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Research Article

Effects of the Aflatoxin B1 Given in Ovo on the Histomorphological Changes of Developing Cerebellar Cortex and the AgNOR Activity of the Purkinje cell Nuclei of Chickens

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Abstract

In this study, the effects of in ovo administrated aflatoxin B1 (AFB1) on the histomorphological changes of developing cerebellar cortex and the AgNOR activity of the Purkinje cell nuclei of chickens in the post-hatching period were investigated. 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 AFB1 doses (2.5, 7.5, 12.5 and 17.5 ng/egg)] then conventionally incubated. Tissue samples were taken from six animals of each control and experimental groups on the hatching day and 10th, 20th and 28th days after hatching. The samples, which were fixed in 4% neutral-buffered formalin, were dehydrated, cleared, and embedded in paraffin. The sections taken from the paraffin blocks were stained with haematoxylin-eosin and AgNOR staining methods. Quantitative computer assisted morphometric study was done on the cortical layer of the cerebellum. Histomorphometric examination revealed that there were no significant differences among the groups in the thicknesses of molecular and granular cell layer at summit and in the fissure of folium of the cerebellar cortex and AgNOR parameters of Purkinje cell nuclei. In conclusion the administration of a low concentration of AFB1 in ovo at the beginning of early embryonic development may not profoundly affect the development of the cerebellar cortex or the damage to the cerebellum, if occurring, might get repaired during subsequent stages of development.

ABBREVIATIONS

AF: Aflatoxin; AFB_1: Aflatoxin B_1; CNS: Central Nervous System; NORs: Nucleolus Organizer Regions

INTRODUCTION

Aflatoxins (AFs) are extremely toxic metabolites of *Aspergillusflavus*and *Aspergillusparasiticus* [1,2]. Although AFs consist of a group of approximately 18 related fungal metabolites, only AFB_1, AFB_2, AFG_1, and AFG_2 have been detected in food and foodstuffs [3]. Among these AFs, AFB_1 is one of the most important food borne mycotoxins due to its biological effects and widespread toxicity [4,5]. AFB_1 induces acute toxicity in most animal species and is known as being one of the most potent genotoxic agents and hepatocarcinogenes [6]. Teratogenic effects of AFB_1 have also been reported in some species [5,7-12].

AFs causeeconomic losses to poultry production because aflatoxicosis results in listlessness, anorexia, poor food utilization, decreased body weight gain, decreased egg production, increased susceptibility to microbial and unspecified diseases and increased mortality [13]. In addition the dietary AF and their metabolites infiltrate and accumulate most of the soft tissues and fat depots of chicken, and they can also be transferred to eggs [14]. In poultry food and food-stuffs, this contamination and residue may have little significance for human health, but it can be a serious veterinary problem in the poultry industry because of the residues in fertilized eggs [15].

The central nervous system (CNS) is well protected by the blood-brain barrier and neurotoxicity often involves peripheral

nervous system effects in adults. However developing CNS at prenatal and early post natal periods is more susceptible to several factors induced neurotoxicity than in adults due to the lack of a protective barrier. A large number of processes occur at the different stages of development such as cell proliferation, migration, differentiation and apoptosis. Chemicals can freely enter the developing CNS and may exert deleterious effects by interfering with these processes [16].The main mechanisms of mycotoxin toxicity include stimulation of lipid peroxidation, apoptosis and inhibition of DNA, RNA and protein synthesis [17].There is a delicate balance among cell proliferation, cell differentiation and apoptosis in the developing embryo; impairment in these mechanisms due to mycotoxins might have been responsible for the anomalies observed [18]. Although the microscopic examination of serial sections of visceral organs may detect cellular alterations due to AFB_1, the histological evaluation associated with the effects of AFB_1 on the developing CNS are still lacking.

The cerebellum is one of the best studied regions of the CNS because of its well-defined cytoarchitecture and pathways of cerebellar cell migration during neurogenesis [19].The key cellular elements of the cerebellar cortex include granule cells and the Purkinje cells. Both cell types have properties that make them attractive to experimental investigations and thus these cells are widely used in many studies of cerebellar development [20].

