Enzyme histochemical and serological investigations on the immune system from chickens treated in ovo with aflatoxin B$_1$ (AFB$_1$)

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SUMMARY

In this study, the detrimental effects of in ovo administrated aflatoxin B$_1$ (AFB$_1$) on the immune system of chickens in the post-hatching period were investigated by enzyme histochemical and serological methods. For this purpose, 730 laying hen eggs were divided into 7 groups [3 control groups (not-treated, drilled-sealed and 30% ethanol (solvent)-injected groups) and 4 assay groups in which eggs were injected with increasing AFB$_1$ doses (2.5, 7.5, 12.5 and 17.5 ng/egg)] then conventionally incubated. The chickens were vaccinated against the Newcastle Disease (ND) and the Infectious Bursal Disease (IBD) viruses on days 2, 5, 20 and on days 10 and 15 post-hatching, respectively. The serum antibody titres were assayed at the 1st and 28th days post-hatching. The proportions of the peripheral blood lymphocytes (PBL) and the ANAE (alpha-naphthyl acetate esterase) or ACP-ase (acid phosphatase) positive lymphocytes / PBL ratios were determined on the days 1, 10, 20 and 28. The AFB$_1$-treatment caused significant decreases in both the ANAE and ACP-ase positivity ratios of PBL depending on the AFB$_1$ dose, during the post-hatching period, but the PBL counts were significantly depressed at the end of the experimental period only in chickens treated in ovo by the highest AFB$_1$ dose (17.5 ng/egg).

The anti-IBD antibody titres measured on the hatching day (maternal antibodies) and the anti-ND and IBD antibody titres measured on the day 28 post-hatching were significantly reduced in chicks treated with 12.5 and 17.5 ng AFB$_1$/egg compared to the controls and have also gradually declined according to the AFB$_1$ dose. The results clearly evidenced that AFB$_1$ transferred into the fertilised eggs has caused deficiency in cellular and humoral immunity and particularly in the embryo transfer of the maternal antibodies, that can partially explain the observed immunosuppressive AFB$_1$ effects in poultry.

Keywords: Aflatoxin B$_1$, chicken, in ovo treatment, lymphocytes, antibody titre.

RÉSUMÉ

Etudes enzymo-histochimiques et sérologiques du système immunitaire chez des poussins traités in ovo par l’aflatoxine B$_1$ (AFB$_1$)

Au cours de cette étude, les effets délétères de l’administration in ovo d’aflatoxine B$_1$ (AFB$_1$) sur le système immunitaire des poussins après éclosion ont été recherchés par des méthodes enzymo-histochimiques et sérologiques. Pour cela, 730 œufs de poules pondeuses ont été répartis en 7 groupes [3 groupes contrôles (non traités, percés et scellés, injectés par le solvant (éthanol 30 %) et 4 groupes expérimentaux dans lesquels les œufs ont été injectés par des doses croissantes d’AFB$_1$ (2.5, 7.5, 12.5 et 17.5 ng/œuf)] puis incubés de façon conventionnelle. Les poussins ont été vaccinés contre le virus de la maladie de Newcastle (ND) 2, 5 et 20 jours après éclosion et contre le virus de la maladie infectieuse de la bourse de Fabricius (MIBF) 10 et 15 jours après éclosion. Les titres en anticorps sériques anti-ND et anti-MIBF ont été déterminés 1 et 28 jours après l’éclosion par respectivement un test d’inhibition de l’hémagglutination et par un test ELISA. Les proportions des lymphocytes sanguins périphériques et celles des lymphocytes ANAE (alpha-naphthyl acetate esterase, lymphocytes T) ou ACP-ase (phosphatase acide, lymphocytes B) positifs ont été mesurées les 1er, 10e, 20e et 28e jours après l’éclosion. Le traitement par l’AFB$_1$ a été responsable de diminutions significatives des proportions des lymphocytes ANAE positifs et des lymphocytes ACP-ase positifs au sein de la population lymphocytaire, dépendantes de la dose de toxine durant la période expérimentale cependant, la numération en PBL n’a été significativement ineffondée 28 jours après éclosion que chez les poussins ayant été traités par la dose la plus élevée d’AFB$_1$ (17.5 ng/œuf). Les titres en anticorps anti-MIBF mesurés le jour de l’éclosion (anticorps maternels) et ceux des anticorps anti-ND et anti-MIBF mesurés 28 jours après éclosion ont été significativement réduits par rapport aux contrôles chez les poussins traités par 12.5 et 17.5 ng d’AFB$_1$/œuf, mais ils ont aussi progressivement diminués en fonction de la dose reçue. Ces résultats montrent clairement que la contamination des œufs fertilisés par l’AFB$_1$ a provoqué une défaillance des réponses immunes cellulaires et humorales, et en particulier du transfert embryonnaire des anticorps maternels, ce qui peut en partie expliquer les effets immunosupresseurs de l’AFB$_1$ chez les poules.

