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Concurrent Dietary Incorporation of Bentonite Clay Reduces Aflatoxin Induced Health Effects In White Pekin Ducks

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Abstract

An experiment was carried out to assess the efficacy bentonite clay supplementation to the diet of white pekin ducks, experimentally fed with aflatoxin B1 contaminated diet (96 ppb) on production performance, and biochemical parameters including oxidative stress indices. The experimental birds (n=72) were provided with standard duck feed and ad libitum water. The grower white pekin ducks, aged 9 weeks, were randomly divided into six groups containing twelve birds in each group. The birds of group I served as healthy controls and was fed with basal diet without addition of toxin or bentonite clay. The birds of group II were provided with a diet with toxin level of 48 µg/kg of feed. The grower ducklings of group III to VI were fed with a diet containing 96 µg of toxin/kg of feed. The experimental feed was provided from 9th week of age (grower stage) for a period of 6 weeks. The birds of group IV, V and VI were provided with feed containing 96 µg aflatoxin /kg of feed along with bentonite clay the dose rate of 1, 2 or 3g of clay/ kg of feed, respectively. The birds fed with aflatoxin-treated diet showed significant (P<0.05) decrease in body weight gain by 4th week of experiment in group II and III. However, the body weight was significantly (P<0.05) higher in bentonite clay supplemented group IV, V, and VI, as compared to toxin-exposed positive group II and III. Feed Conversion Ratio (FCR) increased significantly in Group II and III by 4th week. At the end of 6th week, group II and III showed significantly higher FCR. Significantly decreased serum triglyceride level and increased cholesterol level was recorded in group II and III in comparison to all other groups on 4th week. There was significant decrease in creatinine level in group IV, V, and VI, compared to group II and III on completion of 4th week of the experiment. The activity of GGT increased significantly in all aflatoxin exposed groups. The feeding of aflatoxin contaminated feed increased the erythrocytic malonaldehyde (MDA) production and activity of superoxide dismutase and catalase. It is concluded from the experiment that bentonite clay supplementation in the diet at the dose rate of 3g/ kg of feed reduced the toxic effects of aflatoxin on production performance and oxidative stress in white pekin ducks.

Keywords: Bentonite Clay, Aflatoxin, White Pekin Ducks, FCR, Oxidative Stress

Introduction

Mycotoxins cause significant economic losses in the poultry industry as those affect nutritional value and palatability of feed. Thus, eradication or inactivation of mycotoxins in poultry feed is essentially required to improve production performance and economics of production. Among the poultry, ducks are most sensitive to aflatoxicosis. The chronic exposure to aflatoxin, even at very low doses, can cause growth retardation, severe damage to various organs including liver, spleen, heart, thymus, bursa of fabricious and kidney, and is associated with hemorrhagic syndrome and mortality. Therefore, cost-effective methods of detoxifying potentially toxin-contaminated grain are required where preventative management strategies in the field have failed to prevent *Aspergillus* species from producing aflatoxin in a crop [1].

Duck farming plays an important role in the agricultural economy in Asian continent and accounts for 82.6% of the total duck meat production of the world. In India, ducks constitute about 10% of the total poultry population and contribute about 6-7% of total eggs produced in the country. Ducks are mostly concentrated in the eastern and southern states of the country, mainly in coastal regions. Ducks are more preferred over chicken for farming by small and medium scale producers because of many advantages. Ducks require lesser management attention and thrive well in

scavenging conditions. Ducks have a long productive and profitable life and lay eggs profitably during second and third year. Ducks supplement their feed by foraging, which reduces the cost of feeding. It lays their eggs during early morning and thus, saves time and enables easy egg collection. White Pekin duck is the most popular breed in the world, known for table purpose. The Pekin duck (*Anas domesticus*) has a large body, orange feet and beak, and creamy white feathers. The breed was developed in China during the time of the Mongols. It has low feed consumption and produces fine quality meat. It attains 2.2 to 2.5 kg body weight by 42 days of age, with feed conversion ratio of 1:2.3 to 2.7.

Ducks are resistant to many diseases, those commonly affect poultry. However, they are affected by diseases like viral hepatitis, pasteurellosis, botulism etc. Among non-infectious diseases, mycotoxicosis, especially aflatoxicosis, is the most important and very common in ducks. It is produced by a potent toxin, called mycotoxins, representing the toxic secondary metabolites produced by fungus species. Mycotoxin family includes aflatoxin, ochratoxin, tricothecenes, zearalenone and many others. Aflatoxin is mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, affecting important agricultural feed and food commodities including dried fruits, oilseed meals, spices and cereals. Aflatoxins are difuranocoumarin derivatives comprising of aflatoxin B1, B2, G1, G2, M1 and M2, where in, AFB1 is the most toxic one. These different forms of aflatoxins are identified according to the fluorescence color; for example, B stands for blue and G for green. M1 and M2 are metabolites of aflatoxin B1, secreted in milk of ruminants, fed with aflatoxin B1 contaminated feed. The warm and humid climate favours the production of aflatoxin. Many factors like substrate, time, CO^2 levels, and other environmental factors are important for AFB1 bio-synthesis; especially temperatures near 30°C and water activity of 0.99 provide ideal conditions [2].