Nucleolus organizer regions (NORs) are loops of DNA that contain genes responsible for the transcription of ribosomal RNA [21]. Since proteins of these regions can be identified as small black dots in the nucleus using silver staining techniques, they are named AgNORs [22]. An increase in the number and size of AgNORs in interphase nuclei highly correlates with cellular hyperactivity and might give a valuable indicate of the proliferation rate, differentiation process and secretory activity of a given cell. In addition these changes appear to be of value in aiding the diagnosis in malignant transformation [23-26].

Neurotoxicity is usually defined as a structural changes or a functionally adverse response of the nervous system, resulting from exposure to a chemical, biological or physical agent [16]. The present study was carried out to evaluate the possible neurotoxic effects of in ovo administrated AFB_1 on the histomorphological changes of developing cerebellar cortex and determine some AgNOR parameters of Purkinje cells of chickens during post-hatching period.

MATERIALS AND METHODS

Preparations of AFB_1 solutions

The test solutions were prepared according to Sur and Celik [27]. Briefly, pure AFB_1 obtained from Makor Chemical Co. (Jerusalem, Israel) was diluted in benzene to prepare a stock solution containing 20 ppm AFB_1. This solution was then transferred into the vials containing the desired concentrations of AFB_1 for each dose group and left overnight for the benzene to evaporate. The AFB_1 residue was dissolved in absolute ethanol (99.9%), which was then reduced to 30% with sterile bi-distilled water. 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 an ultraviolet-visible recording spectrophotometer (UV 2100; Shidmadzu, Nakagyo/KU, Kyoto, Japan) using standards.

Animals and embryonic exposure to AFB_1

For the experiment, 730 fertile eggs of laying hens (Bowans-White) were used. The eggs were fumigated with 80 g potassium permanganate in 130 ml 40% formaldehyde solution/m^3 for 20 minutes. The eggs were 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_1/egg (assay 1, 101 eggs), 7.5 ng AFB_1/egg (assay 2, 114 eggs), 12.5 ng AFB_1/egg (assay 3, 115 eggs) or with 17.5 ng AFB_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.

Treatments 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 space [28] using micropipettes (Sealpette, Jencons, Finland) with sterile tips. After the injections, the holes were immediately sealed with melted paraffin. The eggs were placed in an incubator (Veyisoglu, Turkey) maintained at 37.8°C, 65% relative humidity (RH) and 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 [29] 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) [29]. 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.

Histological investigations

Tissue samples were taken from six animals of each control and experimental group on the hatching day and 10^{th}, 20^{th} and 28^{th} days after hatching. The samples, which were fixed in 4% neutral-buffered formalin, were dehydrated, cleared, and embedded in paraffin. The sections taken from the paraffin blocks were stained with haematoxylin-eosin [30] and AgNOR [31] staining methods.

AgNOR staining

The staining solution was prepared by mixing one volume of 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate. The sections were stained at 37°C in the dark for 20-30 min to reach the optimal results. At the end of this period, the sections were 3 times rinsed with distilled water, dehydrated through graded ethanol to xylene and covered with entellan (Merck).

Evaluation of specimens

All specimens were examined light microscope (Leica DM-2500 model with DFC-320 camera attachment giving digital

images). Morphometric analyses of cerebellar cortex including thickness of molecular and granular cell layer at summit and in the fissure of folium were measured from five different areas of the sections from each animal.

To determine AgNOR parameters, 25 Purkinje cells with clearly visible nucleoli per section in five different fields from each animal were examined. The areas of the nucleus, and the areas and number of AgNORs per cell nuclei were determined. Also, the percentage of the AgNOR area relative to the whole nuclear area was calculated. All measurements were performed with IM-50 image analysis programme.

Statistical analysis

The parameters were analyzed by one-way analysis of variance and followed by post hoc Duncan multiple comparisons test using the Statistical Package for Social Sciences (SPSS version 10.0; SPSS Inc. Corp., USA). Results were considered at significant at $p<0.05$.

RESULTS

General histologic structure and histomorhology

The cerebella cortex completed its development in all animals of all groups. It was divided into three layers. The outmost molecular cell layer was composed of small neurons and glia cells. Granular cell layer adjacent to medulla was made of small, tightly packed neurons characterized by their great number of dark nucleus and small amount of cytoplasm. The middle layer, the Purkinje cells layer, formed the border between the molecular and granular layers. The mean thickness of molecular and granular layer at summit and fissure are shown in (Figure 1). The molecular layer was thicker in the fissure than at the summit of folium and thickness of granular layer was thicker at the summit than in the fissure of folium. Also the mean thickness of the molecular and granular layers increased through the entire experimental period. However there was not any significant difference in morphological development and the measured parameters of the cerebellar cortex among the groups.

