PRODUCTION OF AFLATOXINS FROM *Aspergillus flavus* AND ACUTE AFLATOXICOSIS IN YOUNG BROILER CHICKS

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In the present study aflatoxins were produced by fermentation of rice. The highest average concentration of aflatoxins B1 was 763.157µg/ g of rice in those flasks having addition of trace metals. Broiler chicks of 7 days of age were offered feed containing 0, 1600, 3200 and 6400 µg/ kg aflatoxin B1 for 7 days. Clinical signs in intoxicated birds included depression, ruffled feathers, decreased interest in feed, increased water intake and soft to watery feces. A significant decrease in body weight was observed in all groups which were in a dose related manner and it was more severe in group fed 6400 µg/kg aflatoxins B1 in feed. Pathological lesions in birds of treatment groups included pallor discoloration of liver and enlargement of liver and kidneys. Hemorrhages were present on different organs of the body. Microscopically congestion of liver parenchyma, cytoplasmic vaculation/fatty change of hepatocytes, necrosis of hepatocytes, newly formed bile ducts, mononuclear and hetrophilic cell infiltration were observed in aflatoxin fed broiler chicks. Kidneys of aflatoxins B1 intoxicated birds were enlarged and microscopically revealed degeneration and necrosis of tubular epithelial cells, congestion and hemorrhages of the parenchyma. Present study revealed that all the clinical, gross and histopathological lesions were dose related.

Key words: Aflatoxicosis, broilers, clinical signs, gross and histopathology

INTRODUCTION

Aflatoxins (AF) are mycotoxins produced by the toxigenic fungi mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. Toxicity of these fungi was first reported in samples of groundnut meal used for livestock and poultry feed (Asplin and Carnaghan, 1961 and Sargeant *et al*., 1961). Aflatoxins ingested by the birds accumulate in different tissue/organs and eggs and entered into human food chain which posses a major risk to human health, as these aflatoxins are not inactivated by dry heating at160 °C for one hour or by steam heating (Carnaghan, 1964) and are unlikely to be affected by cooking. In poultry the severity of clinical disease and lesions of aflatoxicosis may vary with varying levels of dietary aflatoxins (Espada *et al.,* 1992). In tropical and subtropical areas, aflatoxin is the number one mycotoxin problem and especially in Pakistan the highest concentration may exceeds 1000 µg/kg (Wei and Cortyl, 2002).

Aflatoxicosis in poultry is primarily a disease of the liver and it showed the typical lesions on it, which ultimately cause production problems and mortality. The main clinical signs in affected birds are decreased feed intake, decreased body weight, poor skin, decreased egg production and decreased immunity. The disease may be fatal and resulted in heavy mortality. The main lesions of aflatoxicosis in birds are also appeared on liver and kidneys which are, Jaundice, generalized oedema and hemorrhages, tan or yellow discoloration of the liver, periportal necrosis with bile duct proliferation and fibrosis and depletion of lymphoid organs. (Charlton *et al*., 2006). and there is a little awareness of harmful effects of aflatoxins and it is a common practice of feed mill owner, that damaged and moldy food grains rejected as unfit for human consumption are mixed in poultry feed. Similarly other cereal grains, oilseeds cake and their products, particularly corn and its bye product contamination with AF are mixed in poultry ration.

In Pakistan, the majority of research work was conducted on the incidence of toxigenic fungi in feed and feed ingredients and their aflatoxins levels (Afzal *et al.,* 1979; Munir *et al.,* 1989; Hanif *et al.,* 2006; Bhatti *et al*., 2001). Continuous reports of heavy contamination of poultry feed ingredient and finished feed with AF contamination, a little work has been done on experimental production of aflatoxicosis in Pakistan. Sporadic work was performed on experimental aflatoxicosis like Khan (1994); Arshad *et al.,* (1992); Rizvi (1990) and even then no one studied the lesions on their intensity. Keeping in view the present study was design to produce experimental aflatoxicosis in broiler birds of 7 days old with AFB1 at varying dose levels.