Mots clés : Aflatoxine B$_1$, poussin, traitement in ovo, lymphocytes, titre en anticorps.

Introduction

Aflatoxins (AF) are toxic metabolites of fungi of the genus Aspergillus, particularly A. flavus and A. parasiticus. Although AF consist of AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$ [23], AFB$_1$ is the most toxic compound [7]. AF is a major problem on livestock health and productivity because aflatoxicosis results in anorexia, weak food utilisation, decreased body weight gain, decreased egg production, increased susceptibility to microbial and unspecified diseases and increased mortality as such in the other mycotoxicosis in poultry industry [27, 33].

The dietary AF and their metabolites infiltrate and accumulate most of the soft tissues and fat depots of chicken [5]. Previous researchers have reported different results for AFB$_1$ contents in the chicken egg. JACOBSON and WISEMAN [13] found 9 ng AFB$_1$/egg in the eggs from animals fed with diets containing 100 ppb dietary AFB$_1$, on day 10. However, SUDHAKAR [30] found 5 ng AFB$_1$/g of egg in the eggs of layers fed with...
a diet containing 600 ppb AFB\textsubscript{1} for 3 weeks, while OLIVEIRA et al. [25] found 6 ng AFB\textsubscript{1} in the eggs from layers fed with a diet containing 500 ppb AFB\textsubscript{1} for 8 weeks. Although the legal upper limits in food for laying hens are 10 μg/kg for AFB\textsubscript{1} and 20 μg/kg for AF [19], these limits were frequently exceeded. Surveys in Turkey have shown that AF concentrations were 5 to 100 ppb in foods and foodstuffs [22]. Moreover, TUNCER [35] also determined extremely high AFB\textsubscript{1} content in egg samples (approximately 120 ng/egg) obtained from Ankara, Turkey.

These results showed that dietary AFB\textsubscript{1} at legal limits might not cause important problems in human health but it may cause important problems in poultry, especially in parent stocks and hatchery. JELINEK et al. [14] determined the embryotoxicity limits for AFB\textsubscript{1} as 0.3 to 30 ng/egg and teratogenic dosages, they observed 33% embryonic mortality, 12% heart abnormalities and 0.04% disclosures of the body wall [14].

The most important problems caused by maternally transferred AFB\textsubscript{1} is functional defects in the immune system of the developing embryo [7, 31]. The functional impairment of the immune system increases the susceptibility to pathogen micro-organisms during growth and maturation of compromised chicks. Such animals develop a deficient resistance to infectious diseases and a reduced immunity after vaccination [2, 4, 9, 27]. Enzyme histochemical studies can be used to evaluate the functional development and maturation of the immune system [7, 31]. A lymphocyte lysosomal enzyme [20], alpha-naphthyl acetate esterase (ANAE) has been demonstrated in mature, immunocompetent circulating T-lymphocytes of many animal species. ANAE positivity has widely been used in mature, immunocompetent circulating T-lymphocytes of many animal species. ANAE positivity has widely been used to identify T and B lymphocytes and monocytes in various species including humans [6], chicken [18], cattle [15], dog [36], and mouse [20]. Acid phosphatase (ACP-ase) is one of the lysosomal enzymes in leukocytes. It was reported that ACP-ase was specific for T-lymphocytes in human [3]. However, some investigators have pointed that ACP-ase positive lymphocytes were derived from bursa of Fabricius in chicken [10, 29].

The present study was designed to evaluate the effects of in ovo administrated AFB\textsubscript{1} on the humoral immune response of chicken vaccinated against Newcastle disease (NDV) and Infectious Bursal Disease (IBD) during post-hatching period using enzyme histochemical and serological methods.