AgNOR parameters of the purkinje cells

AgNORs were observed as black dots having eccentric locations in cell nuclei (Figure 2). The number of the AgNORs per Purkinje cell nuclei 1 or 3. The mean area of the Purkinje cell nucleus and the AgNOR area increased through the entire experimental period. However there were no differences in the mean area of the Purkinje cell nucleus, AgNOR area, the mean ratio of AgNOR area to Purkinje cell nucleus area and AgNOR counts among the groups (Figure 3).

DISCUSSION

AFB_1 is able to cross the maternal placental barrier to reach the fetus and offers a potential threat to animal and human health in view of their teratogenicity [10,11]. The teratogenic, embryotoxic effects and developmental toxicity of AFB_1 have been reported in some species [5,7-12]. In poultry, the AFB_1 carry over from food to the fertilized egg leads to serious economic

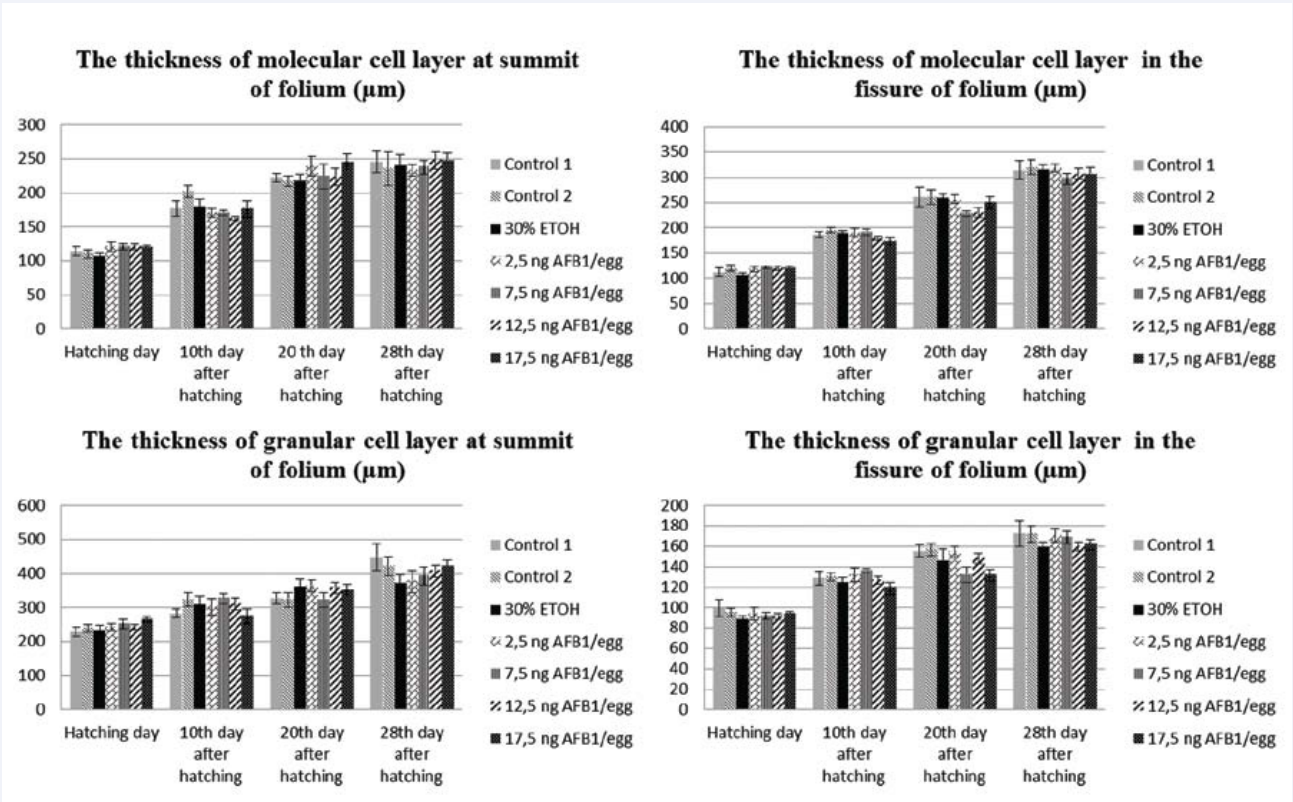

Figure 1: The mean thickness of different cerebellar cortex layers. Results are presented as means, and bars are SD. The differences among the groups are not significant ($p>0.05$).

