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Immunotoxic Action of Aflatoxin B1 Against Lymphoid Organs is Coupled with the High Expression of Bcl-2 by Reticulo-Epithelial Cells in Broiler Chickens

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Abstract: Aflatoxins are a significant threat to chicken welfare and production due to their presence in contaminated feedstuff. This study aimed to evaluate any immunosuppressive and proto-oncogenic effects of Aflatoxin B1 in experimentally dosed chickens. The 46 days old Ross 308 broiler chickens were treated with Aflatoxin B1 (50 μg kg^{-1} BW diluted in sunflower oil, per os) for up to 28 days. Every 7 days, five treated and five control chickens were euthanized to determine changes in lymphoid organs, particularly and the thymus and bursa of Fabricius. Tissue changes were evaluated by conventional histology and electron microscopy as well as by immunohistochemistry for Bcl-2 expression. The Aflatoxin B1 exposure induced significant reductions in body weight and in the weight of the lymphoid organs at the end of the experiment. In parallel, severe thymocyte and lymphocyte depletion, considered due to apoptosis was evidenced in the thymus and bursa of Fabricius whereas reticulo-epithelial cells proliferated and strongly expressed the Bcl-2 protein. These results demonstrate the severe immunosuppressive effects of Aflatoxin B1 on B and T cells in chickens and its proliferative action on the reticulo-epithelial cells involved in lymphocyte maturation, suggesting a proto-oncogenic action.

Key words: Aflatoxin, poultry, immunosuppression, immunohistochemistry, electron microscopy

INTRODUCTION

Aflatoxins are a group of heterocyclic metabolites predominantly synthesized by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and may cause severe economic losses in poultry due to their presence in feedstuff as contaminants. The toxicity of aflatoxins in broiler chickens has been widely investigated by determination of their carcinogenic, toxigenic, mutagenic, teratogenic (Wild *et al*., 2000; Wild and Turner, 2002; Verma *et al*., 2004; Kusumaningtyas *et al*., 2006) growth inhibitory and immunotoxic effects (Ehrich *et al*., 1988; Oguz and Kurtoglu, 2000; Bokhari *et al*., 2003). Leukocyte changes, depressed antibody formation (Wangikar *et al*., 2005) and increased susceptibility to diseases have been related to the rates of metabolism and elimination of aflatoxin. The bursa of Fabricius and thymus are susceptible to aflatoxicosis. The bursa of Fabricius in contrast to other lymphoid organs, decrease in weight with increased aflatoxin dose (Ehrich *et al*., 1988; Kumar and Balachandran, 2009). Until now the mechanism of these changes is not clear.

The chicken *Bcl-2* gene is very similar to that of human *Bcl-2* gene. The expression of human *Bcl-2* gene is de-regulated by t (14;18) translocation in most of follicular lymphoma (Eguchi *et al*., 1992). It was reported that expression of Bcl-2 not only suppresses apoptosis but induces cell proliferation. Alternatively, Bcl-2 can be associated with nuclear matrix (Wang *et al*., 1998) suggesting its possible role in genomic organization, function and regulation linked through nuclear architecture (Huang and Cidlowski, 2002).

Overexpression of Bcl-2 causes tissue hyperplasia and promotes tumor development (Vaux *et al*., 1988; Strasser *et al*., 1990). Bcl-2 were expressed in the majority in the thymomas and the staining reaction was stronger in the clinically malignant ones (Engel *et al*., 1998). Many tumors harbor genetic, epigenetic or post-transcriptional alterations that result in overexpression of the anti-apoptotic protein Bcl-2 (Tsujimoto *et al*., 1985; Cimmino *et al*., 2005; Gao *et al*., 2010).

The study aimed to identify that immunosuppressive effect of Aflatoxin B1 (AFB1) on lymphoid organs and immunocompetent cells is associated with overexpression

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of Bcl-2 and proliferative action on reticulo-epithelial cells of lymphoid organs, suggesting a proto-oncogenic effect.

MATERIALS AND METHODS

Animals and experimental protocol: A total of 40 Ross 308 broiler chickens, 6 days old were used in the study. After 1 week period of acclimation to the provided living conditions, birds were randomly divided in 2 groups (Experimental (E) and Control (C)) with similar average weights (79.03±0.73 g in the group E and 80.1±0.63 g in the group C). Chicks were reared on sawdust litter and specific microclimate conditions adapted to their age were provided: the room temperature gradually decreased from 32-24°C and permanent lighting was established. Commercial-type food, free of AFB1 was administered *ad libitum*.