**Materials and Methods**

**ANIMALS AND EMBRYONIC EXPOSURE TO AFB\textsubscript{1}**

A total of 730 fertilised eggs of laying hens (Bowans-White) fed with standard diet obtained from a commercial hatchery regularly controlled for mycotoxins contaminations were used for experiments. The eggs were fumigated (80 g potassium permanganate in 130 mL 40% formaldehyde solution per m\textsuperscript{3} for 20 minutes) and divided into 7 groups as follows: non-treated controls (control 1, 95 eggs), drilled-sealed group (control 2, 93 eggs), solvent 30% ethanol-injected group (control 3, 96 eggs), injected with 2.5 ng AFB\textsubscript{1}/egg (assay 1, 101 eggs), 7.5 ng AFB\textsubscript{1}/egg (assay 2, 114 eggs), 12.5 ng AFB\textsubscript{1}/egg (assay 3, 115 eggs) or with 17.5 ng AFB\textsubscript{1}/egg (assay 4, 116 eggs). In the drilled-sealed group, the egg shells were drilled and immediately sealed with melted paraffin in a sterile cabinet.

The test solutions were prepared according to SUR and CELIK [32]. Briefly, pure AFB\textsubscript{1} obtained from Makor Chemical Co. (Jerusalem, Israel) was diluted in benzene to prepare a stock solution containing 20 ppm AFB\textsubscript{1}. This solution was then transferred into the vials containing the desired concentrations of AFB\textsubscript{1} for each dose group and left overnight to evaporate the benzene. The AFB\textsubscript{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\textsubscript{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 wavelength, and by a UV-VIS Recording Spectrophotometer (Shimadzu-UV 2100) using standards.

Injections of the test solutions were performed just prior to placing the eggs into the incubator. After drilling the shell at the blunt ends of the eggs, 20 μL of test solution was injected into the air sac [7]. 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 (Veyisoglu, Turkey) at 37.8°C, 65% relative humidity (RH). The eggs were turned every 2 hours. The chicks were housed in heated batteries under fluorescent lighting and consumed diets and water ad libitum. The possible mycotoxin contamination of the basal diet was determined before feeding by the method of HOWEL and TAYLOR [12] and it was found no detectable aflatoxin levels in diets (detection limit: 1 μg AF/kg food with 95% of recovery by the extraction method) [12]. Measurements were performed by TLC-densitometer (Perkin Elmer MPF 43-A). The hatched chicks received human care according to criteria outlined in the “Guide for the Care and Use of Experimental Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health.

**ENZYME HISTOCHEMICAL ANALYSIS**

On the hatching day and 10\textsuperscript{o}, 20\textsuperscript{o} and 28\textsuperscript{o} days after hatching, cardiac blood samples were taken into heparinised tubes (10 IU mL/blood). From each sample, 6 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 [32], two of the smears were used for ACP-ase demonstration [31] and the remaining ones were stained with May Grünwald-Giemsa [16] for the determination of peripheral blood lymphocytes (PBL) percentages.

All specimens were examined under a light microscope (Leica DM2500, Leica Microsystems GmbH, Wetzlar, Germany). In blood smears, the cells with lymphocyte morphology and having...
1-3 large, reddish-brown granules were classified as ANAE-positive T-lymphocytes while the lymphocytes having one to three reddish brown granules were evaluated ACP-ase positive B-lymphocytes. The positivity rates were determined by counting 200 lymphocytes. Similarly, PBL percentages were also determined by counting 200 leukocytes on the May Grünwald-Giemsa stained smears.

**SEROLOGICAL ANALYSIS**

Before vaccinations, the maternal antibody titres were detected in sera prepared from blood samples taken at the 21st day of incubation (one day old chicks). All chicks were vaccinated on the day of hatching with Newcastle Disease (ND) vaccines (B1 La Sota strain, Intervet International B.V. Holland) by aerosol-spray. On the 5th and 20th days after hatching the chicks were orally vaccinated with ND vaccines (La Sota strain, Intervet International B.V. Holland). All chicks were orally vaccinated with Infectious Bursal disease (IBD) vaccines on the 10th and 15th days after hatching. The final orally vaccinated with Infectious Bursal disease (IBD) vaccine strain, Intervet International B.V. Holland). All chicks were orally vaccinated with ND vaccines (La Sota strain, Intervet International B.V. Holland). All chicks were vaccinated on the day of hatching with Newcastle Disease (ND) and the Infectious Bursal Disease (IBD) were determined by Haemagglutination-Inhibition Test (HI) and ELISA respectively as described by ERGANIS and ISTANBULLUOGLU [8].