Ducks are the most susceptible species to AFB1 among all poultry species [3]. Oral LD50 of aflatoxin is 0.5- 1.0 ppm in duck embryo and 0.3- 0.6 ppm in neonates [4]. The cumulative body weight gain in Pekin ducklings decreases by 169 g per bird from 3 to 14 d with an increase of every 0.10 mg of AFB1 contamination /kg of feed. AFB1 at very low concentrations (≤0.3 mg/kg) can significantly impair the duck's liver function and innate immune characteristics [5]. AFB1 is a potent inhibitor of protein synthesis, thus, it is very likely that dietary protein concentration has a role on the degree of aflatoxicosis by altering the supply of protein synthesis substrates [6, 7].

The ultraviolet radiation, heat, oxidizing agents such as hydrogen peroxide, sodium hypochlorite, or exposure to alkaline substances like ammonia, sodium bisulfate and sulphur dioxide have been used for the degradation of aflatoxins [8-10]. Other approaches include mycotoxin separation from contaminated feeds, detoxification and inactivation [11]. Detoxification and inactivation methods are attained by use of binders or sequestering-agents, added to feed as an approach to reduce toxicity of mycotoxins by reducing reactivity of bound mycotoxins and reducing their intestinal absorption. Substances used as mycotoxin-binders include indigestible adsorbent materials such as silicates, activated carbons, complex carbohydrates and others. The use of binders offers an approach over salvaging feeds with low levels of mycotoxins to protect animals and birds from the background levels of mycotoxins that routinely occur, although in low concentrations, and cause chronic disease problems reducing growth performance.

Bentonite is chemical absorbent, aluminum phyllosilicate clay consisting of mostly montmorillonite. It originates from weathering of volcanic ash. It may be referred as sodium, potassium, calcium, or magnesium bentonite depending on the presence of interchangeable ions. Its adsorption properties are attributed to the lamellar crystalline micro-structures which also accounts for its marked swelling abilities when added to water. This adsorbent compound has varied utility in industrial, engineering, agricultural and beverage fields. It has also been introduced into poultry sector as a binding and lubricating agent in pellet foods. Its supplementation has been seen to increase rate of egg laying, feed efficiency, egg size and shell quality, feed efficiency and decreased mortality in layers and even in pullets. Its adsorbent quality has been exploited against 5 toxic compounds, including. parquet toxicity in rats and cats and *Lantana camara* poisoning in cattle. The present investigation examined the effect of Sodium bentonite clay incorporation in the diet on growth performance, biochemical changes and oxidative stress indices in experimentally induced aflatoxicosis in ducks.

Materials and Methods

The present investigation was conducted on White pekin grower ducks. The experimental birds (n=72) were provided with standard duck feed and ad *libitum* water, and were vaccinated against duck cholera and duck plague, and were randomly divided into six experimental groups containing twelve birds in each group. The 12 grower birds of group I served as healthy controls and was provided with basal diet without addition of toxin or bentonite clay. The birds of group II were provided with a diet with toxin level of 48 µg/kg of feed. The grower ducklings of group III to VI were fed with a diet added with 96 µg of toxin/kg of feed. The toxin added and bentonite clay supplemented feed was used from 9th week of age (grower stage) for a period of 6 weeks.

Purified toxin, procured from commercial sources, was added to the feed at desired proportions (48 or 96 µg/kg of feed) though premix making. Handling of toxins was done with strict biosafety measures. The experimental protocol was conducted ethically with the approval of Institutional Animal Ethical Committee. The birds of group IV, group V, group VI were provided with feed containing 96 µg aflatoxin /kg of feed along with bentonite clay at the dose rate of 1, 2 or 3g of clay/ kg of feed, respectively.

Feed Preparation: Proximate analysis of the feed samples was done before feeding to the ducks. Medicated ration was prepared on weekly basis and kept in closed containers for feeding of ducks. The required amount of aflatoxin was dissolved in acetone. This was sprayed over a small amount of feed which was thoroughly mixed with bulk feed to get the required quantity of feed with desired level of aflatoxin. The procedure was followed separately for both doses.

Production performance: Body weight of birds were recorded at weekly interval using calibrated digital balance starting from day one up to completion of the experiment.

Collection of samples: The blood samples were collected from brachial vein (wing vein) using commercially available clot activator vials and EDTA vials for estimation of serum biochemical parameters and oxidative stress indices in experimental ducks.

Clinical signs and symptoms: The birds were observed daily for any changes in behavior. The clinical signs and symptoms in ducks were studied daily throughout the day, especially during noon hours when the sun is at peak.

Serum Biochemical parameters: The blood samples were allowed to stand in slant position in the clot activator vials at room temperature for about 3-4 hours for separation of serum. The blood clot was separated from the

walls of the test tube by carefully running a clean applicator stick around the inner surface of the tube with utmost care to avoid haemolysis. The supernatant serum was then collected with an auto pipette, and stored in a deep freezer at a temperature of -20°C in properly capped and labeled vials for further analysis. Biochemical parameters like ALT, AST, GGT, ALP, BUN, creatinine, triglyceride, and cholesterol etc. were estimated by spectrophotometer using commercial reagent kits (CoralR).