In the E group, the Aflatoxin B1 (AFB1-Sigma Chemicals Co.) dissolved in sterilized sunflower oil at a dose of 50 μg kg^{-1} BW was daily administered to birds by gavage for 28 days. The control group received only the solvent (sterilized sunflower oil).

At the end of each week, the chickens were individually weighed. Five chickens were randomly selected from each group and were euthanized by an overdose of thiopentone sodium at 7th, 14th, 21st and 28th days of the experiment. The whole body weight and that of lymphoid organs was recorded. All animal experimental procedures had been conducted in accordance with Romanian legislation.

Histopathology: Histopathological investigations were performed on thymus and Fabricius bursa fragments from each experimental group. The tissue fragments were fixed in 10% formalin solution, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin-eosin.

Immunohistochemistry: Fixed samples were trimmed, embedded in paraffin, sectioned at 5 μm then dewaxed and the epitopes were revealed by heating at 95°C in 10 mmol citrate acid buffer pH 6 for 10 min in a microwave and then they were left at room temperature for 20 min. The slides were then washed twice in PBS (pH 7.5) for 5 min. Tissue sections were incubated with primary anti-Bcl-2 antibodies (diluted 1:100) at room temperature in a humid chamber for 1 h. After being washed with PBS, slides were incubated with the secondary antibody, HRP Goat anti Mouse IgG. for 1 h in a humid chamber at 4°C then washed with PBS and incubated with the DAB substrate for 7 min and counter-stained with Harris hematoxylin, clarified in xylene and mounted. Using a light microscope (Olympus CX 41) four samples from each chicken were randomly evaluated (at a magnification of 90x, 10 fields).

Electron microscopic investigations: Cell ultrastructure was assessed in fixed liver samples. Briefly, control and treated liver tissues were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, post-fixed in 2% OsO_4, dehydrated in an ethanol series and embedded in Epoxy 1510. Ultrathin sections were prepared on a Reichert OmU3 (Austria) microtome, stained with 1% uranyl acetate and 1% phosphotungstic acid, viewed and photographed with a Phillips CM 100 transmission electron microscope. From each sample, three or more 300-mesh copper grids were examined in a blinded fashion and apoptotic changes in lymphocytes from the thymus and Fabricius bursa of chickens dosed with AFB1 were evidenced.

Statistical analysis: In order to compare the control group to experimental group, student test was applied. The minimum level of significance was fixed at 0.05.

RESULTS

During the experiment the body weight and weight of lymphoid organs (thymus, Fabricius bursa) was significantly reduced in AFB1 dosed chickens (Table 1). The histological profile of the control chickens showed normal thymic and Fabricius bursal structure and Bcl-2 expression was undetectable or rarely detectable by immunohistochemistry during the experiment (Fig. 1a, b).

After 7th day of AFB1 dosing, thymus changes were not evident only a clear distinction between cortex and medulla was observed in hematoxylin-eosin staining. The staining of Hassall's corpuscles was typical as control slides demonstrated the same pattern. After 14th day of AFB1 dosage, the thymus cortex and medulla become equally atrophic with hydropic degeneration of

Table 1: Body weight and weight of lymphoid organs of chickens dosed with AFB1 (group E) or control chickens (group C)

Weight (g)*	Groups	7th day	14th day	21st day	28th day
Body	C	210.50±1.12	420.10±1.02	793.90±1.31	1653.10±1.20
	E	128.50±1.79**	275.30±2.21**	428.20±2.31**	898.30±1.90**
Thymus	C	0.11±0.02	1.19±0.06	2.29±0.04	1.68±0.12
	E	0.08±0.02	0.22±0.02**	1.42±0.02**	0.94±0.06**
Fabricius bursa	C	0.45±0.06	1.33±0.03	2.61±0.08	1.45±0.03
	E	0.16±0.02**	0.85±0.32**	0.37±0.26**	0.16±0.03**