**STATISTICAL ANALYSIS**

The proportions of PBL, and ACP-ase and ANAE positive lymphocytes were analysed with one-way ANOVA (SPSS, 10.0) following arc-sin transforming [37]. The serological results were statistically analysed using Man Whitney-U and Duncan for ND and IBD, respectively. Results were considered as significant when *P* values were less than 0.05.

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<th>Groups</th>
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<td>17.5 ng AFB₁/egg</td>
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Under each value the same superscripts indicate no significant differences between groups.

Different superscripts a,b,c in a same column indicate significant differences (*P* < 0.05) between groups.

Table I. Serum antibody titres against the viruses of the Newcastle disease (ND) and the Infectious Bursal disease (IBD) measured on the hatching day (day 1) and 28 days later in chickens from eggs incubated with various AFB₁ doses (2.5, 7.5, 12.5 and 17.5 ng AFB₁/egg) or not AFB₁ treated (control 1: not treated eggs, control 2: drilled-sealed eggs and control 3: solvent 30% ethanol treated eggs). Results are expressed as median for anti-ND antibodies and as mean ± standard error for anti-IBD antibodies.

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*Results*

The serum anti-ND and anti-IBD antibody titres determined on the hatching day and 28 days later in chickens from eggs incubated with the various AFB₁ doses were summarized in Table I. In control and assay groups, chickens exhibited maternal antibodies measured on the hatching day. However, the titres of the anti-IBD maternal antibodies gradually and significantly declined according to the AFB₁ dose injected in eggs (for 12.5 and 17.5 ng AFB₁/egg: *P* < 0.05). Although the differences between groups were not significant, the same tendency was also noted for the anti-ND maternal antibodies. Whatever the group, chickens have responded to vaccinations as shown by the observed increases in the serum antibody titres 28 days after hatching but the significantly lowest titres of anti-ND and anti-IBD antibodies compared to controls were obtained in birds hatched from eggs inoculated with 12.5 and 17.5 ng/egg (*P* < 0.05). Although there was no statistical difference in the antibody titres against to ND and IBD between the groups treated with 2.5 and 7.5 ng AFB₁/egg, the decreasing in the antibody titres was even conspicuous.

As shown in the Table II, the variation profiles of the proportions of the total peripheral blood lymphocytes and of the ANAE positive cells according to time were similar whatever the egg groups, with maximal values reached 10 and 20 days after hatching. However, the AFB₁ treatment at the dose of 17.5 ng/egg has dramatically depressed the whole proportions of lymphocytes during the whole hatching period compared to the control groups (*P* < 0.05) and to chickens stemming from eggs inoculated with the 2 lowest doses (2.5 and 7.5 ng/egg) (*P* < 0.05). Chickens stemming from eggs inoculated with 12.5 ng AFB₁/egg exhibited also a significant decrease in the PBL proportions 10 days after hatching compared to the controls (*P* < 0.05). In parallel, the ANAE positive lymphocytes / PBL ratios were also markedly decreased on the whole hatching period in chickens treated in ovo by 17.5 ng AFB₁/egg (*P* < 0.05). Furthermore, this parameter was also significantly altered in chickens treated with 12.5 ng AFB₁/egg compared to the controls (*P* < 0.05 except for the 20th day after hatching) but not so deeply than in the group treated with the higher dose (12.5 ng/egg vs. 17.5 ng/egg: *P* < 0.05). Interestingly, 28 days after hatching, the ANAE positive lymphocytes / PBL ratios were not significantly lower than those in the controls (*P* < 0.05)
AFB1 has not only significant depressive effects on the cell-mediated immunity but also has adverse effects on humoral immunity in chicken. It was stated that chickens with feeding with AFB1 containing diet exhibited increased susceptibility against ND and IBD. OGUZ et al. [24] have reported low antibody titres of IBD (Table I). Tressler et al. [28] have demonstrated the embryotoxic effects of AFB1 in food and foodstuffs might have detrimental effects on the lymphoid organs of the developing embryo. Especially, inhibition of the embryonic development of thymus and bursa of Fabricius cause functional deficiencies in the immune system. SUR and CELIK [31, 32] have demonstrated the embryotoxic effects of in ovo administrated AFB1 on bursa of Fabricius and thymus. The same investigators [32] have reported that the administration of AFB1 prior to the incubation resulted in decreasing T-cell counts. Moreover, QURESHI et al. [27] have observed a significant reduction in the antibody titres against sheep red blood cells (SRBCs) and Brucella abortus in chickens from hens consuming AFB1-amended diet. Similarly, NELDON-ORTIZ and QURESHI [21] have shown that AFB1 administered at the early embryonic period caused inhibition of phagocytic and microbicide activities of macrophages in hatching chickens. In the present study, it was observed that the in ovo administration of AFB1 prior to the incubation, the ANAE- and ACP-ase positive lymphocyte counts and antibody titres against ND and IBD were found significantly lower compared to the controls after hatching period. As the ACP-ase positive lymphocytes are considered in chickens as B-lymphocytes able to differentiate into antibody producing plasma cells after antigenic stimulation [28], the decrease in their counts may mediate the reduction of the antibody titres against ND and IBD.

