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Metabolic Effects of Low Aflatoxin B_1 Levels on Broiler Chicks†

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The effects of daily ingestion of aflatoxin B_1 (AFB_1) on growth, feed intake, plasma glucose, plasma cholesterol, plasma amino acids, plasma albumin, plasma ceruloplasmin, muscle amino acids, liver lipid, and bone strength were studied. For 3 weeks, beginning at an age of 2 days, broiler chicks were dosed daily per os with 50 or 100 μg of AFB_1 per kg of body weight. Body weight and feed consumption were recorded daily, and metabolic responses were determined at 3 weeks. Treatment with AFB_1 did not significantly alter body weight or feed intake. Relative liver weight showed a significant increase at the highest dose, with a significant concomitant increase in liver lipid and decrease in hepatic zinc. Relative spleen and heart weights were not affected by the toxin. Plasma glucose and cholesterol were significantly elevated at the highest dose. AFB_1 significantly decreased plasma lysine and histidine and significantly increased muscle histidine, arginine, and valine. AFB_1 decreased plasma albumin and markedly increased plasma ceruloplasmin. Dimensions of the long bones (femur and tibiotarsus) were not altered by the toxin. However, AFB_1 caused a significant linear decline in the resistance of bone to breakage ("bone breaking strength"). The results indicate that low levels of AFB_1 reduced bone strength in broiler chicks. The alterations in blood parameters indicated that AFB_1 can disrupt metabolism even at low levels.

Aflatoxins are structurally related toxic bisfuranocoumarins produced as secondary metabolites by strains of *Aspergillus flavus* and *A. parasiticus* during growth on a wide variety of agricultural commodities, including peanuts, cottonseed, and corn (32). Aflatoxin B_1 (AFB_1), the most toxic and predominant naturally occurring aflatoxin, is classified as highly to extremely toxic: for most species, it has a 50% lethal dose of <15 mg/kg of body weight (24). In addition, AFB_1 is one of the most potent hepatocarcinogens (7). AFB_1 at acutely toxic levels causes severe growth depression, weight loss, icterus, and death. The more insidious effects of long-term, low-level ingestion on several species have been studied to some extent (18, 21), but research directed specifically toward the effects of AFB_1 on avian metabolic processes has been limited to levels of >625 ppb (>625 ng/g) in feed (15). We report here the effects of low AFB_1 levels on selected chick tissue parameters.

MATERIALS AND METHODS

AFB_1 and animal treatment. Commercial AFB_1 (Sigma Chemical Co., St. Louis, Mo.) was used. A stock solution of the toxin was prepared with propylene glycol-ethanol (9:1 [vol/vol]). The treatments consisted of 0, 50, and 100 μg of AFB_1 per kg of body weight per day. Treatments were initiated at 2 days of age and continued for 3 weeks. Chicks were weighed each afternoon (1:30 to 2:30 p.m.) and dosed per os with a pipette. The daily volume stock solution intubated was adjusted to provide the required dosage. Controls received a volume of the carrier mixture equivalent to the mean daily volume used for the highest dose.

Husbandry and necropsy. Male broiler chicks (Hubbard × Hubbard) from Whitworth Feed Mill, Lavonia, Ga., were housed in an electrically heated battery brooder with wire mesh floors. The unit was maintained in a temperature- and humidity-controlled animal room and exposed to light for 24 h. Feed and water were provided ad libitum. A standard corn-soybean diet formulated according to National Research Council nutrient specifications (20) was fed to the chicks. At the end of the study, blood samples were collected in dry heparinized tubes by frontal cardiac puncture. The tubes were held in an ice bath during blood collection. Plasma was separated by centrifugation within 30 min of collection, and each sample was divided into subsamples for various chemical determinations. Plasma samples not immediately processed were stored at −20°C until used. The birds were sacrificed by cervical dislocation, and livers, hearts, and spleens were examined, excised, and weighed. Livers and pectoralis muscle samples were frozen for chemical analyses. The right femurs and tibiotarsi were dissected, and the

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soft tissue was removed. Bone length and diameter were measured with calipers. After removal, femurs were kept moist with a 0.9% sodium chloride solution and maintained at 4°C for 24 h. Strength of the femur was measured by quasistatic compression to fracture with an Instron Testing Instrument (model 1122; Instron Corp., Canton, Mass.) operating at a crosshead speed of 30 mm/min.

