 and 40 ng/egg AFB_1 groups.

Results of ANAE histochemistry

ANAE-positive lymphocytes were first observed in the medullary regions of developing thymic lobules from the control animals on d 12 of incubation, whereas ANAE positive lymphocytes were first observed at a low level (2%) in the peripheral blood on d 18 of incubation (Figure 3). On day 21 of incubation, percentages and ANAE-positivity of PBL in 5 and 10 ng/egg AFB_1 groups were significantly ($P<0.05$) lower than those of the non-treated control, drilled-sealed and solvent (30%) injected groups. However, the differences between the 5 and 10 ng/egg AFB_1 groups were not significant (Table 1).

DISCUSSION

AF levels ranging from 5 to 100 ppb in broiler food have been detected in different countries (Hegazy *et al.,* 1991; Kichou and Walser, 1993; Nizamlıoğlu, 1996; Pitet, 1998). The levels mostly accumulate to less than 50 ppb, but AFB_1 is found at relatively lower levels. Özpınar *et al.* (1988) found 0.2-30.4 ppb AFB_1 in poultry feed. With a carry-over ratio of 1/2000 and an AFB_1 level of 10 ppb, an egg will contain 1.3 ng AFB_1 when the hen has consumed 130 g of the food daily and laid every other day. However, with a 20 ppb

Table 1. Peripheral blood lymphocyte percentages and ANAE-positive lymphocyte rates of control and experimental groups on the day of hatching.

Treatment	n	Peripheral blood lymphocyte percentages on the day of hatching (%) (X±S)	ANAE (+) lymphocyte rates on the day of hatching (%) (X±S)
Group 1 (Non-treated control)	6	16.33±3.56[a]	37.18 ±1.50[a]
Group 2 (Drilled-sealed)	6	15.83±2.32[a]	35.35±0.94[a]
Group 3 (%30 ETOH)	6	15.50±1.87[a]	35.28±0,33[a]
Group 4 (5 ng/egg AFB_1)	6	11.50±1.97[b]	34.95±1.44[b]
Group 5 (10 ng/egg AFB_1)	6	11.34±1.86[b]	34.87±0.98[b]

[a-b] Values within a column with no common superscripts are significantly different ($p<0.05$).

AF limit, the AFB_1 content of the egg would increase up to 2.15 ng, since natural AF consist of 83.06% AFB_1, 12.98% AFB_2, 2.84% AFG_1 and 1.12% AFG_2 (Oğuz, 1997). Although the estimated level of AFB_1 may not cause important problems in human health, it can cause serious problems in poultry. Using the chick embryotoxicity screening test-I (CHEST-I), Jelinek *et al.* (1985) determined the embryotoxicity limits for AFB_1 as 0.3 to 30 ng/egg, and the teratogenicity limits as 3 to 30 ng/egg. In the present study relatively higher doses - 5, 10, 20, and 40 ng AFB_1/egg levels - were used since the limits are frequently exceeded. Moreover, previous researchers (Sudhakar, 1992; Oliveira *et al.,* 2000) have reported different results for AFB_1 levels in the chicken egg. Sudhakar (1992) found 5 ng/g AFB_1 in the eggs of layers fed a diet containing 600 ppb AFB_1 for 3 weeks, while Oliveira *et al.* (2000) found 6 ng AFB_1 in the eggs from layers fed a diet containing 500 ppb AFB_1 for 8 weeks. In addition, Jelinek *et al.* (1985) observed 33% embryonic mortality, 12% heart abnormalities, and 4% disclosures of the body wall at embryotoxic and teratogenic levels.

