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EFFECTS OF ALUMINOSILICATES ON LIPID PEROXIDATION AND ANTIOXIDANTS IN AFLATOXIN B_1-INDUCED TISSUE INJURY IN CHICKENS

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ABSTRACT. Aflatoxins in poultry cause biochemical changes in major organs, which can assist in the diagnosis of toxication. Producers and researchers have attempted to develop an effective decontamination technology to deal with this feed-borne toxin. Aluminosilicates(clays and zeolites) were preferred because of their high binding capacitiyforaflatoxins and their reducing effect on aflatoxin-absorption from the gastrointestinal tract. The purpose of the present study was to evaluate the toxic effects of aflatoxin B_1 (AFB_1) by biochemical examination of liver, kidney, spleen, erithrocytes, and pancreas of broiler chickens, and to determine the possible preventive role of ATN-dietary aluminosilicates (mixture of clinoptilolite and bentonite) on the investigated values. In total, 84 broiler chicks were divided into two treatment groups: control-basal diet and basal diet plus 5 g aluminosilicate/kg diet. After 21 days, twelve hours prior to sacrifice, 21 chicks from each group received one dose of AFB_1 orally. Lipid peroxidation was significantly increased in the liver and kidney suggesting oxidative stress in these organs. Supplementation withATN decreased these negative effects. No effects due to AFB_1 were observed in enzyme activity and lipid peroxidation inthe pancreas, spleen or red blood cells. This data suggest that a single dose of AFB_1 could provide a toxin alleviating effect on biochemical indices of liver and kidney in broiler chicken. Therefore, ATN protects broiler chickens against the harmful effects of AFB_1.

Keywords: *aflatoxin B_1, antioxidative enzymes, clay, kidney, liver, zeolite*

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INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi that may be injurious to animals upon ingestion, inhalation, or skin contact. Aflatoxins (AF) are polyketide products (difuranocoumarins) of a number of *Aspergillus* species [1, 2, 3]. They have received greater attention than any other mycotoxins because of their demonstrated potent carcinogenic effects and acute toxicological effects in animals and humans. The biochemical, hematological, immunological and pathological toxic effects of AF have been well described [4, 5, 6, 7, 8]. AF contamination of agricultural products is one of the most important factors determining product quality, and has caused significant financial losses for producing countries [9, 10]. AF are a group of 15-20 closely related compounds. The most common forms in nature are AF B_1, B_2, G_1, and G_2. These abbreviations indicate the colour (blue or green) and relative migration distance, 1 and 2 (higher and lower), of the compounds as seen by thin-layer chromatography under ultraviolet light [1, 8].

Aflatoxin B_1 (AFB_1) is considered the most toxic AF. It is metabolized mainly by the liver to AFB_1-8, 9-exo-epoxide and 8, 9-endo epoxide. The exo-epoxide binds to DNA to form the predominant 8, 9-dihydro-8-(N_7-guanyl)-9-hydroxy AFB_1 (AFB_1-N_7-Gua) adduct. Furthermore, AFB_1-N_7-Gua may be converted to two secondary forms [3].

Several approaches have been investigated to reduce exposure of animals to AF in contaminated feeds, including: solvent extraction, ammoniation, ozonolysis and other chemical, physical and biological treatments [9, 10, 11, 12]. Binding agents such as activated carbon, clays and zeolites have been added to contaminated feeds in an attempt to reduce or prevent AF exposure in animals. An effective sequestering agent is one that tightly binds the mycotoxin in contaminated feed without disassociating in the gastrointestinal tract of the animal [10, 11, 13, 14].

Clays and zeolites are hydrated and composed mostly of aluminium and silica: belonging to the group of aluminosilicates. Phyllosilicate clays are hydrated, crystalline aluminosilicateswith a layered structure. Montmorillonite, the main constituent of the phyllosilicate ore bentonite, is a trimorphicphyllosilicate formed by 2:1 condensation of layers with aluminium sandwiched between two layers of silica. Montmorillonite possesses exchangeable sodium or calcium cations and has expandable sheets [15]. Natural zeolites are hydrated aluminosilicate minerals characterized by cage-like structures, with high internal and external surface areas, and high cation-

exchange capacities. The basic building blocks of natural zeolites are electrostatically charged tetrahedra of silica and aluminium, with the negative charge balanced by alkaline or alkaline earth cations. The stacking of these tetrahedral gives rise to various three-dimensional honeycomb structures containing tunnels or channels of uniform diameter [16, 17].