Chemical analyses. Plasma samples used for the determination of free amino acids were processed immediately after centrifugation. Each 2.0 ml of plasma was deproteinized with 0.2 ml of 36% sulfosalicyclic acid (3). We added 0.2 ml of 1 mM norleucine as an internal standard and centrifuged the mixture at 27,000 × *g* for 15 min. The supernatant was used for amino acid analysis. Muscle samples were delipidated with 20 volumes of chloroform-methanol (2:1 [vol/vol]) (13) and freeze dried. The lyophilized samples were digested in 6 N hydrochloric acid in vacuo at 100°C for 24 h. Amino acid profiles of plasma and muscle were determined by ion-exchange chromatography with an automatic amino acid analyzer (model K-8000 VG; Phoenix Precision Instrument Co., Phoenix, Az.) and sodium citrate buffers for elution (30). Plasma glucose was measured by the *o*-toluidine method (9), and plasma cholesterol was measured by a colorimetric method with ferric acetate and uranyl acetate (23). Plasma ceruloplasmin activity was determined by the procedure of Rice (26), with *p*-phenylenediamine as the substrate. The procedure was modified for chicken plasma by using 0.2 ml of plasma and increasing the incubation period to 1 h (31). The packed cell volume of the blood of each bird at 3 weeks of age was determined by the microhematocrit method. Serum albumin was determined by the bromocresol green method (kit no. 630; Sigma). Copper, manganese, and zinc contents of the livers were determined by atomic absorption spectrometry (model 503 spectrophotometer; The Perkin-Elmer Corp., Norwalk, Conn.) after wet oxidation with a concentrated nitric acid-perchloric acid mixture (3:1 [vol/vol]).

Statistical analysis. The three treatments were allotted in a randomized complete-block design, with four blocks per treatment. Each quarter of the battery brooder constituted a block. Within a block, each treatment was allocated to a pen of five chicks. Data were analyzed by analysis of variance. The significance of the difference between means was determined by the least-squares method. Probability values of <0.05 were considered significant. All results are expressed as means ± pooled standard errors of the means.

RESULTS

Broiler chicks showed no significant differences in body weight, feed efficiency, and relative spleen and heart weights due to daily dosing with 50 or 100 μg of AFB$_1$ per kg of body weight. The effects of AFB$_1$ on liver weight and composition, plasma metabolites, hematocrit, and bone measurements are presented in Table 1. Plasma cholesterol was significantly depressed, and plasma glucose and ceruloplasmin were significantly elevated only at the highest dose (100 μg/kg). Serum albumin, however, was

TABLE 1. Effects of AFB$_1$ on chick tissue parameters[a]

AFB$_1$ dose (μg/kg of body wt)	Plasma metabolites				Liver wt (g)	Liver make-up[b]		Bone size (mm)				Bone breaking strength (kg)
	Cholesterol (mmol/liter)	Glucose (mmol/liter)	Albumin (%)	Ceruloplasmin (IV)	Hematocrit (%)		Total lipid (%)	Zinc (μg/g)	Femur		Tibiotarsus	
									Length	Diam	Length	Diam
0	4.18^A	6.88^C	1.29^E	0.38^G	36.0	2.22^K	16.65^M	164.9^O	45.1	60.3	5.9	5.1	3.58^Q
50	4.12^A	7.07^C	1.11^F	0.74^G	35.5	2.25^K	16.69^M	131.7^P	45.6	61.7	6.0	5.5	3.06^{Q,R}
100	3.50^B	8.31^D	1.13^F	1.98^H	37.0	2.64^L	19.18^N	128.8^P	44.6	60.8	6.1	6.4	2.78^R
SEM	±0.18	±0.23	±0.04	±0.20	±0.67	±0.04	±9.52	±4.95	±0.87	±1.11	±0.30	±0.23	±0.145

[a] For each set of values obtained for one parameter, values with differing superscript capital letters are significantly different ($P < 0.05$).