MATERIAL AND METHODS

The study was completed in two phases. In Phase one a toxic fungus was cultured in the laboratory and aflatoxins were produced on rice. Aflatoxins contents of rice were analyzed and quantified. In phase two

quantified Aflatoxins were mixed in experimental feed and aflatoxicosis was induced in broiler chicks. This trials was conducted at the age of 7 days and acute aflatoxicosis was induced by doses of AFB1 (1600, 3200, 6400 µg/kg) in feed for one week. Different parameters like, gross, clinical pathology and histopathology were studied in detail.

Aflatoxins production and quantification

Aflatoxins were produced with lypholized spores of *Aspergillus flavus*, link: Fries. A (NRRL 6540 and CECT 2687) obtained from the Culture Collection Center University De Valencia Spain, on rice following the method described by Shotwell (1966) with or without 25 µL trace metals solution ($ZnSO_4 \cdot 7H_2O$1.0 g, $CuSO_4 \cdot 5H_2O$, 0.50 g and Distilled water 100 ml). After 5 days these flasks were autoclaved at 121^o C for 15 minutes and aflatoxin B1 level of their contents was determined by High Pressure Liquid Chromatography (HPLC) method 990.33 (AOAC, 2000).

Extraction and mixing of AF in feed

The amount of AFB1 to be mixed in feed of each group was calculated. The quantities of fermented rice containing required amount of AFB1 were weighed and extracted by soaking into three fold quantity of chloroform (100:300) for overnight and filtered through cotton cloth. All the chloroform was evaporated under rotary evaporator. The concentrated residues were re-suspended into 100 ml Polyethylene glycol (PEG). This suspension was evenly mixed in 0.5 kilogram, than in two kilogram and finally in the required quantity of feed. A corn soy meal based feed having 22% total protein and 3000 Kcal/kg metabolizable energy was prepared without addition of any toxin binder, vitamins, mineral supplement and antibiotic. Prior to use, each batch of the basal feed was analyzed for aflatoxin, ochratoxin and zearalenone (Howel and Taylor, 1981).

Experimental plan

Broiler chicks of 7 days of age were randomly divided into four groups (A-D) having 80 birds in each. Aflatoxin B1 was mixed in the feeds at 0, 1600 µgm/ kg, 3200 µgm/ kg and 6400 µgm/ kg and offered to birds of these groups for 7 consecutive days. Afterward birds were offered basal feed for remaining period. Six birds were killed from each group at days 1, 2, 3, 5 and 7 of the experiment. Individual body and relative organ weights were also recorded at the time of each sampling. Mortality in each group was also recorded. All remaining birds were killed at the end of the experiment. Duration of the experiment was 35 days (42 days of age).

Study of Clinical signs, behavioral alterations, gross and histopathological lesions

Clinical signs and behavioral alterations in birds of each group were subjectively recorded by observing twice daily for dullness, attraction to feed, attraction to water, condition of feces, appearance of feathers. The presence or absence of nervous signs was also observed. Each clinical signs was subjectively evaluated and designated as normal, mild, moderate and severe change

At necropsy the visceral organs of the birds were examined and lesions present were recorded as no change, mild, moderate and severe. Different organs were weighed. About 5 mm thick pieces from liver and kidneys of each bird were fixed in 10 % neutral buffered formalin for histopathological examination (Bankroft and Gamble 2007).

Statistical Analysis

The data obtained in all the groups were subjected to statistical analysis by analysis of variance. The means of different groups were compared by Duncan's Multiple Range Test using MSTATC statistical software. The level of significance was $p \leq 0.05$.

RESULTS

Experimental Production of Aflatoxins

Aflatoxins production was achieved through fermentation of rice. This part of experiment was conducted in different batches. The results of different batches are shown in Table 1. Flasks showing formation of spores yielded lesser AF than those where spore formation had not occurred. Results of fermentation with trace metal showed high production of AF compared with those batches where no trace metal mixture had been incorporated in the rice. Highest AF was produced in batch 5, which were 763.157µg/ g µg/ g.