Oxidative stress indices: About 3ml of blood as collected in non-vacuum tubes containing heparin (10 IU/ml of blood) as anticoagulant for preparation of RBC suspension and RBC hemolysate. The plasma and buffy coat were removed after centrifugation at 3000 rpm for 10 min. Then, red blood cells were washed thrice in ice-cold isotonic sodium chloride solution (NSS). The RBC pellet was diluted with ice-cold distilled water in 1:10 ratio for the preparation of 10 % hemolysate for the estimation of superoxide dismutase (SOD) and catalase activity and lipid peroxidation (LPO). The remaining RBC pellet was diluted with ice-cold NSS in 1:1 ratio to get RBC suspension for GSH estimation. Haemoglobin concentration of the RBC hemolysate was estimated by cyano hemoglobin method [12]. Briefly, 20µl of hemolysate was mixed with 5 ml of (1:25 dilution) Drabkin's solution, and allowed to stand for at least 3min. The absorbance was read at 540 nm using Drabkin's solution as blank.

The oxidative stress biomarkers such as malondialdehyde (MDA) production, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), NBT activity, reduced glutathione (GSH) were estimated using prepared hemolysate. Reduced glutathione (GSH) in RBC suspension was estimated by DTNB method of Prins and Loos (1969). Two hundred µl of packed RBC suspension was added to 4ml of 0.08N H_2SO_4 and mixed carefully. After 10 min of standing at room temperature, 0.5ml of tungstate solution was added to the clear brown hemolysate. The tubes were stopered and the mixture was shaken vigorously for 5 min. The stopper was removed and the suspension was allowed to stand for 5 min in order to avoid crust formation on the top of the supernatant fluid in the subsequent centrifugation. The suspension was then centrifuged for 20 min at 2000 rpm at room temperature. After centrifugation, 2 ml of the clear supernatant was added to 2.5 ml of tris buffer (pH 8.0). DTNB reagent (0.2ml) was added, mixed well just before taking reading. When colour development was complete, optical density was measured at 412 m against blank. Simultaneously, a reagent blank was also prepared in which 2 ml of water was added instead of the supernatant. GSH concentration in the test sample was calculated by employing the molar extinction coefficient of DTNB-GSH conjugate as 13100/M/cm.

Glutathione peroxidase activity (GPx): GPx activity in hemolysate was measured by the method of Rotruck et al. (1973) using H_2O_2 as substrate in the presence of GSH. GPx enzyme catalyzes decomposition of H_2O_2 or other peroxides (- OOH) with simultaneous oxidation of GSH into GSSG. This method is based on the principle that µmol of GSH is oxidized in reducing the µmol of H_2O_2. The GPx enzyme preparation allowed the splitting of H_2O_2 utilizing the GSH. Reaction was stopped at a particular time by addition of TCA and remaining GSH was measured by developing the color complex with DTNB. GPx activity was expressed in µmol GSH oxidized/min/g Hb. The assay mixture consisted of 0.4 ml Tris-HCL buffer, 0.2 ml of GSH, 0.1 ml of sodium azide, 0.1 ml of H_2O_2, appropriately diluted enzyme preparation and distilled water in a total volume of 1.0 ml. Reaction was started by the addition of H_2O_2. After incubation at 370C for 10 min, reaction was stopped by addition of 0.5 ml TCA. Reaction mixture was centrifuged at 3000 rpm for 10 min. Then 0.5ml of supernatant was added to 2.0 ml of dibasic solution and 0.5 ml DTNB. The colour was read at 420 nm against suitable reagent blank.

Lipid peroxidation: The lipid peroxides level in the RBC hemolysate was determined by the method of Placer (1967) [37]. The method depends on formation of a coloured complex between the products of lipid peroxidation and thiobarbituric acid (TBA). Briefly, 0.2 ml of the RBC hemolysate was added to 1.3 ml of Tris–KCl buffer (0.2 mol/L Tris, 0.16 mol/L KCl, pH 7.4) after which, 1.5 ml of TBA reagent was added and mixture was heated in a boiling water bath for 10 min, with glass marbles as condenser. After cooling, 3ml of pyridine- n- butanol (3:1 v/v) and 1 ml NaOH (1N) were added and mixed by shaking. A blank was run simultaneously by incorporating 0.2ml distilled water instead of the RBC hemolysate. The absorbance was read at 548 nm using a spectrophotometer against the blank. The concentration of malondialdehyde (MDA) per mg haemoglobin was calculated using the extinction coefficient of 1.56x105 /M cm [13].

Catalase activity: Catalase activity in the haemolysate was estimated by using H2O2 as a substrate as per the method of Bergmayer (1983). Briefly, 20µl of the stock haemolysate was mixed with 1980µl of 50 mM phosphate buffer (pH 7.0) to make 2 ml of working hemolysate which was added to 1 ml of phosphate buffer-H_2O_2 solution taken in a cuvette of 3 ml capacity and mixed properly. Absorbance was taken immediately at 240 nm for 1 min using a spectrophotometer against phosphate buffer. Initial (0 sec) and final absorbance (after 60 sec) were noted down and difference between two was calculated. Catalase activity in the assay mixture was expressed in µmol H_2O_2 decomposed/ min/mg Hb. Catalase activity (µmol H_2O_2 decomposed/min/mg Hb) = (Final Abs - Initial Abs) x total volume of assay mixture / (0.067 x volume of actual sample x mg Hb/ml).