*Mean±Standard deviation; **p<0.05

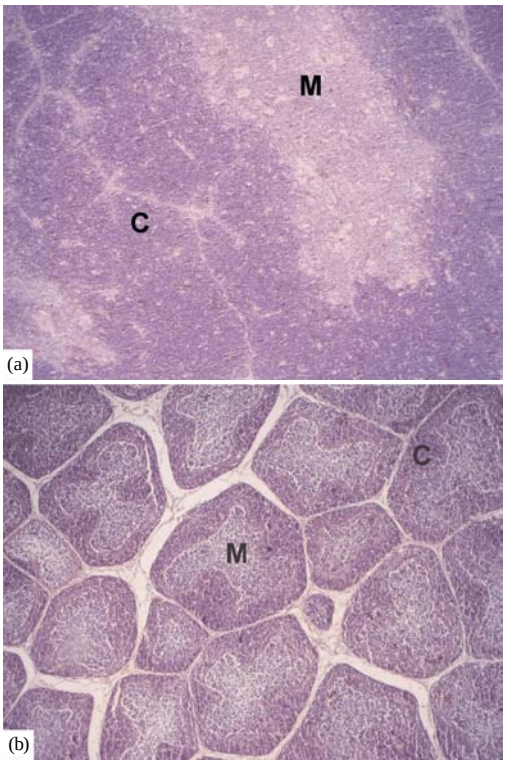

Fig. 1: a) Thymus in control group at 28th day. Cortical zone (C) and Medullar zone (M) Bcl-2 IHC staining x200 and b) Fabricius bursa in control group at 28th day. Cortical zone (C) and Medullar zone (M) Bcl-2 IHC staining x200

reticulo-epithelial cells and necrotic foci. Severe lymphoid depletion was seen in the thymus cortex whereas the reticulo-epithelial cells between medullary and cortical zones had characteristics of proliferation and were positively Bcl-2 immunolabelled. The thymus changes consisting in a severe atrophy of the cortex and presence of small necrotic foci were obvious after day 21st and became more and more severe up to day 28th of AFB1 dosage (Fig. 2).

Also, a marked proliferation of the reticulo-epithelial cells in the thymic reticulum network and in the medullar zone and an abundance of mucous cells, zone of necrosis, small mucous cysts and cystiform Hassal's were observed. In the thymic cortex, a high number of Bcl-2 positive cells were observed (Fig. 3a and b). The number of reticulo-epithelial cells immunohistochemically Bcl-2 positive in thymus from the group receiving AFB1 increased progressively during the weeks of the experiment (Table 2).

In Fabricius bursa, a mild to severe atrophy of the medullar lymphoid follicles coupled to small necrotic foci

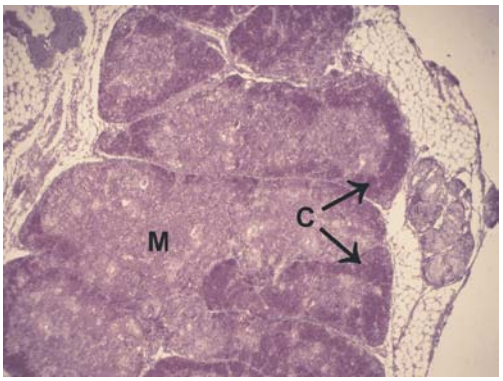

Fig. 2: Thymus injury after 28th day of AFB1 intoxication in chickens. Small necrotic foci in medulare zone and severe cortical atrophy. Cortical zone (C) and Medullar zone (M). Haematoxilin-Eosin staining x60