The unexpected results obtained from the present study were the maternal antibody titres of IBD (Table I). Tressler
and Roth [34] have reported that the transfer of the maternal antibodies from yolk to the embryonic circulation corresponds to a receptor-mediated endocytosis process similar to that observed in other species including mammals. This event occurs at pH 6.0, which is the yolk pH. One of the most acceptable reasons for the lower maternal antibody titres against to IBD observed in AFB1-treated eggs in the present study may be an unfavourable pH resulting from the in ovo AFB1 administration prior to incubation. On the other hand, AFB1 has caused retarded development of embryonic cells coupled to the inhibition of some protein and enzyme synthesis. In this way, the receptor-mediated endocytosis process starting on the embryonic day 7 may be impaired. Another hypothesis for low maternal antibody titres may be the decreased consumption of the yolk which contains the maternal antibody. Indeed, OZAYDIN [26] has shown that the yolk consumption was decreased under various circumstances such as heat stress, embryotoxic agent... Moreover, in this study, the AFB1 transmission from dams to eggs was imitated. In natural occurrence of aflatoxicosis, these results might be more serious because the intoxication firstly affects the dam immune system. GRINDSTAFF et al. [11] reported that the maternal physiological condition has profound effects on the offspring immunity and they found a significant positive correlation between immunoglobulin concentrations in maternal circulation and immunoglobulin as a significant positive correlation between immunoglobulin concentrations in maternal circulation and immunoglobulin concentrations in eggs. Since the newly hatched chicks have not fully developed immune system, maternal antibodies have an important role in protecting them against specific or non-specific diseases [1]. Especially, the IgG in newly hatched chicks is thought to be maternally derived because IgG-secreting cells are firstly noted at the 6th day post hatching in chickens [17].

Myco toxin contamination in poultry food and foodstuffs may be a serious veterinary problem because of the residues in fertilised eggs. Especially, the AFB1 carry-over from food to the fertilised egg leads to economic losses because of the reduction in the embryo viability and hatchability [27]. The administration of a low concentration of AFB1 in ovo at the beginning of early embryonic development may not profoundly affect the embryonic development of the bursa and thymus but decrease the proportions of ANAE and ACP-ase positive peripheral blood lymphocytes and antibody production against to ND and IBD during the post-hatching period. It is suggested that breeder diets should be investigated for aflatoxins and specifically AFB1, in cases of low hatchability and flock immunity, because low concentrations of AFB1 in fertilised eggs might trigger more serious troubles until the problem was really evidenced.

As a conclusion, results obtained in this study suggest that in ovo administrated AFB1 reduces the immune response and decrease the response to vaccines, increasing the chicken susceptibility to infections. However, in the present study, as the immunoglobulin types which were lower in the contaminated eggs compared to the control eggs were not identified, it was difficult to adequately explain the significant decrease in the maternal antibodies against the IBD in AFB1 treated eggs. Consequently, further studies may be conducted to detect immunoglobulin types and their transport route in order to elucidate the disruption in maternal antibody transport mechanisms during aflatoxicosis.


22. NIZAMLIOGLU F.: Determination of aflatoxin B1, B2, G1 and G2 in feeds and feedstuffs which were brought to Konya province laboratory. *Veterinarium*, 1996, 7, 42-45.