Super oxide dismutase (SOD): Superoxide dismutase activity was estimated as per the method described by Madesh and Balasubramanian (1998) [36]. It involves generation of superoxide by pyrogallol autooxidation and the inhibition of superoxide-dependent reduction of the tetrazolium dye MTT [3-(4-5 dimethyl thiazol 2-yl) 2, 5 diphenyltetrazolium bromide] to its formazan. The reaction was terminated by the addition of (DMSO), which helps the formed formazan to become soluble. The colour evolved is stable for many hours. The assay mixture consisted of 1280 µl of PBS (1M, pH 7.4), 60 µl of MTT, 20 µl of hemolysate and 150 µl of pyrogallol. Pyrogallol was freshly prepared and added after addition of all other reagents. The mixture was incubated for 5 minutes at 30oC and reaction was terminated by addition of 1500 µl of DMSO. The absorbance was measured at the wavelength of 570 nm. A blank was run by using distilled water only. A control was run by using all the reagents except hemolysate. The values were expressed as µmol MTT formazan formed per mg haemoglobin.

Respiratory burst activity (NBT Assay): The respiratory burst activity was measured by the reduction of nitro blue tetrazolium (NBT) by intracellular superoxide radicals. Fifty µl of heparinized haemolysate from each group was mixed with 50 µl of 0.2% NBT (Sigma, USA) solution, incubated for 30 min at 25°C. Fifty µl from the above mixture was added with 1 ml of N, N diethylmethyl formamide (Qualigens, India), and then centrifuged at 6000 X g for 5 min. The OD of the supernatant was measured at 540 nm.

Statistical analysis: The biochemical and oxidative stress parameters were analyzed by applying unpaired student 't' test and two way analysis of variance (ANOVA) followed by Bonferroni's post test using the Graph Pad Prism v4.03 software program (San Diego, CA, USA), and the differences between the experimental and control groups were considered statistically significant at $P \leq 0.05$.

Results

Production parameters: The ducks fed with aflatoxin-treated diet showed decreased body weight in comparison to the control group (Table 1). There was significant (P<0.05) decrease in body weight by 4th week of experiment in group II (2204.12±13.45g) and III (2104±10.56g) in comparison to control group I (2308.14±10.94g). However, body weight was significantly (P<0.05) higher in bentonite clay supplemented group IV (2184.21±10.31), V (2295.84±10.56), VI (2329.36±10.34) from toxin exposed positive group II and III (Table 1). Feed Conversion Ratio (FCR) increased significantly in group II (2.38±0.099) and III (2.33±1.002) in comparison to control group as recorded on 4th week. However, FCR on 4th week in group VI (2.23±0.085) was statistically comparable to control group (Table 3). At the end of 6th week, group II (2.27±0.0.096) and III (2.38±0.0.099) showed significantly higher FCR in comparison to control group (2.06±0.095). Decrease in FCR was significantly lower by 6th week in group VI (2.15±0.092) in comparison to other toxin-exposed groups.

Table 1: Experimental Design

Group	No. of ducks	Level of toxin given through feed (in ppb)	Bentonite clay to be given (g/kg of feed)
Group-1 (healthy control)	12	Basal diet	0
Group -2	12	48	0
Group -3	12	96	0
Group -4	12	96	1
Group -5	12	96	2
Group -6	12	96	4

Serum biochemistry: Significantly decreased serum triglyceride level was recorded in group II and III in comparison to all other groups on 4th week (Table 2). However, the triglyceride level in group IV, V, VI was significantly higher as compared to group II and III. Similarly, there was significant increase in triglyceride level in group IV, V and VI in comparison to group II and III on completion of 6th week of the experiment. However, group VI showed significantly higher triglyceride level compared to all other groups on 6th week. The cholesterol level increased significantly in group IV, V and group VI in comparison to all other groups on 4th week. However, cholesterol level was significantly higher in group VI in comparison to all other groups on 4th week. It was observed that group II (95.11± 5.458) and III (88.45± 6.473) showed significantly lower serum cholesterol level in comparison to control group (125.88± 5.012). Significant increase in cholesterol level was observed in group VI (128.94± 9.590) in comparison to other groups on 6th week and it showed very low significant difference as compared to control group (125.88± 5.012mg/dl of serum).

There was significant decrease in creatinine level in group IV, V, VI in comparison to group II and III on completion of 4th week of the experiment,. However, creatinine level was significantly lower in group VI. The aflatoxin treated groups showed increased creatinine level in comparison with the control group. All aflatoxin exposed bentonite clay supplemented groups showed decreased creatinine level, as compared to only aflatoxin treated group. Blood urea nitrogen (BUN) level was significantly increased in group II and III in comparison to all groups as recorded on 4th week. However, BUN level was significantly lower in group VI (5.59±0.231 mg/dl) in comparison to group II (5.84±0.200 mg/dl) and III (5.96±0.119 mg/dl) on 4th week. Group II (6.07±0.107 mg/dl) and III (6.61±0.225 mg/dl) showed significantly higher serum BUN level as compared to control group (5.51±0.204 mg/dl).