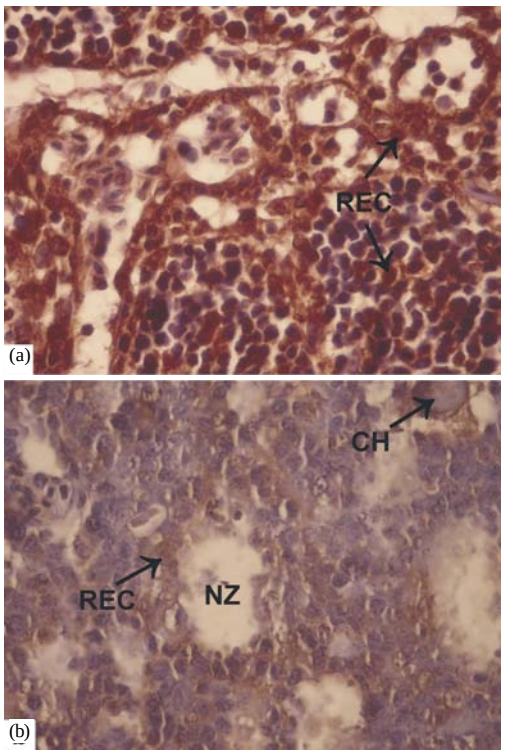

Fig. 3: a) Thymus injury after 21st day of AFB1 exposure. The Reticulo-Epithelial Cells (REC) were proliferated and Bcl-2 immunoreactive (arrows). Bcl-2 IHC staining x900 and b) Thymusinjury after 28th day of AFB1 exposure. The Reticulo-Epithelial Cells (REC) were proliferated and Bcl-2 immunoreactive (arrows), Necrotic zone (NZ), small mucous cysts and Cystiform Hassal's (CH). Bcl-2 IHC staining x900

Table 2: Effect of AFB1 on detection of Bcl-2 positive reticulo-epithelial cells in thymus (immunohistochemistry)

Day of AFB1 dosage	Groups	Reticulo-epithelial cells number*	
		Cortical	Medullary
7th	C	10.8±2.49	10.00±1.70
	E	22.4±3.47	55.20±3.55**
14th	C	9.2±1.93	10.80±2.04
	E	29.7±4.88	87.90±3.84**
21st	C	10.2±2.70	10.30±1.77
	E	47.0±3.74**	169.50±5.68**
28th	C	11.2±4.18	12.90±2.63
	E	77.0±6.34**	349.50±7.18**

*Mean±Standard deviation; **p<0.05

Table 3: The variation of number of non-lymphoide cells immunohistochemically positive in Fabricius bursa

Day of experiment	Groups	Non-lymphoide cells number*		
		ECL	Cortical	Medullary
7th	C	2.3±0.95	8.8±1.69	11.2±1.81
	E	18.8±9.09**	18.2±5.71**	25.3±9.09**
14th	C	2.1±0.99	10.7±2.50	10.4±1.90
	E	35.7±2.21**	30.4±2.99**	48.0±3.65**
21st	C	2.0±0.94	9.8±2.74	11.3±1.77
	E	55.6±6.88**	91.9±23.8**	71.3±8.26**
28th	C	2.4±0.97	11.8±2.89	12.9±2.40**
	E	67.3±6.88	112.7±6.61	97.8±9.80**

*Mean±Standard deviation; **p<0.05

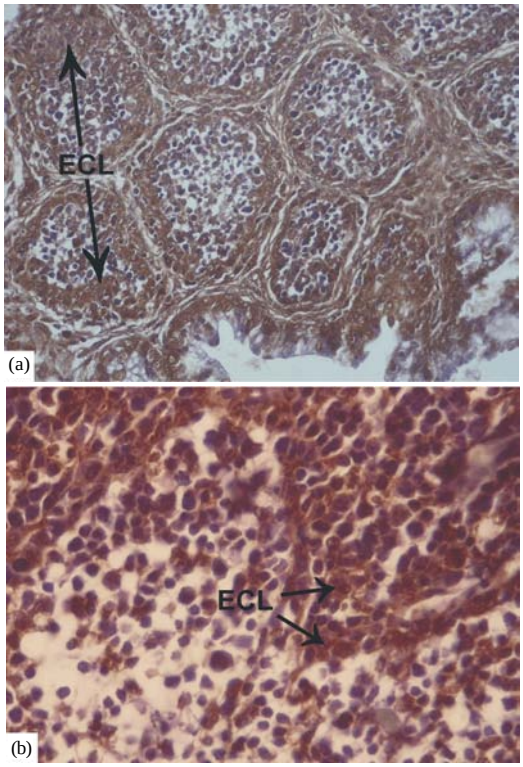

Fig. 4: a) The lesions of Fabricius bursa after 28th day of exposure to AFB1. Severe atrophy of the cortical and medullar lymphoid follicles and proliferation Epithelial Cell Line (ECL). Bcl-2 IHC staining x200 and b) Lesions of Fabricius bursa after 28th day of exposure to AFB1 chronic intoxication in chickens. Bcl-2 immunopositive positive non lymphoide cells. Epithelial Cell Line (ECL) separating the cortex from the medulla. Bcl-2 IHC staining x900