Clinical Signs and Behavioral Alterations

The results of clinical signs and behavioral alterations are shown in Table 2. Birds in group A (control), did not exhibit any abnormal signs and their behavior remained normal throughout the length of the experiment. All the birds rushed towards feed and water.

The birds remained active and became alert upon tapping of the cage wall. Birds of Groups B, C and D showed depression which was progressive with dietary concentration of aflatoxin being highest at day 7 of the experiment and then gradually decreased. Attraction towards feed decreased in a dose related manner and was lowest on day 7 of the experiment. Water intake

Table 1. Aflatoxin production by fermentation of rice

Fermentation without spore formation	Fermentation with Spore formation	Fermentation without spore formation	Fermentation with Spore formation	Fermentation without spore formation	Fermentation with Spore formation
Batch 1*		Batch 2		Batch 3	
AFB1 Contents (µg/g) of flasks with out trace metal					
96.358	25.221	107.268	36.664	98.279	20.265
AFB1 Contents (µg/g) of flasks with trace metal					
Batch 4		Batch 5		Batch 6	
516.231	41.256	763.157	50.759	613.746	48.143

*****Six flasks in each batch

Table 2. Score of clinical signs and behavior of broiler chicks given different levels of feed aflatoxin B1 for 7 days at age of 7 days

Clinical signs and behavior	Groups (aflatoxin B1 µg/kg feed)			
	A (0)	B (1600)	C (3200)	D (6400)
Alertness Normal – depressed	0	Mild	Mild	Severe
Attraction to feed Normal – less interest	0	Mild	Severe	Severe
Attraction to water Normal – more/less interest	0	Moderate	Moderate	Moderate
Faeces consistency Normal Formed – watery	0	Mild	Mild	Moderate
Feather Normal Shiny – ruffled & Broken	0	Mild	Moderate	Moderate
Nervous derangement No – present	0	No	No	No

A. Control B. 1600 µg/kg aflatoxin B1 in feed C. 3200 µg/kg aflatoxin B1 in feed D. 6400 µg/kg aflatoxin B1 in feed

increased in all experimental groups in a dose related manner being highest in group D followed by C and B. Feces of the birds of group A were formed and solid, whereas those of other groups passed semisolid (soft) and loose feces. Feathers of birds of group A were shiny throughout the length of the experiment. Birds of group B, C and D exhibited a decrease in shine and cleanliness of feathers. In Group C and D birds had ruffled and broken feathers. None of the birds in any of the experimental groups showed signs of nervous derangement. Clinical signs in all the groups gradually returned to normal and the behavior of the birds became same as in group A after withdrawal of aflatoxin from the feed.

Mortality

Mortality of the birds given different dietary levels of aflatoxins at 7 days of age has been presented in Fig. 1. No bird died in control group (group A). Mortality was 0 %, 10 %, 20 % and 36.25 % in groups, A, B, C and D, respectively.

Body weights

Bodyweights (g) of broiler chicks given different levels of AFB1 in feed for 7 days have been shown in Table 3. There was non-significant difference in the body weights of groups A, B, C and D on days 1 and 5 of the experiment. On day 7, the body weight of Group D was significantly lower from groups A, B and C. On day 35 body weight of group B was non-significant while those of groups A and D were significantly lower from that of groups A, B, and C.

Relative organ weights

Results of relative organs weight have been presented in table 4.

On day 7 relative organ weights of livers**,** kidneys and spleens in all treatment groups had significantly higher values than those of group A. Relative weight of Bursa of Fabricius of all groups was non significantly different from each other on day 7 of the experiment.