Table 2: Composition of Basal Diet Fed to the Ducks

MERCK feed ingredient	Duck grower
DORB (kg)	96
Soyabean (kg)	
Fish meal (kg)	6
Oysters shell grit (kg)	0
DCP (kg)	1.5
Calcite (kg)	1.5
Trace min. (kg)	1
DL-Metheonine (g)	150
L-Lysine (g)	195
Vit.A,D3,B2 &K (kg)	45
Vit.E & Se (g)	90
B Complex (g)	60
Toxin binder (g)	450
Ch. Chloride (g)	300
Wheat (kg)	159
Total (kg)	300

Table 2 shows the effects of Bentonite clay addition in feed on the level of serum AST in ducks exposed to aflatoxin. On 4th week post exposure to aflatoxin, there was significant increase in AST level in group II and III. AST level was significantly lower in group IV, V and VI, in comparison to group II and III, On completion of 6th week, the aflatoxin treated groups showed increase in AST level in comparison with the control group. All aflatoxin exposed bentonite clay supplemented group showed significant decrease in AST level, as compared to only aflatoxin treated group. The activity of ALT increased significantly in all aflatoxin exposed and aflatoxin exposed Bentonite clay supplemented groups on 4th week as compared to the control group. On completion of 6th week, there was significant increase in ALT level in group II and III. However, ALT level markedly decreased in group IV, V and VI. Group VI showed significant decrease in ALT level as compared to other treatment groups. The activity of GGT increased significantly in all aflatoxin exposed groups as compared to control group 1 and group IV on 4th week. On completion of 6th week, there was significant increase in GGT level in group II and III. This level markedly decreased in group IV, V and VI. However, the GGT level of group VI was statistically comparable to control group. The mean ALP activity was significantly ($P<0.05$) higher in group II and III (272.0±12.04 and 268.4±15.41, respectively) in comparison to control group (215.5±7.80) on 4th week. However, group IV, V, VI showed significant decrease in ALP level, and significantly decreased ALP level was seen in group IV, V, and VI in comparison to toxin exposed group without bentonite clay added to feed (group II and III). A significantly decreased ALP level in serum was observed group VI compared to other groups on 6th week.

Table 3: Effects of Bentonite Clay on Production Indicators in Ducks Exposed to Aflatoxin

		Treatment groups					
	wk	I	II	III	IV	V	VI
Body weight (grams)	0	2290.61±11.65^{a}	2410.21±10.23^{a}	2341.21±11.89^{a}	2294.54±12.74^{a}	2300.84±11.45^{a}	2346.21±11.32^{a}
	4	2308.14±10.94^{a}	2204.12±13.45^{a}	2104±10.56^{b}	2184.21±10.31^{b}	2295.84±10.56^{a}	2329.36±10.34^{b}
	6	2324.41±10.11^{a}	2145±11.33^{b}	1936.27±14.25^{b}	2214.56±11.57^{b}	2306.45±12.35^{c}	2389.46±11.63^{c}
FCR	0	2.04±0.093^{a}	2.09±0.087^{a}	2.14±0.077a	2.06±0.097^{a}	2.18±0.072^{a}	2.11±0.086^{a}
	4	2.09±0.079^{a}	2.24±0.090^{b}	2.33±0.056^{b}	2.30±0.071^{b}	2.29±0.082^{b}	2.23±0.085^{ab}
	6	2.06±0.095^{a}	2.27±0.0.096^{b}	2.38±0.099^{b}	2.27±0.066^{b}	2.25±0.094^{b}	2.15±0.092^{a}

3.3. Oxidative stress indices: The feeding of aflatoxin contaminated feed increased the erythrocytic malonaldehyde (MDA) production in comparison to the control group by 4th week of exposure. All aflatoxin-exposed and bentonite clay supplemented m group showed decreased MDA level compared to only aflatoxin exposed groups. There was significant decrease in MDA level in Group IV, V, VI in comparison to Group II and III. However, MDA level was significantly lower in Group VI (Table 3) supplemented with 3g of bentonite clay/ kg of feed. On completion of 4th week, there was a significant increase in superoxide dismutase activity in group II and III as compared to the control group, and a significant decrease in superoxide dismutase activity level was seen in group IV, V and VI as compared to group II and III as recorded on day 42. However, superoxide dismutase activity level significantly reduced in group VI in comparison with group IV and V. On completion of 4th week of the experiment, there was a significant decrease in GSH level in group II and III as compared to the control group and there was a significant increase in activity of GSH in group IV, V and VI as compared to group II and III. There was significant increase in GSH level in group IV, V and III as compared to the aflatoxin-exposed group on day 42. However, there was no significant difference in GSH activity between control group and group VI (Table 3).

On completion of 4th week, there was a significant decrease in catalase activity in group II and III as compared to the control group and a significant increase in catalase activity in group IV, V and VI as compared to group II and III. There was significant increase in catalase activity in group IV, V and III as compared to the aflatoxin treated group on day 42. However, catalase activity significantly increased in group VI in comparison with group IV and V. On completion of 4th week, there was a significant decrease in GPx level in group II and III as compared to the control group and a significant increase in GPx level in group IV, V and VI as compared to group II and III. There was significant increase in GPx level in group IV, V and III as compared to the aflatoxin treated group on day 42. However GPx level significantly increased in group VI in comparison to group IV and V.