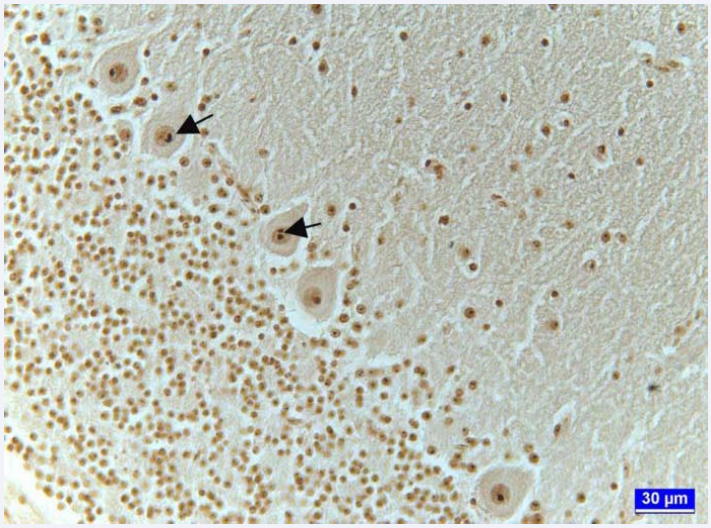

Figure 2: Histological section from the cerebellum of 28-day-old chicken from the control group. Arrows indicate AgNORs in the Purkinje cell nuclei. AgNOR staining.