[b] Liver lipid is expressed on a dry-matter basis; liver zinc is expressed on a fat-free dry-matter basis.

TABLE 2. Effects of AFB_1 on plasma and muscle amino acid levels

AFB_1 dose (μg/kg of body wt)	Amino acid level[a]				
	Lysine	Histidine	Arginine	Valine	Phenylalanine
Plasma					
0	11.87^{B}	1.91^{D}	3.22	2.52	1.27^{F}
50	5.86^{C}	1.46^{E}	4.49	2.22	$1.38^{G,F}$
100	5.30^{C}	1.59^{E}	4.72	2.32	1.42^{G}
SEM	±1.06	±0.07	±0.46	±0.30	±0.03
Muscle					
0	8.37	2.92^{H}	6.16^{J}	4.56^{L}	3.68
50	8.36	2.88^{H}	$6.37^{J,K}$	4.55^{L}	3.86
100	8.35	3.32^{I}	6.73^{K}	4.86^{M}	4.07
SEM	±0.15	±0.07	±0.14	±0.08	±0.18

[a] In plasma, levels are expressed as milligrams per 100 ml. In muscle samples (defatted and lyophilized), levels are expressed as milligrams per 100 mg. For each set of values obtained for one amino acid in one type of sample, values with differing superscript capital letters are significantly different ($P < 0.05$).

significantly depressed at both levels of AFB_1. Hematocrit was unaffected at either level. AFB_1 at the highest level significantly increased liver weight and lipid content. Hepatic zinc was significantly depressed even at the lowest level of AFB_1, and hepatic manganese and copper declined with increased AFB_1 ingestion. Bone measurements indicated that AFB_1 had no significant effect on linear or appositional growth of tibiotarsi and femurs. Breaking strength of wet femurs was significantly reduced at 100 μg/kg.

Table 2 summarizes the data on plasma and muscle amino acids. In general, with the exceptions of arginine and phenylalanine, the concentrations of free amino acids in plasma decreased with increased AFB_1 dosage. Plasma lysine and histidine were significantly reduced, whereas phenylalanine was significantly increased. In contrast, most muscle amino acids were elevated, and histidine, arginine, and valine were significantly reduced.

DISCUSSION

We examined the effects of low levels of AFB_1 on chick growth and selected tissue parameters. Although body weight gain and feed efficiency were not adversely affected, there were disruptions in the normal profiles of plasma metabolites, liver lipid, and bone breaking strength. In our study, chicks were given a single daily dose of AFB_1. In comparing our results with others, differences in toxicokinetics should be kept in mind. Steady-state kinetics are obtained by putting a toxin in the food, and mixed kinetics are obtained by administration of a single dose (11).

Plasma glucose showed a linear increase with increased AFB_1 dosage, a result similar to that observed for a single dose of 2.7 mg/kg (25). This increase may be ascribed to impaired glucose utilization, since aflatoxicosis has been shown to reduce the activities of the enzymes involved in glucose metabolism (29). In agreement with earlier reports (8, 33), AFB_1 at the highest dose caused liver fat accumulation. Our observation that plasma cholesterol was reduced at 100 μg of AFB_1 per kg of body weight is consistent with the general reduction of lipogenesis (8) and impaired lipid transport (33) in chicks and specific inhibition of hepatic cholesterol biosynthesis (17) in rats by AFB_1. Hypoalbuminemia was evident even at the lowest dose of AFB_1, confirming the sensitivity of serum albumin to AFB_1 (34).