Table 4: Effects of Bentonite Clay on Serum Biochemical Parameters in Ducks Exposed to Aflatoxin

Parameters		Treatment groups					
	wk	I	II	III	IV	V	VI
Triglyceride (mg/dl)	0	100.6±4.5^{a}	102.7±3.3^{a}	99.9±3.9a	102.1±4.8^{a}	100.8±3.3^{a}	101.5±6.0^{a}
	4	101.0±5.2^{a}	95.6±2.2^{ab}	92.2±2.6^{b}	94.9±2.8a^{b}	95.4±2.9a^{b}	96.0±2.2^{b}
	6	101.3±4.5^{a}	93.3±4.0^{b}	89.1±4.8^{b}	95.7±3.4bc	97.5±3.3c	98.6±2.9^{c}
Cholesterol (mg/dl)	0	123.3± 5.4^{a}	125.1± 5.4^{a}	125.5± 4.5^{a}	123.2± 4.9^{a}	124.1± 5.3^{a}	124.1± 4.9^{a}
	4	125.3± 4.3^{a}	108.5± 6.5^{ab}	99.6± 5.4^{b}	105.9± 7.2^{b}	112.5± 7.8^{ab}	115.8± 4.2^{ab}
	6	125.9± 5.0^{a}	95.1± 5.5^{b}	88.5± 6.5^{b}	109.1± 4.2^{bc}	120.5± 8.1^{bc}	128.9± 9.6^{c}
Creatinine (mg/ dl)	0	0.45±0.07^{a}	0.58±0.07^{a}	0.57±0.07a	0.47±0.08^{a}	0.52±0.09^{a}	0.43±0.06^{a}
	4	0.48±0.06^{a}	0.85±0.09^{ab}	1.55±0.09^{b}	0.86±0.08^{b}	0.74±0.07^{b}	0.58±0.06^{a}
	6	0.41±0.09^{a}	1.54±0.12^{b}	2.14±0.14^{b}	0.68±0.19a^{b}	0.59±0.12^{a}	0.48±0.05^{a}

BUN (mg/dl)	0	5.14±0.14^{a}	5.16±0.15^{a}	5.12±0.22^{a}	5.05±0.20^{a}	4.93±0.24^{a}	5.10±0.19^{a}
	4	5.02±0.21^{a}	5.84±0.20^{ab}	5.96±0.12^{ab}	5.66±0.12^{b}	5.61±0.20^{b}	5.59±0.23^{ab}
	6	5.51±0.20^{a}	6.07±0.11^{b}	6.61±0.23^{b}	5.57±0.13^{bc}	5.35±0.12^{c}	5.14±0.12^{c}
AST(U/L)	0	131.56±13.1^{a}	131.9±12.8^{a}	133.1±9.6^{a}	132.8±8.6^{a}	132.0±11.8^{a}	132.6±12.6^{a}
	4	132.24±11.7^{a}	175.3±8.8^{ab}	205.6±12.1^{b}	179.3±8.4^{ab}	175.5±6.2^{ab}	164.2±5.2^{ab}
	6	132.4±10.5^{a}	202.0±10.1^{b}	241.3±8.5^{b}	162.0±7.8^{b}	160.2±9.6^{bc}	152.1±6.8^{c}
ALT(U/L)	0	2.83±0.15^{a}	2.53±0.03^{a}	2.62±0.46^{a}	2.64±0.30^{a}	2.74±0.29^{a}	2.59±0.18^{a}
	4	2.90±0.23^{a}	7.41±1.01^{b}	15.28±1.10^{b}	12.36±0.18^{b}	11.0±1.18^{b}	9.51±0.16^{b}
	6	2.71±0.12^{a}	11.35±1.09^{b}	20.76±2.18^{b}	9.57±0.20^{b}	8.22±0.10^{c}	6.28±0.13^{ab}
Data are expressed as mean ± SE; n=12; data bearing superscript not in common differ significantly ($p<0.05$) within a parameter between periods of observation							

Table 5: Effects of Bentonite Clay Supplementation Oxidative Stress Indicators in Ducks Exposed to Aflatoxin