The objective of the present study is to explore the possibile use of feed additives based on natural occurring hydrated aluminosilicates (Antitoxic nutrient-ATN) in preventing or minimizing the oxidative stress induced by acute administration of AFB_1 in broiler chickens.

RESULTS AND DISCUSSION

The liver is considered to be the main target organ for AF. The hepatotoxic effects of AFB_1 have been well-documented in a variety of animal species [18, 19, 20]. AFB_1-induced free radical production has been referred to as a possible contributing factorin hepatotoxicity [21, 22, 23, 24, 25]. Table 1 shows the effect of ATN on AFB_1-induced LP and antioxidant status in the liver of broiler chickens. The significant increase in LP seen in the AFB_1 group was maintained at normal levels by ATN treatment. A significant decrease in the activity of glutathione S-transferase (GST) was observed in the liver of AFB_1 treated chickens. Oral intake of AFB_1 and ATN alone or in combination did not cause inhibition of the other selected enzyme activities: superoxide dismutase (SOD-1), catalase (CAT), pyrogallol peroxidase (PPx), and guaiacol peroxidase (GPx) in the liver of broiler chickens. ATN alone did not induce any significant changesin the activities of these measured enzymes, and did not induce lipid peroxidation in liver tissue.

Table 1. Effect of aflatoxin B_1 (AFB_1)exposure alone and in combination with ATN on the activities of endogenous antioxidant enzymes and lipid peroxidation in the liver of broiler chickens

	Experimental group			
	Control	ATN	AFB_1	ATN + AFB_1
SOD-1 [IU/mg protein]	18.37 ± 0.52[a]	19.41 ± 0.87[a]	18.05 ± 0.55[a]	19.11 ± 0.74[a]
CAT [IU/mg protein]	22.75 ± 0.95[a]	23.87 ± 2.12[a]	22.00 ± 0.80[a]	25.07 ± 0.57[a]
GPx [IU/mg protein]	2.11 ± 0.08[a]	2.09 ± 0.06[a]	1.95 ± 0.06[a]	1.96 ± 0.06[a]

	Experimental group			
	Control	ATN	AFB_1	ATN + AFB_1
PPx [IU/mg protein]	64.02 ± 2.88[a]	59.21 ± 1.68[a]	57.18 ± 1.87[a]	56.92 ± 2.07[a]
GST [IU/mg protein]	393.14 ± 11.36[a]	378.84 ± 13.21[a]	298.17 ± 7.32[b]	354.68 ± 9.98[a]
Lipid peroxidation [nmol MDA/mg protein]	2.01 ± 0.11[a]	1.94 ± 0.06[a]	2.58 ± 0.05[b]	1.77 ± 0.05[a]

The data are mean values ± standard error

[a, b] values without the same superscript within each row differ significantly ($P< 0.05$)

SOD-1, superoxid dismutase; CAT, catalase; GPx, guaiacol peroxidase; PPx, pyrogallol peroxidase; GST, glutathion S-transferase

LP is one of the main manifestations of oxidative damage initiated by ROS and has been linked with altered membrane structure and enzyme inactivation. It is initiated by the abstraction of a hydrogen atom from the side chain of polyunsaturated fatty acids in the membrane [26]. The present data reveals that AFB_1 administration produces a marked oxidative impact as evidenced from the significant increase in LP (Table 1). This increase in lipid peroxides might result from increased production of free radicals and a decrease in antioxidant status. The oxidative stress observed in our study is in agreement with other reports, where it has been implicated in AFB_1-induced hepatotoxicity in various animal species: broiler chickens [23, 27], ducklings [28], laying hens [29], mice [22, 25, 30] and rats [20, 21, 24]. In this study, ATN treatment significantly reduced AFB_1-induced LP, presumably by its ability to scavenge molecules of AFB_1 in the gastrointestinal tract.