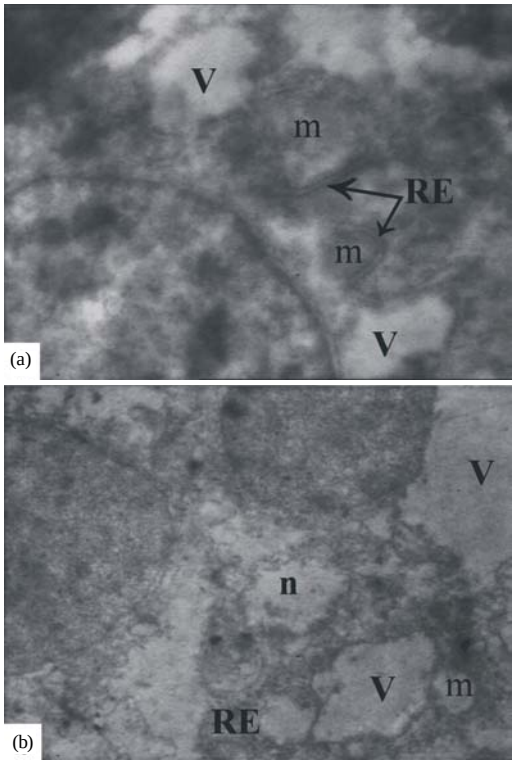

Fig. 5: a) Ultrastructural changes in thymocytes from a chicken intoxicated with AFB1 for 21st day. Dilated and fragmented Reticular cistern (RE), numerous Vacuoles in cytoplasm (V) and chromatin condensation. Balonizedmithocondria (m). Magnification x16000 and b) ultrastructural changes in lymphocytes of the Fabricius bursa from a chicken intoxicated with AFB1 for 28th day. Dilated and fragmented Reticular cisterns (RE), Vacuoles in the cytoplasm (V) and chromatolysis in nucleus (n). Balonizedmithocondria (m). Magnification x8000

in follicle associated epithelium and hydropic epithelial degeneration were observed from the 14th day of AFB1 exposure and these lesions were maximal after the 21st and 28th day and featured a lack of cortico-medullar differentiation and a generalized lymphoid depletion (Fig. 4a). Fibrous tissue and the interfollicle epithelium gradually increased and large mucoid cysts became evident. Glandular transformation of isolated follicles and hyperplasia with cystic changes were also observed

mainly after the 21st and 28th days of AFB1 exposure. In parallel, a marked and progressive intense proliferation of the epithelial cell line separating the cortex from the medulla was found and a strong Bcl-2 immunoreactivity of the reticulo-epithelial cells was observed (Fig. 4b).

The number of reticulo-epithelial cells Bcl-2 positive in the bursa of Fabricius from the experimental group gradually increased during the exposure time. Exceptions are reticulo-epithelial cells of the follicle in control and experimental group in the 1st week of investigation for the experimental group in the 7th day which showed a high variability (Table 3).

The comparison of the statistical values for Bcl-2-positive reticulo-epithelial cells in the thymus and bursa of Fabricius revealed significant differences ($p<0.05$) between control and experimental groups in each of the key moments of the experiment (Table 2 and 3). The thymuses from the AFB1 dosed chickens showed marked ultra-structural changes in cell nuclei from focal chromatin condensation to partial lysis (Fig. 5a). Also, progressive degenerative changes in centrioles were occasionally observed.

Rough Endoplasmic Reticulum (RER) was enlarged surrounding the mitochondria, cisterns showed irregularities and loss of ribosomes attached to the membrane surface leading to RER fragmentation and various degrees of proliferation of the endoplasmic reticulum, depending on the dose of AFB1. Focal degeneration and rupture of the lymphocyte membranes were often observed. In some cells, there was a paucity of specialized cytoplasmic organelles and a decrease in the electron density of the cytoplasm giving them a pale appearance.

These apoptotic changes were also evidenced in lymphocytes from the bursa of Fabricius in chickens dosed with AFB1. Dilatation and fragmentation of the cisterns of the endoplasmic reticulum associated with abundant vacuoles were seen. Some free ribosomes were found in the cytoplasm. Hyperchromatosis was observed in the periphery of the nucleus and lipid inclusions were seen between the two layers of the nuclear membrane looking like vesicles attached to the nuclei (Fig. 5b). In the two primary lymphoid organs, apoptosis of thymocytes and lymphocytes was mainly apparent after AFB1 dosage for 21 days.