Parameters	Wk	Treatment groups					
		I	II	III	IV	V	VI
LPO (nmol MDA/mg Hb)	0	1.95 ± 0.06a	1.93 ±0.07a	1.90±0.07a	1.87±0.08a	1.93±0.09a	1.90±0.06a
	4	2.02±0.06a	2.81±0.09ab	3.08±0.09b	2.54±0.08ab	2.36±0.07ab	2.19±0.06ab
	6	2.11±0.09a	2.97±0.12b	3.25±0.14b	2.19±0.13ab	2.10±0.12ab	2.06±0.12a
SOD (U/mgHb)	0	47.48±2.19a	42.79±2.08a	41.71±2.46a	45.36±1.97a	48.92±2.79a	47.65±2.56a
	4	49.46±2.16a	36.45±1.66b	34.56±3.66b	40.38±2.48ab	42.46±3.21ab	47.21±2.76a
	6	49.82±2.69a	33.12±1.84b	31.24±2.57b	42.15±2.65bc	45.92±1.22c	50.45±1.63c
Catalase (µmol H2O2 decomposed/min/mg Hb)	0	0.31±0.02a	0.29±0.02a	0.28±0.016a	0.34±0.021a	0.32±0.016a	0.31±0.013a
	4	0.33±0.14a	0.27±0.03b	0.28±0.02b	0.29±0.03b	0.33±0.03ab	0.36±0.01ab
	6	0.32±0.012a	0.25±0.015b	0.24±0.018b	0.32±0.015b	0.35±0.022c	0.40±0.017c
GPx(U/g Hb)	0	15.32 ±1.64a	14.65±1.06a	14.02±0.89a	15.04±1.23a	14.92±0.84a	15.63±1.14a
	4	15.67±1.55a	13.87±0.93b	12.65±0.73b	14.31±0.76b	15.26±0.78a	16.27±0.63ab
	6	15.98±1.44a	13.21±1.02b	11.86±1.51b	18.02±0.95bc	17.28±0.89c	20.45±0.91c
NBT (nmol /min/mg Hb)	0	0.120±0.005a	0.124±0.006a	0.128±0.006a	0.127±0.005a	0.124±0.008a	0.125±0.005a
	4	0.126±0.007a	0.162±0.009ab	0.208±0.012b	0.141±0.008ab	0.129±0.007ab	0.115±0.006a
	6	0.127±0.012a	0.195±0.016ab	0.241±0.015b	0.132±0.014ab	0.119±0.012ab	0.098±0.013a
GSH(µmol/mL of packed RBC)	0	2.84±0.163a	2.79±0.128a	2.81±0.134a	2.86±0.175a	2.84±0.081a	2.90±0.074a
	4	2.86±0.169a	2.34±0.100b	2.28±0.094ab	2.58±0.181ab	2.73±0.075ab	2.86±0.061a
	6	2.87±0.123bc	2.51±0.134a	2.01±0.132a	2.76±0.098ab	2.82±0.101ab	2.94±0.123c
Data are expressed as mean ± SE; n=12; data bearing superscript not in common differ significantly ($p<0.05$) within a parameter between periods of observation							

Discussion

Aflatoxin has varied inherent toxic properties on several body systems like immunotoxicity, carcinogenicity, blood clotting, protein utilization. Bio-activation of AFB1 predominantly occurs in hepatocytes after absorption in the small intestine, especially from the duodenum. Cytochrome P450 isoenzyme system metabolizes aflatoxin B1 into reactive and electrophilic exo-AFB1 8, 9-epoxide (AFBO), that forms adducts with DNA, RNA and protein, mediating the toxic effects of aflatoxin. In the present experiment, experimental contamination of diet with aflatoxin caused a significant reduction in body weight of the grower ducks on completion of 4th and 6th week in a dose dependant manner. The present doses of exposure were decided from the study revealing significant reduction in body weight gain in growing Alabio ducks after feeding on diets containing 50 µg of aflatoxin B1 equivalent or more /kg of feed [14]. A dose-dependent reduction in weight gain and feed consumption was also observed when broiler chicks were fed with a diet contaminated with increasing level of purified AFB1 [15]. Han et al. (2008) also recorded a significant reduction in body weight gain and feed intake in cherry valley ducks maintained in aflatoxin contaminated diet. Negative impact of aflatoxin on body weight gain have also been reported earlier [5, 17-21]. In the present experiment, the feed conversion ratio (FCR) increased significantly in groups maintained on diet containing only aflatoxin. Increased feed to gain ratio and liver damage were earlier reported upon feeding of diets containing different levels of AFB1 in Cherry Valley commercial ducks and in broilers [16, 22]. However, contradictory findings of decreased feed conversion ratio in the aflatoxin-treated group than the controls have been documented in male Cherry Valley ducklings [23]. The ducks treated with aflatoxin plus bentonite clay showed a significant decrease in the feed conversion ratio (FCR) by the end of 4th and 6th week. This may be due to the prophylactic effect of bentonite clay as a toxin adsorbent.

In vivo experiments were carried out to assess efficacy of bentonite against aflatoxicosis. Addition of bentonite (levels not more than 0.5%) in swine diets containing 800ppb AFB showed improvement in average daily gain and daily feed intake and also liver and kidney biochemical enzymes [24]. Schell et al. (1993) used 1% sodium bentonite and 0.5% calcium bentonite separately against aflatoxicosis that showed a potential effect on partial restoration of performance and liver function without influencing mineral metabolism. Sodium bentonite (SB) has been found to reverse diminishing body weight gain induced by aflatoxin in broilers [25]. The addition of different amounts of sodium bentonite to growing swine diets containing aflatoxin B1 could improve the average daily body weight gain and average daily feed intake [24]. This may be due to the adsorption of aflatoxin by bentonite clay in gut which reduces the availability of aflatoxin [26].