GST plays a critical role in the protection of tissues from the deleterious effects of activated AFB_1. GST catalyzes the conjugation of AFB_1-8, 9-epoxides with glutathione (GSH) to form AFB_1-epoxide-GSH conjugates, thereby decreasing intracellular glutathione content [31]. This observation supports our findings, where we observed a significant decline in the activity of GST in AFB_1-induced chickens (Table 1). This agrees with other reports on experimental AF in mice[30], and rats [20, 31]. There was no significant difference in liver GST activityin controlsorin animals treated with ATN or ATN along with AFB_1. This result indicates that ATN provides full protection

tothe liver of broiler chickens exposed to harmful AFB_1 treatment. Our previous study also demonstrates that oral intake of ATN does not provoke inhibition or stimulation of liver GST in broiler chickens[32].

Unlike other authors [20, 21, 25, 30, 31], we did not observe any change in the activity of other measured antioxidative enzymes in the liver following AFB_1 intoxication (Table 1). A single dose of AFB_1 is not enough to induce a shift in CAT, SOD-1 or peroxidase activities in the liver of broiler chickens.

Table 2 shows the effects of AFB_1, ATN, and AFB_1 plus ATN on lipid peroxidation and the activities of CAT, SOD-1, PGx, and GPx in the kidney of broiler chickens. No significant alterations between the control and ATN group were observed. The level of lipid peroxidation was significantly higher vs. the control group in AFB_1 treated chickens. Oral administration of ATN along with AFB_1 ameliorates AFB_1-induced lipid peroxidation. Oral administration of AFB_1 or ATN did not induce CAT or GPx activity in the kidneys. SOD-1 activity is an indicator of ROS production. In the kidneys of broiler chickens treated with AFB_1, SOD-1 and PPx activities were significantly decreased.

Table 2. Effect of aflatoxin B_1 (AFB_1)exposure alone and in combination with ATN on the activity of endogenous antioxidant enzymes and lipid peroxidation in the kidney of broiler chickens

	Experimental group			
	Control	ATN	AFB_1	ATN + AFB_1
SOD-1 [IU/mg protein]	10.68 ± 0.42[a]	11.10 ± 0.34[a]	6.89 ± 0.45[b]	9.89 ± 0.31[a]
CAT [IU/mg protein]	64.08 ± 4.20[a]	62.42 ± 3.21[a]	69.25 ± 6.92[a]	67.96 ± 6.05[a]
GPx [IU/mg protein]	15.10 ± 1.00[a]	16.56 ± 1.18[a]	7.64 ± 1.66[a]	15.10 ± 1.76[a]
PPx [IU/mg protein]	49.84 ± 2.15[a]	47.22 ± 2.44[a]	38.09 ± 2.81[b]	37.18 ± 2.02[b]
Lipid peroxidation [nmol MDA/mg protein]	2.31 ± 0.10[a]	2.58 ± 0.08[a]	3.33 ± 0.06[b]	2.95 ± 0.16[a, b]

The data are mean values ± standard error
[a, b]values without the same superscript within each row differ significantly ($P< 0.05$)
SOD-1, superoxide dismutase; CAT, catalase; GPx, guaiacol peroxidase; PPx, pyrogallol peroxidase

Under oxidative stress, SOD can behave in two different ways: initially and when stress is moderated, cells act by suppressing SOD-1; but if the stress lasts for a long time and favors increased production of ROS, the enzyme is exhausted and its concentration falls. The low activity of SOD could also be due to inactivation of the enzyme by crosslinking or DNA damage [33]. In our case, the decreased SOD-1 activity observed could be explained by the massive production of superoxide anions, which override enzymatic activity and lead to a fall in its concentration in kidneys. These results are in agreement with the results of other authors, in studies mostly performed on rats [20, 21] and mice [22, 25]. ATN successfully restored SOD-1 activity to control levels, but did not restore PPxactivity.

Table 3 shows the activity of measured antioxidant enzymes, hemoglobin (Hb) concentration and MDA levels in the erythrocytes of broiler chickens. Activities of protective antioxidative enzymes and MDA levels in the pancreatic tissue of broiler chickensare presented in Table 4. It was observed that oral intake of ATNand AFB_1alone or in combination did not cause impairment of the selected enzyme activities or the level of lipid peroxidation in the red blood cells and pancreas of broiler chickens. Although chronic exposure of mice to AF could provoke oxidative stress in erythrocytes [24], a single oral dose of AFB_1 does not induce that effect in broiler chickens.