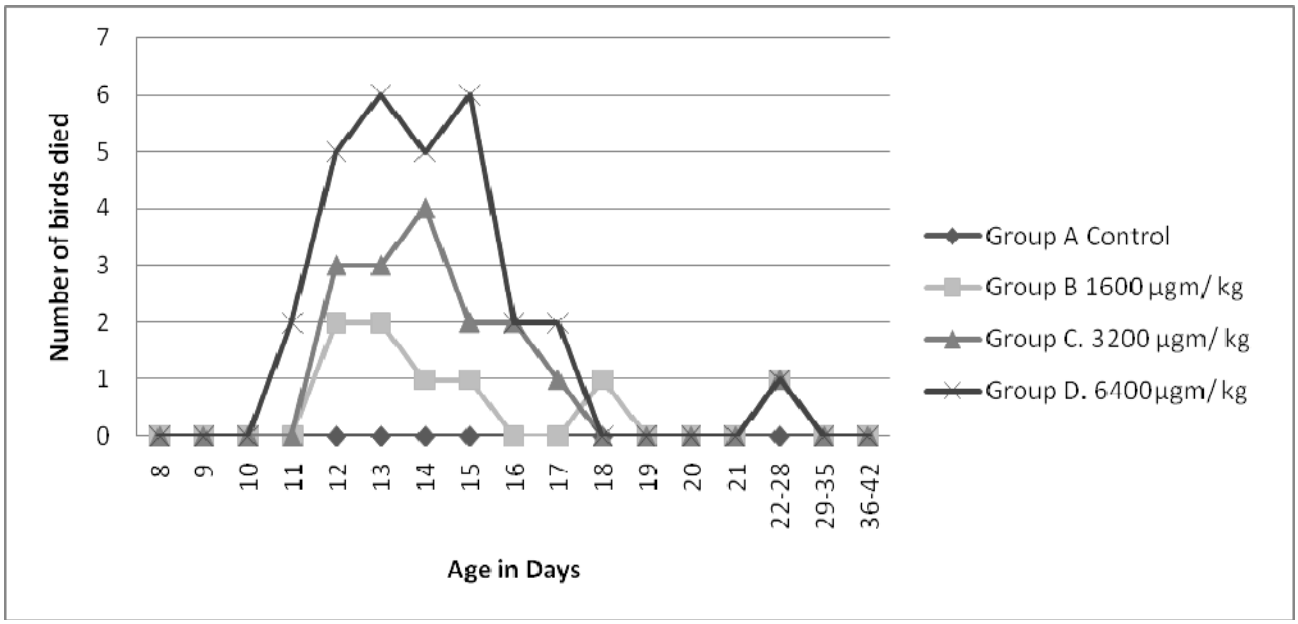

Fig 1. Mortality of broiler chicks given different feed levels of aflatoxin B1 for 7 days at age of 7 days