The changes in the Bcl-2 expression in lymphoid cells were associated with cellular degenerative lesions such as intense dilatation and fragmentation of the endoplasmic reticulum, strong vacuolization of the condensed cytoplasm and condensation and fragmentation of the chromatin all being suggestive of apoptosis. Furthermore, the marked degenerative changes of the endoplasmic reticulum highly suggest that the membranes per se are the major targets of AFB1 injury.

DISCUSSION

In the present study, profound changes were seen in immunological and associated tissue types. In the thymus, cortical lymphocytes were dramatically depleted during the AFB1 poisoning whereas hyperplasia of the reticulo-epithelial cells was observed in the thymuses from all aflatoxin-treated chickens and particularly in those treated for 28 days. Similarly, atrophy and lymphoid depletion were also evidenced in the bursa of Fabricius.

These findings were in agreement with previous studies that have described thymus aplasia in aflatoxicosis (Methenitou *et al*., 2001; Sur and Celik, 2003). Other researchers observed mild depletion of lymphocytes, cystic degeneration and fibrous tissue proliferation in the bursa of Fabricius from 2 weeks old Japanese quails orally exposed to AFB1 (1 ppm) for 8 weeks (Parlata *et al*., 2001; Jakhar and Sadana, 2004).

Oguz and Kurtoglu (2000) and later Ortatatli *et al*. (2005) reported that the dietary supplementation with clinoptilolite (15 g kg^{-1}) partially reversed the growth inhibitory and immunotoxic effects of low aflatoxin doses (50 and 100 ppb) in broilers. These results suggest that AFB1 exposure dramatically reduces the lymphocyte B and T populations by interfering with their maturation. Dietary AFB1 in chickens has been shown by other researchers to result in degeneration of follicle associated epithelium in the bursa of Fabricius, destruction of thymic cortex and decrease of splenic T cells (Celik *et al*., 2000; Wang *et al*., 2013).

Normally, the thymus has distinct cortical and medullar regions that demonstrate serial stages of thymocyte maturation. The complex developmental process responsible for generating T cells within the thymus depends on signals from stromal cells that direct the maturation, clonal expansion and selection of T cell precursors (Anderson and Takahama, 2012). Defects in the thymic microenvironment can prevent the development of the adaptive immune system cause severe immune deficiencies and inhibit immunological surveillance which is essential for recognizing and removing pathogens and malignant cells in vertebrates (Poliani *et al*., 2009). It has been shown that several infectious pathogens (bacteria, virus, parasites and fungi) can cause atrophy of the organ (Savino, 2006; De Meis *et al*., 2012). It is not completely understood how this atrophy occurs and the mechanism may vary. In most infectious diseases causing thymic atrophy, the major biological event associated with thymocyte loss is cell death by apoptosis (De Meis *et al*., 2012).

The vast majority of thymocytes within the cortex were Bcl-2 negative, only rare cells displaying positivity whereas within the medulla, strong Bcl-2 thymocyte positivity was evidenced. Thymocytes undergo positive selection on the basis of their T-cell receptor specificities, the majority of cortical thymocytes die by apoptosis and the mature thymocytes that survive accumulate in the medulla (Hockenbery *et al*., 1991). On the other hand, Chen *et al*. (1996) reported that the Bcl-2 reactivity of thymus carcinoma was significantly higher than that observed in both non-invasive and invasive thymomas. Also, it has been suggested that because all medullary thymomas exhibited Bcl-2 reactivity, the Bcl-2 expression could not be used as an indicator of the invasive behaviour of the tumour when applying the classification by Marino and Muller-Hermelink (1985). Studies confirmed that the Bcl-2 expression was observed only in type A, AB and C thymomas. In addition, the intensity of reactivity in type A and AB thymomas was weak whereas that of type C thymoma was strong (Hiroshima *et al*., 2002).

Overexpression of Bcl-2 causes tissue hyperplasia and promotes tumor development. Aflatoxin binding to DNA and damage the level of different proteins from cell cycle and apoptotic pathways such as c-Myc, p53, pRb, Ras, Protein Kinase A (PKA), Protein Kinase C (PKC), Bcl-2, NF-kB, CDK, Cyclins and CKI contribute to the life or death decision making, process that may contribute to the deregulation of the cell proliferation leading to cancer development (Li *et al*., 1993; Jacotot *et al*., 2000; Vermeulen *et al*., 2003).