Table 3. Effect of aflatoxin B_1 (AFB_1) exposure alone and in combination with ATN on hemoglobin concentration, activity of endogenous antioxidant enzymes and lipid peroxidation in the erythrocytes of broiler chickens

	Experimental group			
	Control	ATN	AFB_1	ATN + AFB_1
Hb [g/l]	144.95 ± 3.71	145.05 ± 3.24	145.44 ± 4.32	147.23 ± 5.11
SOD-1 [IU/mg Hb]	467.16 ± 13.23	485.57 ± 14.55	470.16 ± 14.22	482.38 ± 11.87
CAT [IU/mg Hb]	8.14 ± 0.53	8.22 ± 0.59	7.31 ± 0.56	7.04 ± 0.62
GPx [IU/mg Hb]	6.47 ± 0.21	6.85 ± 0.19	6.50 ± 0.20	6.82 ± 0.32
PPx [IU/mg Hb]	14.44 ± 0.50	14.96 ± 0.48	14.35 ± 0.43	14.20 ± 0.55
GST [IU/mg Hb]	124.04 ± 2.81	131.08 ± 2.95	116.12 ± 2.71	114.30 ± 2.66

Lipid peroxidation [nmol MDA/mg Hb]	1.17 ± 0.10	1.39 ± 0.06	1.07 ± 0.12	1.29 ± 0.12
The data are mean values ± standard error There were no statistically significant differences among values within the same row Hb, hemoglobin; SOD-1, superoxide dismutase; CAT, catalase; GPx, guaiacol peroxidase; PPx, pyrogallol peroxidase; GST, glutathione S-transferase				

Table 4. Effect of aflatoxin B_1 (AFB_1) exposure alone and in combination with ATN on the activity of endogenous antioxidant enzymes and lipid peroxidation in the pancreas of broiler chickens

	Experimental group			
	Control	ATN	AFB_1	ATN + AFB_1
SOD-1 [IU/mg protein]	4.14 ± 0.13	4.25 ± 0.21	4.02 ± 0.09	4.26 ± 0.10
CAT [IU/mg protein]	7.67 ± 0.45	7.40 ± 0.34	8.71 ± 0.34	7.80 ± 0.32
GPx [IU/mg protein]	0.35 ± 0.02	0.39 ± 0.03	0.36 ± 0.01	0.36 ± 0.01
PPx [IU/mg protein]	2.51 ± 0.22	2.15 ± 0.14	2.13 ± 0.27	2.46 ± 0.15
Lipid peroxidation [nmol MDA/mg protein]	3.21 ± 0.35	2, 96 ± 0.08	2.88 ± 0.11	2.78 ± 0.11
The data are mean values ± standard error There were no statistically significant differences among values within the same row SOD-1, superoxide dismutase; CAT, catalase; GPx, guaiacol peroxidase; PPx, pyrogallol peroxidase				

The activities of enzymatic antioxidants, such as SOD-1, CAT, GPx, and PPx, and levels of lipid peroxidation in the spleen are presented in Table 5. The spleen is the principal peripheral lymphoid organ and plays an important role in protective immune reactions. It is involved in humoral and cellular immune responses through its role in the generation, maturation and storage of lymphocytes. Dietary intake of AF can increase apoptotic percentages of splenocytes, which may relate to DNA damage and

mitochondrial lesions caused by increased oxidative stress [25, 34, 35, 36]. We found that a single oral dose of AFB_1 is not capable of disturbing the normal activity of SOD-1 and peroxidases or inducing oxidative stress in the spleen.

Table 5. Effect of aflatoxin B_1 (AFB_1)exposure alone and in combination with ATN on the activity of endogenous antioxidant enzymes and lipid peroxidation in the spleen of broiler chickens

	Experimental group			
	Control	ATN	AFB_1	ATN + AFB_1
SOD-1 [IU/mg protein]	3.82 ± 0.14[a]	3.71 ± 0.19[a]	3.85 ± 0.15[a]	4.00 ± 0.15[a]
CAT [IU/mg protein]	5.63 ± 0.15[a]	5.14 ± 0.14[a]	6.85 ± 0.21[b]	6.52 ± 0.31[a, b]
GPx [IU/mg protein]	0.58 ± 0.03[a]	0.62 ± 0.03[a]	0.59 ± 0.03[a]	0.61 ± 0.02[a]
PPx [IU/mg protein]	16.23 ± 1.05[a]	14.36 ± 0.71[a]	14.75 ± 0.97[a]	16.39 ± 2.35[a]
Lipid peroxidation [nmol MDA/mg protein]	2.62 ± 0.06[a]	2.61 ± 0.08[a]	2.75 ± 0.12[a]	2.31 ± 0.15[a]
The data are mean values ± standard error SOD-1, superoxide dismutase; CAT, catalase; GPx, guaiacol peroxidase; PPx, pyrogallol peroxidase				