Among the many adverse effects caused by AF are hepatotoxicity, nephrotoxicity, cytotoxicity, mutagenity, genotoxicity and teratogenity (Wong and Hsieh, 1976; Smith and Moss, 1985). Biotransformation of AFB_1 leads to the more active metabolites AFB_1-2,3 and AFB_1-8,9 epoxy derivatives by means of a cytochrome p-450 dependent hepatic or extrahepatic mixed function oxidase (MFO) system in the presence of molecular oxygen and NADPH (Kaden *et al.,* 1987; Kärenlampi, 1987). In particular, the metabolite AFB_1-8, 9 epoxide is considered to be responsible for the carcinogenic effect, due to its high ability to react with nucleophilic sites in macromolecular components (Leeson *et al.,* 1995). These derivatives have

the ability to form DNA-aflatoxin adducts resulting in malignant transformations and deletions (La Farge and Frayssinet, 1970). The formation of DNA-aflatoxin adducts results in malignant transformations and deletions (La Farge and Frayssinet, 1970) and sister chromatid exchanges (Potchinsky and Bloom, 1993). These findings may evidence the carcinogenity of AF. The immunotoxigenity of AF is the most important and well documented factor (Smith and Hamilton, 1970; Thaxton *et al.,* 1974; Giambrone *et al.,* 1978; Giambrone *et al.,* 1985a, b).

AFB_1-DNA adducts have also been shown to inhibit the synthesis of ribonucleic acid (Jeffery *et al.,* 1984), which results in inhibition of the synthesis of enzymes and other proteins such as antibodies (Hatch, 1988). Reddy and Sharma (1989) showed that AFB_1 depressed DNA synthesis in cultured splenic cells. Inhibition of protein and interleukin synthesis will result in inhibition of interaction and communication between cells of the lymphoid system (Dugyala and Sharma, 1996). AF have significant depressive effects on the cell-mediated immunity in the chicken, as measured by graft-versus-host and delayed hypersensitivity skin reactions (Giambrone *et al.,* 1978). The detrimental effects of AFB_1 on the histological structure of lymphoid organs may also cause a significant decline in the peripheral blood T-lymphocyte levels of the chicken (Ghosh *et al.,* 1991; Çelik *et al.,* 2000b). Neldon-Ortiz and Qureshi (1992a) and Çelik *et al.* (1996) reported that low levels of AFB_1 caused inhibition of phagocytic and microbicidal activity of peritoneal macrophages in the chicken. Moreover, Neldon-Ortiz and Qureshi (1992b) showed that AFB_1 administered in the early embryonic period caused similar effects on the macrophages of hatching chickens. Giambrone *et al.* (1978) reported that in chickens fed a diet of 2.5 mg/kg AF, the delayed-type hypersensitivity skin reactions to tuberculin were reduced. Ghosh *et al.* (1991) showed a significant decline in skin sensitivity and graft versus host reaction, which were used to evaluate cell mediated immunity in broiler chickens, even when a non-toxic dose was administrated. They also reported significant decreases in phagocytic activity of the splenic macrophages.

AFB_1 in food and foodstuffs may have detrimental effects on the lymphoid organs of the developing embryo. In chickens, the depression of the immune system functions via the decreasing number of B lymphocytes as a result of bursal damage (Ghosh *et al.,* 1991; Çelik *et al.,* 2000b). In the present study, in ovo administration of AFB_1 prior to incubation substantially diminished embryonic development of thymus, and also decreased ANAE-positive lymphocyte counts on the 21st day of incubation compared to the controls, although there were no histological differences between the thymuses of the groups.

The mechanism of the effects of AFB_1 in adult chickens has been clarified, but little is known about the toxicity on embryonic cells and the detoxification mechanisms of the cells in this period of the life. The liver and kidneys are not functionally developed before the 6th day of incubation (Hamilton *et al.,* 1983). Moreover, cell division and differentiation occur at high speed during this critical stage of morphogenesis (Kucera and Burnand, 1987; Çelik *et al.,* 2000a). Therefore, chicken embryos are very sensitive to the harmful effects of environmental agents in the early stages of embryonic development (Etches, 1996). The accumulation of embryonic death in the early embryonic stages observed in this study may show that the non-metabolized, intact AFB_1 molecule is also a highly toxic substance.

From the present study it was concluded that low level administration of AFB_1 in ovo at the beginning of early embryonic development profoundly affected the embryonic development of the thymus and decreased the ANAE positive peripheral blood lymphocyte percentages during the early embryonic period. Consequently, the AFB_1 in breeder diets should be questioned because AFB_1 transferred into the fertilized eggs may cause serious problems such as lower flock immunity, lower hatchability and lower viability of chicks.

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