Plasma amino acids are altered in liver diseases, and the pattern of change varies with the disease (27). Plasma amino acids may be elevated because of impaired hepatic catabolism, release from necrosed hepatocytes, or muscle catabolism. In chronic liver diseases, there is an increase in the amino acids normally removed by the liver and a decrease in the amino acids principally taken up by extrahepatic tissue (19). The amino acid pattern resulting from AFB_1 administration, i.e., the significant increase in the aromatic amino acid phenylalanine and the decrease in the branched-chain amino acids isoleucine, lysine, and valine, is comparable to the common amino acid pattern observed in chronic liver damage. The increase in plasma phenylalanine suggests a decrease in the activity of phenylalanine-4-hydroxylase early in hepatic impairment by AFB_1. Our results provide no evidence of a decrease in all free amino acids (35). The

significance of the changes in muscle amino acids is not known.

Depressed plasma albumin and histidine, in conjunction with elevated ceruloplasmin, indicates a possible disruption of copper metabolism. Ceruloplasmin is the major vehicle for copper transport, and copper complexes of albumin and histidine provide an auxiliary transport system (14). The level of ceruloplasmin is normally very low in chickens but is elevated sixfold when chicks are infected or stressed by ACTH (adrenocorticotropic hormone) or hydrocortisone (31). The precise role of AFB_1 in elevating plasma ceruloplasmin in chicks is not known but may be mediated by cortical steroids (31), metabolites of AFB_1 which mimic estrogen (2), or resialylation of asialo-ceruloplasmin by sialyl transferase (12). The last possibility is an attractive hypothesis, since AFB_1 is known to impair protein synthesis (5) and hypoalbuminemia was evident in the present study even at the lowest dose of AFB_1. The possible effect of AFB_1 on copper metabolism may also be responsible for the hepatocarcinogenicity of the toxin, since copper has been shown to protect against certain hepatocarcinogens (10).

Growth-inhibiting levels of AFB_1 have been shown to have a deleterious effect on chicken bone breaking strength, even after the effect on bone diameter is negated (16). The reduction in bone breaking strength observed in our experiment contradicts an earlier report (16) which indicated that bone breaking strength is not significantly altered at 125 μg of AFB_1 per g of feed. Bones from copper-deficient chicks fracture with less deformation (28), whereas bones from birds suffering from aflatoxicosis break with considerably more deformation (16). This implies that bones from copper-deficient chicks are brittle and bones from AFB_1-dosed chicks are pliable. These data suggest that copper metabolism alterations induced by AFB_1 cannot explain the reduction in bone breaking strength. The changes in the material properties of bone during aflatoxicosis (16) may result from changes in cholecalciferol metabolism, since AFB_1 is both hepato- and nephrotoxic. Assays of chick bone ash have demonstrated an interaction between AFB_1 and cholecalciferol resulting in bone calcification (1). However, this may be discounted because AFB_1 does not alter the hydroxylation of cholecalciferol in the livers or kidneys of chicks (4).

Hypoalbuminemia caused by AFB_1 may partially explain the reduction in hepatic zinc (5): since albumin serves as a zinc carrier, less zinc would be brought to the hepatocytes for processing (6). The effect of AFB_1 on protein synthesis may also be reflected in decreased production of metallothionein, which has been shown to be involved in zinc homeostasis in chicks (22). Either mechanism would result in lowered sequestration of zinc by the liver.

Our results confirm previous reports on the effects of AFB_1. In addition, they clearly show that low levels of AFB_1 disrupt metabolic processes without producing overt symptoms. The apparent impairment of copper metabolism could have important consequences, since copper metalloproteins are involved in diverse physiological processes. It is conceivable that some of the symptoms associated with chronic aflatoxicosis are the result of interference with metabolic schemes involving trace element metalloproteins.

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