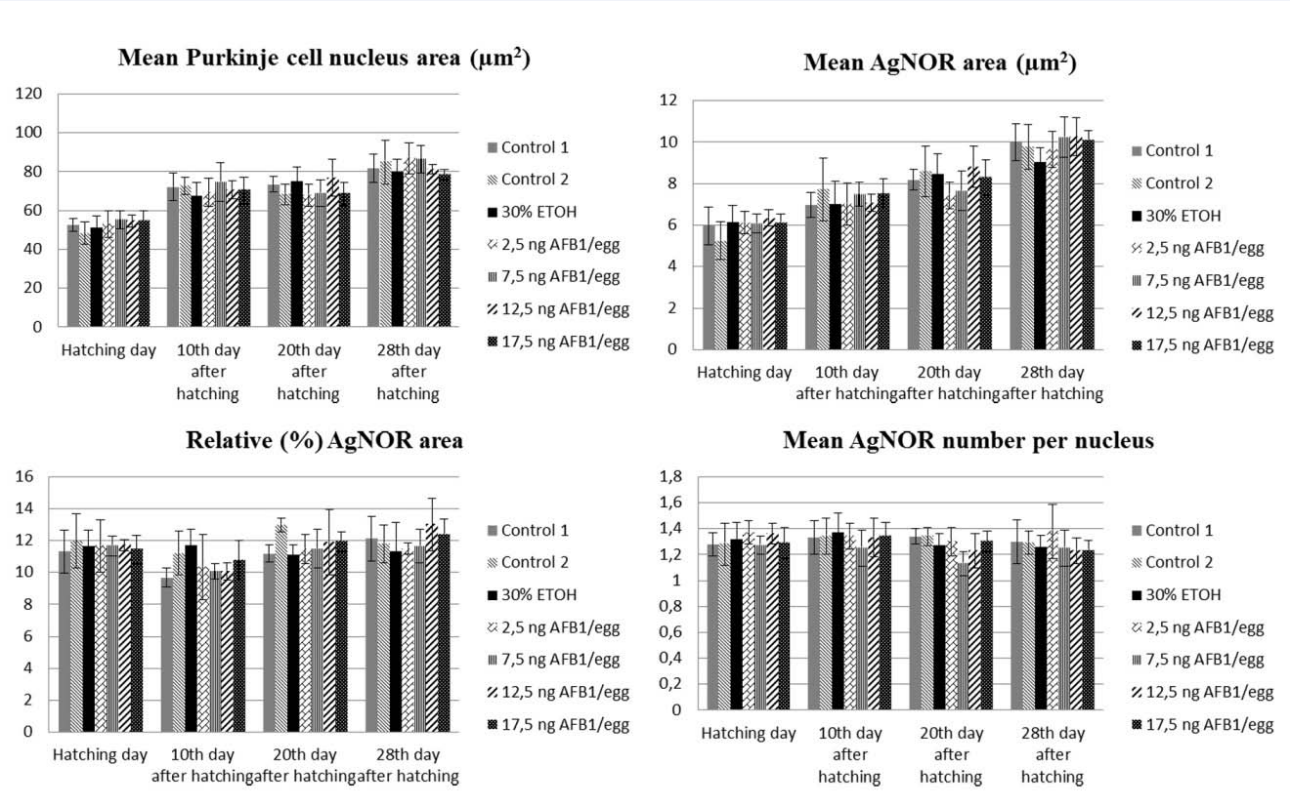

Figure 3: AgNOR parameters of Purkinje cell nuclei. Results are presented as means, and bars are SD. The differences among the groups are not significant (p>0.05).

loss by decreasing embryo viability and hatchability [32] and by causing organ malformations [33]. Previous studies in our laboratory demonstrated that the administration of AFB_1 in ovo at the beginning of early embryonic development induced immune depression [2,27,32,34], and muscle and skeletal malformations [35-37] in developing chick embryos.

Jelinek *et al.*, [38] determined the embrytoxicity limits for AFB_1 as 0.3 to 30 ng/egg and teratogenicity limits as 3 to 30 ng/egg using the Chicken Embryotoxicity Screening Test-I (CHEST-I). Furthermore, at these doses, they observed 33% embryonic mortality, 12% heart abnormalities and 4% non-closure of the body wall at embryotoxic and teratogenic concentrations [38]. In

the present study, AFB_1 administration in ovo at the beginning of early embryonic development was performed with doses of 2.5, 7.5, 12.5 and 17.5 ng/egg. Our previous studies [2,37] showed that the high doses of AFB_1 caused embryonic death and /or decreased hatching performance. Also, if we had administrated excessively high doses, our results would have not reflected the field situations. Besides, we know the legal upper limit in our country. When this knowledge was taken into account, we preferred the low doses of AFB_1 to evaluate the effects of AFB_1 on the post-hatching development of the cerebellum. Consequently, we thought that the doses of AFB_1 using in this study were more reasonable for us.

The behavioral teratogenic effects of AFB_1 have been also studied in rat offspring of dams injected with a subteratogenic dose of AFB_1 during mid or late organogenesis and it was suggested that prenatal exposure to AFB_1 produced a delay of early response development, impaired locomotor coordination, and impaired learning ability in the rat offsprings [4]. The cerebellum regulates of equilibrium of the body by making co-ordination of somatic motor activity and by regulating the muscle tone [39]. In addition, it is involved in some non-motor cognitive functions such as sensory discrimination, attention, learning and memory [40]. In this study, the effects of in ovo administrated AFB_1 on the histomorphological changes of cerebellar cortex of chickens during post-hatching period was evaluated. The mean thickness of the molecular and granular layers increased through the entire experimental period. However there was not any significant difference in morphological development and the measured parameters of the cerebellar cortex among the groups. Also there weren't any behavioural changes to be observed with the chicks.

The mechanism of teratogenicity caused by AFB_1 has not been fully elucidated. It was suggested that the toxin arrested mitosis and induced teratogenesis due to reduction in cell proliferation during early morphogenesis [10]. Russel *et al*., [41] reported that the occurrence of AgNORs reflect proliferative activities of the cell. However, they claimed that an increase in the size of AgNOR clusters rather than their number was the major feature. Akar and Sur [19] suggested that investigating Purkinje cells as a model by applying the AgNOR technique offers a good approach to the functional study of structures in the central nervous system. In the present study, the nuclei of Purkinje cells and the AgNOR area increased through the entire experimental period. However there were no significant differences among the groups in AgNOR parameters.

CONCLUSION

The administration of a low concentration of AFB_1 in ovo at the beginning of early embryonic development may not profoundly affect the development of the cerebellar cortex or the damage to the cerebellum, if occurring, might get repaired during subsequent stages of development. There is a dose-related increase in the detrimental effects of AFB_1 [34].Therefore further studies, utilizing various doses higher than those used in the present study, may be conducted to evaluate the possible neurotoxic effects of AFB_1 on development of cerebellar cortex.

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