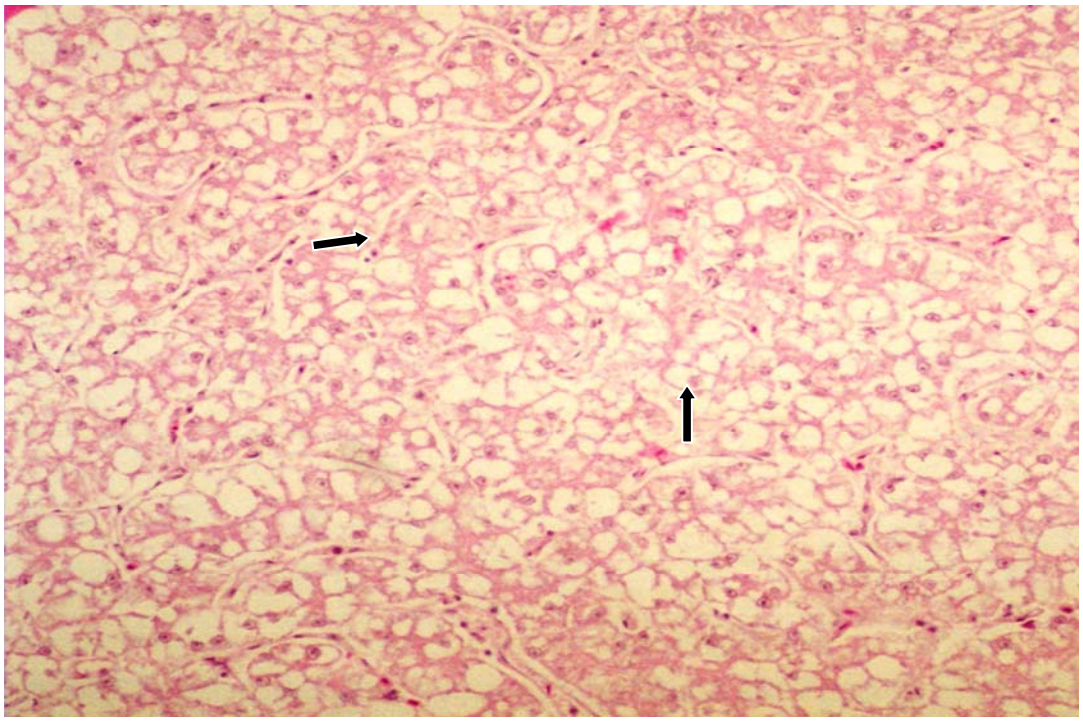

Fig. 2. Photomicrograph of Liver showing fatty changes fed 6400 µg / kg AFB1 for 7 days (H& E stain 200X)

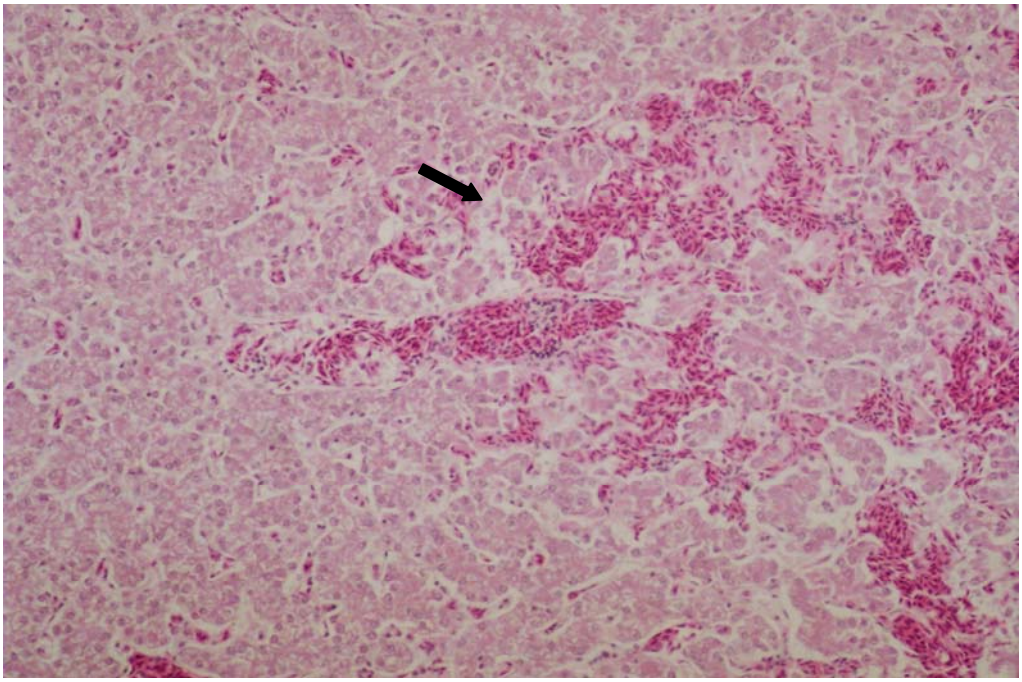

Fig. 3. Photomicrograph of liver of a broiler chick fed 6400 µg / kg AFB1 for 7 days. There is congestion and hemorrhages in the parenchyma. (H& E stain 200X)