CONCLUSIONS

In conclusion, co-administration of naturally occurring aluminosilicates (ATN) offers significant protection against AFB_1-induced oxidative stress in the liver and kidney tissue of broiler chickens. A single dose of AFB_1 did not induce any adverse effectsin the pancreas, spleen or erythrocytes. ATN has the ability to absorb AFB_1 in the lumen of the digestive tract, and thus could be used as a supplementary agent in animal feeds.

EXPERIMENTAL SECTION

Chickens and diet

Eighty-four 1-day-old, unvaccinated broiler chicks of both sexes were obtained from a commercial hatchery. Individually weighed chicks were divided at random into four groups. There were seven replicates of three broiler chicks for each dietary treatment. The chicks were housed in electrically heated batteries under fluorescent lighting and received a commercial basal diet (maize and soybean meal diet 220 g protein, 13.00 MJ ME kg^{-1}) formulated to contain National research Council (1994) requirements. Food and water were available *ad libitum* and lighting was continuous.

Experimental design

The experimental design consisted of two dietary treatments: 1. Control: basal diet; 2. ATN: basal diet plus 5.0 g ATN kg^{-1} diet. ATN (Antitoxic nutrient) is a fine powder containing mostly zeolitic ore (with > 90% of clinoptilolite) and bentonite (with > 83% of montmorillonite), together with small amounts of activated charcoal (ratio 60:20:1/zeolite:bentonite:charcoal). After 21 days, twelve hours prior to sacrifice, 21 broiler chickens from each group received one dose of AFB_1 from *Aspergillusflavus*(Sigma, Germany)orally (AFB_1 and AFB_1 + ATN groups).AFB_1 was dissolved in distilled waterand every chicken received 1 mg of AFB_1/kg body weight.

Blood sampling and slaughter

Twelve hours after intoxication, the feeding trial was terminated and all broiler chickens were bled by cardiac puncture. Heparin was used as an anticoagulant and non-coagulated blood was used for separation of erythrocytes. Hemoglobin (Hb) concentration in red blood cells was determined by the cyanomethemoglobin procedure [37]. All 84 broiler chickens were sacrificed by cervical dislocation and liver, kidney, spleen, and pancreas were removed. Homogenates of these organs with phosphate buffer (pH=7.0) were used for further biochemical analysis.

Biochemical analysis

Activity of antioxidant enzymes: superoxide dismutase (SOD-1), catalase (CAT), guaiacol peroxidase (GPx), pyrogallol peroxidase (PPx), and lipid peroxidation were measured in erythrocytes, liver, kidney, spleen and pancreas. Glutathione S-transferase (GST) activity was evaluated in erythrocytes and liver homogenates. Protein content in homogenates of liver, spleen, and pancreas were determined according to the method of Bradford

[38], using bovine serum albumin as a protein standard. SOD-1 activity was determined in samples according to McCord and Fridovich[39]. The CAT activity was assayed by the method of Clairborne[40]. Utilization of hydrogen peroxide by CAT in the samples was measured spectrophotometrically as the decrease in optical density at 240 nm. GPx activity was measured by following the H_2O_2 dependent oxidation of guaiacol at 470 nm Agrawal and Laloraya[41]. The activity of PPx was measured using pyrogallol as the substrate according to Chance and Maehly[42]. The formation of purpurogallin was followed at 430 nm. GST activity in samples was evaluated using 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate as previously described by Habig*et al.* [43]. The formation of the adduct of GSH-CDNB (2, 4-dinitrophenyl glutathione) was monitored by measuring the increase in absorbance at 340 nm against a blank with a spectrophotometer. MDA levels were analyzed with 2-thiobarbituric acid, monitoring the change in absorbance at 532 nm with a spectrophotometer [44].

Statistical analysis

Results are expressed as the mean of determinations of 3 independent samples made in triplicates. Statistical significance was tested by analysis of variance followed by comparison by Duncan's multiple range test ($P< 0.05$) calculated using STATISTICA for Windows version 9.0 (StatSoft, Tulsa, OK, USA).

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