The germinal center of the lymphoid follicles appears to provide a microenvironment for the generation of memory B and plasma cells. Following stimulation with antigen, B-cell blasts seed the primary follicle which matures to a secondary germinal center with well-defined anatomic zones. Within human tonsils the most intense staining for Bcl-2 was in the follicular mantle, composed of re-circulating IgM/IgD cells. In striking contrast, the majority of cells within the germinal center were negative for Bcl-2 (Hiroshima *et al*., 2002). The expression of human *Bcl-2* gene is deregulated by translocation in most of follicular lymphoma (Hockenbery *et al*., 1991). Bcl-2 inhibits apoptosis from a number of stresses including DNA damage, microtubule perturbation and oncogene activation by binding the BH_3 (Bcl-2 homology-3) domain of pro-apoptotic factors (Cheng *et al*., 1997).

The expression of the chicken *Bcl-2* gene was analysed in various tissues. In the adult broilers, Bcl-2 transcripts were detected in spleen, kidney, heart, ovary, brain and mainly in thymus. However, the bursa of Fabricius which is the site of the early B-cell development, expressed much lesser amounts of Bcl-2 RNA. On the other hand in chicken embryos, the gene is extensively expressed in the bursa as well as in muscle and the above tissues. These findings indicate that a homologue of the human *Bcl-2* gene does exist in the chicken and that its expression is developmentally regulated in some tissues (Eguchi *et al*., 1992).

In the present study, the Bcl-2 immunoreactivity of thymocytes was dramatically depressed in AFB1-dosed chickens, this depletion being earlier and more pronounced in the thymus cortex than in the medulla. In parallel, the epithelial cell line separating the cortex from the medulla have proliferated and highly expressed the Bcl-2 protein in thymus and in the bursa of Fabricius.

The RER disorganization is consistent with the reported decrease in protein synthesis induced by the AFB1 administration. Aflatoxin was shown to inhibit RNA polymerase activity and prevented DNA dependent RNA synthesis leading to inhibition of protein synthesis (Wild *et al*., 2000; Ortatatli *et al*., 2005). Aflatoxins act, after bioactivation in the liver by binding of molecules such as essential enzymes, blocking RNA polymerase, lipid peroxidation and ribosomal translocase (inhibiting protein synthesis) and formation of DNA adducts (Richard, 1991; Ortatatli *et al*., 2005). AFB1-exo-8, 9-epoxide can bind with DNA to form the predominant trans-8, 9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB1 (AFB1-N^7-Gua) adduct which causes DNA lesions then can bind covalently to various proteins which may affect structural and enzymatic protein functions (Richard, 1991). AFB1 is capable to produce as well as promote directly or indirectly the genotoxic and apoptotic process (Brahmi *et al*., 2011).

Immunosuppression is an important reported toxic effect of aflatoxin (Wild *et al*., 2000). Aflatoxin causes impaired immunogenesis in livestock and leads to various disease outbreaks, sometimes even after vaccination (Michael *et al*., 1973; Hussain *et al*., 2008). Thaxton *et al*. (1974) observed depression of antibody titer to sheep erythrocytes in chicks treated with aflatoxin. Moreover, aflatoxin also affects cell mediated immunity. Several studies have demonstrated that macrophages were found to be less phagocytic in aflatoxicosis (Robens and Richard, 1992; Stern *et al*., 2001). This study has shown that at the ultrastructural level, the cytoplasm of lymphocytes developed vacuoles of different sizes. The cytoplasmic vacuolisation implies increased permeability of cell membranes leading to an increase of intracellular water. As Assaf *et al*. (2004) showed, the loss of membrane integrity of cellular organelles may be the result of specific inhibition of protein synthesis by AFB1 and lipid peroxidation associated with degenerative changes

in lymphocytes. Bcl-2 and other anti-apoptotic molecules play a central role in preserving mitochondrial structure and function. The cell death could be a consequence of lipid peroxidation and reactive oxygen species which in association with caspase and Bcl-2 induce mitochondrial membrane permeabilization and cytochrome C release from mitochondria, a mechanism identified by Rincheval *et al*., 2012).

CONCLUSION

AFB dosing of chickens resulted in intense histological lesions in the thymus and the bursa of Fabricius, characterized by lymphoid necrosis associated with apoptotic changes. The proliferation of non-lymphoid cells in the thymus and bursa of Fabricius cells was associated with a high Bcl-2 expression suggesting a proto-oncogenic activity of AFB1 in this host species.

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