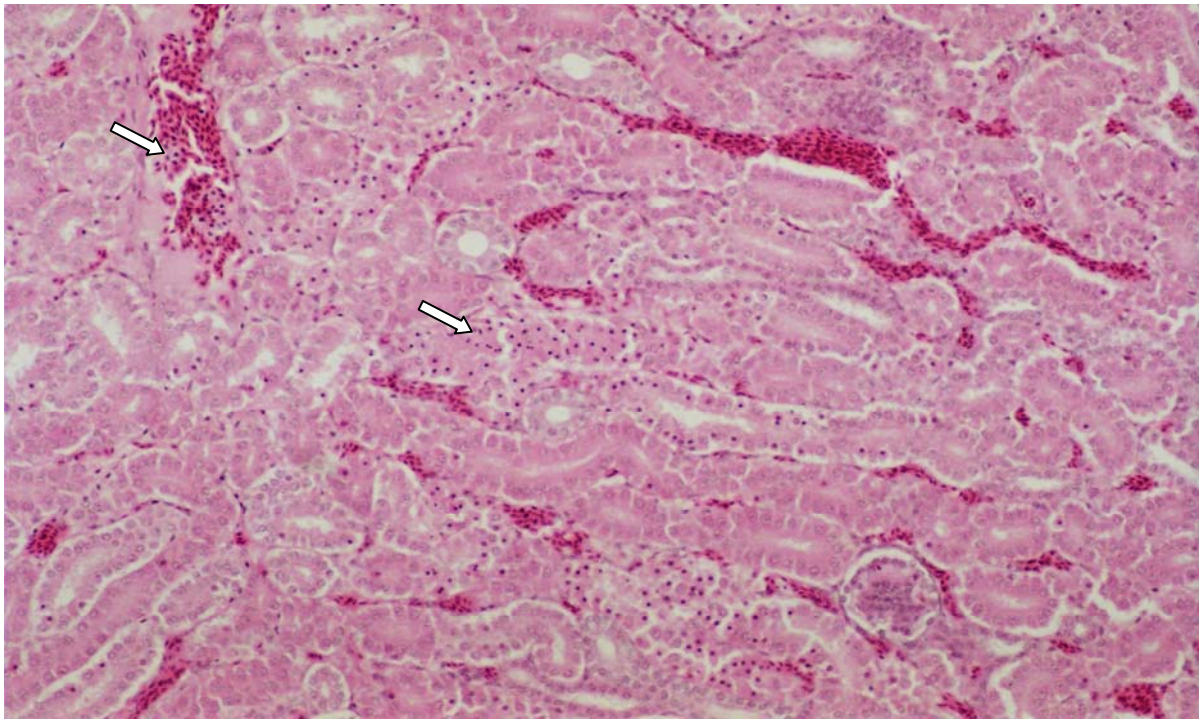

Fig. 4. Histopathological picture of a kidney of a broiler chick fed 3200 µg / kg AFB1 for 7 days. There are congestion and pyknotic nuclei of tubules (H& E stain 200X)

Gross Lesions

Variation in color, size of different organs, and friability of liver and frequency of hemorrhages in different organs/tissues of birds of different groups were evaluated by visual examination on each slaughtering and has been presented in Table 5. Birds in group A did not show any gross deviation from the normal anatomical patterns in any organ throughout the course of the experiment. Liver was normal in size, color and consistency. Kidneys, heart, spleen, thymus, intestine and subcutaneous tissue were normal in appearance throughout the length of experiment. In group B no changes were present on day 1, 2 and 3 of the experiment. On day 5 a moderate enlargement of liver and kidneys was present while other organs appeared normal. On day 7 liver and kidney were enlarged. Livers were lighter in color and had friable consistency. Mild to moderate punctuate and echymotic hemorrhages were present on subcutaneous tissue, muscles, liver, intestine, mesentery, heart and kidneys. On day 10, livers were larger in size, lighter in color and friable in consistency. Kidneys were also moderately enlarged. On day 15 and 35 no gross morphological abnormality was present in the organs.