Current study showed a dose dependant decrease in the level of serum triglyceride, cholesterol in aflatoxin B1 fed ducks as compared to the healthy control birds. Aflatoxin B1 at levels of up to 0.3 mg/kg decreased serum cholesterol levels in broilers [27, 28]. The triglyceride and cholesterol value increased significantly in aflatoxin exposed and bentonite supplanted groups as compared to only aflatoxin treated group. The BUN and creatinine value increased with increasing dose of aflatoxin in ducks as compared to the control group. This increase could be attributed to the damaging effects of aflatoxin to the renal corpuscles and increase in catabolism of protein. A linear increase in serum urea nitrogen concentrations with increasing AFB1 and elevated serum urea nitrogen levels by 2 mg of AFB1/kg of feed have been reported in broiler birds [5]. Birds fed with aflatoxin contaminated feed supplemented with bentonite showed a marked decrease in the level of BUN and creatinine as compared to the birds fed with aflatoxin B1 alone. This reduction was significant by both 4th week and 6th week of feeding bentonite clay. The treatment with sodium bentonite caused decrease in urea nitrogen in swine fed with aflatoxin [24]. A significant increase in the serum AST and ALT values was seen by feeding of aflatoxin B1contaminated feed for a period of 4 week and 6 weeks. This maight be due to the inherent hepatotoxic effect of aflatoxin B1 in ducks. A significant increase in serum ALT value in all aflatoxin B1 treated groups compared to controls has been reported earlier [29]. An elevated serum AST and ALT level indicates cellular (hepatocyte) damage in aflatoxin B1 intoxicated white Pekin ducklings [5]. Han et al. (2008) also recorded significant increase in the activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in AFB1-contamined groups. A marked increase in serum aspartate aminotransferase (AST) or glutamic oxalocetic transaminase (SGOT) levels was recorded in chickens receiving 10 ppm AFB1 indicating substantial liver damage [15]. Serum AST and ALT values decreased in ducks fed with aflatoxin contaminated diet and upplanted with at the end of the 4th week. Serum level of GGT increased significantly in aflatoxin intoxicated ducks. This could be attributed to the damaging effect of aflatoxin on hepatocytes [5]. A significant reduction in serum GGT level was seen in ducks fed with aflatoxin plus bentonite by 4th week. Similar result was earlier recorded in pigs [25]. Feeding of aflatoxin contaminated diet with 0.5% sodium bentonite reduced serum level of GGT as compared to aflatoxin intoxicated pigs [24]. However, a higher concentration of the adsorbent was recommended for ducks to reduce GGT level.

The present experiment in ducks with exposure to two different levels of aflatoxin induced oxidative stress as evidenced by increased level of MDA and auto up regulation of activities on erythrocytic antioxidant enzymes,. There was a significant elevation of malondialdehyde (MDA) level in aflatoxin exposed ducks at the end of 4th and 6th week of exposure. This could be attributed to the cytotoxic effect of aflatoxin which causes release of free radicals, which in turn results in lipid peroxidation [30, 31]. Increase in the content of hepatic mitochondrial malondialdehyde (MDA) in aflatoxin treated groups as compared to the control group justifies that AFB1 significantly induced dysfunction of hepatic mitochondrial antioxidant [32]. The MDA level decreased in ducks fed with diet supplemented with bentonite clay as recorded on 4th week and a significant decrease was recorded in 6th week of treatment. This may be due to the absorptive and ion exchange properties of bentonite clay which absorbed the toxin present in feed (Winfree and Alfred, 1992). The amount of reactive oxygen species (ROS) or superoxide was determined by measuring nitroblue tetrazolium assay (NBT). The NBT level was significantly high in groups fed with aflatoxin alone as compared to control group. Intracellular increase in amount of reactive oxygen metabolites was recorded earlier in bovines exposed to different levels of aflatoxins [30]. The cytotoxic outcomes in aflatoxin fed birds may be associated with intracellular ROS generation, leading to membrane damage and DNA strand break [33].

However, the NBT level was decreased in groups exposed to aflatoxin plus supplemented with bentonite clay by completion of 4th and 6th week of experiment. The amount of GSH level decreased significantly in ducks intoxicated with aflatoxin on completion of 4th and 6th week of the experiment. Umberto et al. (2011) recorded decrease in intracellular GSH level in bovines fed with aflatoxin. This causes cytotoxicity by indirectly increasing the ROS level as GSH acts as ROS scavenger and prevents oxidative damage of proteins [34]. On 4th week post exposure, the superoxide dismutase (SOD) activity decreased significantly in aflatoxin fed birds. Shi et al. (2011) in his experiment in aflatoxin intoxicated ducklings also found a similar result which showed that aflatoxin B1 @ 0.1 mg/kg body weight can significantly reduce the activities of mitochondrial superoxide dismutase (SOD). Chen et al. (2014) also found a dose dependant decrease in SOD value in broilers fed with aflatoxin B1 [35]. Treatment of feed with bentonite clay in aflatoxin contaminated feed showed marked increase in the level of SOD in ducks. The current study showed a significant decrease in the activity of enzyme catalase in aflatoxin intoxicated ducks on 4th and 6th week of post exposure.

This decrease was ameliorated significantly in ducks fed with diet containing aflatoxin plus bentonite on 4th and 6th week post exposure. The glutathione peroxidase (GPx) level decreased significantly in groups fed with increasing dose of aflatoxin as compared to control group on 4th and 6th week of experiment. A significant decrease in glutathione peroxidase level was recorded in bovines fed with diets containing aflatoxin [30]. However, on 4th and 6th week, ducks provided with diet added with bentonite clay showed a increase in GPx level. This cytoprotective effect may be due to the adsorbing of toxin by bentonite clay which reduces the exposure of cells to aflatoxin. Bentonite is postulated to bind irreversibly with toxin in gut preventing absorption of toxin across the intestine wall providing prophylactic action. It is concluded that addition of bentonite clay to the diet reduces deleterious health effects of feeding aflatoxin contaminated feed [36-38].

Conflicts

No conflicts of Interest

Funding

Internal Resources of the Institute

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