Birds in group C showed no gross alteration in any organ on day 1 and 2 of the experiment. On day 3 livers were moderately enlarged and lighter in color. On day 5 a moderate enlargement of liver and kidneys was present while other organs appeared normal. On day 7 livers were larger than on day 5, lighter in color and friable in consistency. Kidneys were also moderately enlarged. Hemorrhages were present on liver, muscles, subcutaneous tissue, heart, intestine, mesentery and kidneys. On day 10 gross pathological pictures was similar to that of day 7. On day 15 and 35 changes reversed and organs were similar in size, color and consistency to those of group A.

Birds in group D showed no gross alterations in any organ on day 1 and 2 of the experiment. On day 3 liver and kidneys showed moderate swelling. Liver was lighter in color and friable in consistency. On day 5 and 7 enlargement of liver and kidneys were more conspicuous and spleen was moderately enlarged. Liver was friable. Different organs and tissues showed multiple petechial and echymotic hemorrhages on liver and kidneys, muscles, heart, intestine, mesentery and subcutaneous tissue. Similar changes were present on day 10 of the experiment. On day 15 enlargements of liver and kidneys was less severe. On day 35 all the organs had normal appearance as those of group A.

Histopathology

Histologicaly livers and kidneys in group A killed during the experiment showed a fairly well preserved lobular texture. No cellular infiltration or circulatory disturbance was observed in hepatic tissue. Nuclei of hepatocytes were centrally placed had one or two nuclei. Cytoplasms of hepatocytes were foamy. No abnormal morphological pattern and cellular accumulation were observed. In kidneys Bowman spaces of glomeruli were clear. Tubules were lined with cuboidal epithelial cells having centrally placed nuclei. There were no cellular or exudative accumulations in the kidney tissue.

Livers of different group's showed, fatty change and hemorrhages of hepatocytes (Fig. 2 & 3) which was sever in group D fallowed by C and B. On day 10, vacuoles of hepatocytic cytoplasm became smaller in size or disappeared in some birds. Some newly formed bile ducts were present in the parenchyma. On day 15, there was shrinkage of cell with pinkish cytoplasm and sinusoidal spaces became wider than those of group A. Hepatocytic necrosis was highest in group D. Mononuclear cell infiltration in group D and C was higher than that of group B. Similarly newly formed bile ducts increased with increase in dietary AF levels. Nuclear size variation attained higher difference in groups D. In kidneys mild to moderate degree of congestion was present on days 1and 2 of experiment. On day 3 pyknosis of epithelial cells of proximal convoluted tubules was prominent along with moderate congestion in group D fallawed by C and B (Fig. 4). On day 5 and 7 necrotic changes in proximal convoluted tubules were wide spread in group D and it was in dose related manner. At days 10 & 15 congestion of parenchyma and necrosis of tubular epithelial cells were still evident. On day 35 the experiment mild congestion was present while necrotic changes were not observed.

DISCUSSIONS

Aflatoxin used in the present study was produced by culturing of *Aspergillus favus* (CECT 2687). This strain has been known to produce both aflatoxin B1 and aflatoxin B2. AFB1 is the most toxic fraction of all aflatoxins and is also produced in excess of the other fractions. Results of present study are in line with Shotwell *et al.,* 1966. Therefore, administration of aflatoxins in the experimental feeds was based upon the concentration of AFB1 only. Many workers produced experimental aflatoxicosis in chicken and swine by using extracts of fungal cultures to prepare AF contaminated feeds and described the level of contamination by AFB1 concentrations only (Del Bianchi *et al.,* 2005; Dilkin *et al.,* 2003; Ortatatli *et al.,* 2002).

Clinical signs of aflatoxicosis in broiler chicks observed in the present study were in line with Ortatatli and Oguz, 2001; Kubena *et al*., 1998; Khan *et al*., 1994 and Asim 1990. They reported, histopathological changes included fatty change, cellular dissociation, necrosis, cellular infiltration, fibrosis and bile duct hyperplasia. Ortatatli and Oguz (2001) also described gross and histopathological lesions on score basis and reported an increase in the relative weights of liver and kidney; and gross-histopathologic hepatic lesions such as paleness, friability, diffuse hydropic degeneration and/or fatty change, bile-duct hyperplasia and periportal fibrosis. Glumerular hypertrophy, increases in the number of mesengial cells and hydropic degeneration of tubuler epithelium in kidneys of chicks fed diet AF alone were also observed. Atrophy and lymphoid depletion were seen in the thymuses and bursa of Fabricius from the chicks fed AF also. Similer lesions were also reported by Ortatatli, et al., 2005 in broilers fed a diet containing low-levels of aflatoxin. They also studied the gross and histopathological changes in the liver, kidneys, spleen, thymus and bursa of Fabricius and calculated the relative organ weights. As compared to controls, significant changes ($P < 0.05$), such as slight to moderate hydropic degeneration and/or fatty change (8 cases of 10), bile-duct hyperplasia (7 of 10) and periportal fibrosis (5 of 10), were found in chicks fed 100 ppb AF-containing diet. No gross–pathological changes were observed in any treatments.

In field outbreaks of aflatoxicosis causes thirstiness, anorexia and mortality as general changes but no consistent clinical symptoms could be attributed to aflatoxicosis. Increased water intake of intoxicated birds as observed in the present study has also been reported in broiler chicks during aflatoxicosis (Rajion and Farrel, 1976).

An increased water intake during aflatoxicosis might be an attempt to avoid dehydration and replenish the body water loss due to loose and watery dropping. In wild birds Robinson *et al*., (1982) described weakness, lack of response to environment, little response to feed and a general depression in snow geese (*Anser caerulescens*) and mallards (*Anser platyrhynchos*) suffering from aflatoxicosis.

In present study changes like clinical signs, mortality, body weights, mortality and gross or microscopic lesions increased in severity of signs with increase in the dietary concentration of AF in the birds of same age. Mortality during experimental and field cases of aflatoxicosis has been reported by many workers in chicken (Shivachandra *et al*., 2003; Abdelhamid *et al*., 1994), turkey poults (Arafa *et al*., 1981) and water fowl (Robinson *et al*., 1982).

A decrease in body weight of birds during experimental aflatoxicosis has been observed by many workers by administering different levels of aflatoxins in the feed for variable periods of time (Bintvihok and Kositcharoenkul 2006; Tessari *et al*., 2006). Lanza *et al.* (1980) reported a significant decrease in body weight of broiler chicks below 21 days of age fed up to 5 mg/kg aflatoxin. Same dietary levels of aflatoxins, however, resulted in a non significant difference in body weight of birds above 21 days of age suggesting an age related development of resistance in chicks towards aflatoxins. In more recent studies Quezada *et al*. (2000) reported a non significant change in body weight of 4 weeks old broiler chicken by feeding AFB1 up to 2.0 µg/g of feed. A variable degree of increased relative weight of the spleen observed in intoxicated birds in the present study, might be due to congestion and hemorrhages in different organs resulting in excessive blood cell lysis. Bursa of Fabricius in the present study also showed a variable response ranging from a decreased to non significant relative weight compared with control. A non significant change in Bursal size or weight during aflatoxicosis in broiler chicks has been described by Ortatatli *et al*. (2005).

It was concluded from present study, that all doses levels produced clinical, gross and histopathological lesions, which are related with dose of aflatoxin in feed. Sever lesions were noted at 6400µg/ kg as compared to 1600µg/ kg AFB1 in feed.

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