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RESEARCH ARTICLE

Effect of Ochratoxin A (OTA)-Contaminated Feed on Several Health and Economic Parameters in White Leghorn Cockerels

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ABSTRACT

This study was designed to evaluate some pathological responses of male White Leghorn (WL) chicks, kept on low level of ochratoxin A (OTA) contaminated feed. For this purpose, 350 a-day old male WL chicks were divided into five groups (A-E). Group A, was kept as control, while Groups B, C, D, and E were fed OTA-contaminated diet at 0.1, 0.5, 1.0, and 1.5 mg/Kg feed, respectively, for 21 days. Feed intake on daily basis and body weight gain of the chicks was recorded on weekly basis. A subjective scoring of clinical signs and gross pathological lesions on visceral organs was performed. Relative weights of liver, kidneys and gizzard were recorded at the end of experiment. Serum biochemical profile and histological alteration in liver and kidneys of chicks was determined to assess the OTA mediated damage. A significant decrease in the feed intake and body weight gain of the chicks was observed in OTA treated groups. Clinical signs exhibited by the chicks included severe diarrhea, dullness, depression, increase water intake and ruffled feathers. Gross pathological lesions on liver and kidneys included lighter in coloration, friable and hemorrhagic. A significant increase in the weight of liver, kidney and gizzard was observed in OTA fed chicks. Histologically, liver and kidneys of chicks showed degenerative and necrotic changes. Serum biochemical profile indicated a severe damage to liver and kidneys in OTA fed chicks. The finding of this study suggested that there were pathological effects from OTA in male WL chicks kept on low doses of OTA-contaminated diet.

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INTRODUCTION

Mycotoxins, the secondary metabolites of some toxigenic fungi, are unavoidable contaminants of human and animal food and feeds (Ahsan *et al*., 2010; Choudhary and Kumari, 2010). It is estimated that 25-30% of the world crop may be contaminated with mycotoxins, and the estimated losses due to mycotoxins in animal industries have been estimated to be as much as several hundred million dollars per annum (Anonymous, 2003). In the long list of >300 known mycotoxins, e.g., aflatoxins, ochratoxins, zearalenone, tricothecene, T-2 toxins, fumonisin, and deoxynivalenol, each are well known for their toxicities (Binder *et al*., 2007).

Ochratoxin A (OTA), among the different classes of mycotoxins, is an important contaminant of cereals intended for use in animal and poultry feed (Saha *et al*., 2007; Saleemi *et al*., 2010), and is produced by seven species of *Aspergillus* and six species of *Penicillium*. *Aspergillus ochraceus*, from which the toxins acquired their name, appears to be the predominant ochratoxin producer (Trenk *et al*., 1971). OTA is characterized by numerous toxicities. Various studies have illustrated its nephrotoxic, hepatotoxic, immunotoxic, teratogenic, genotoxic, mutagenic, and/or neurotoxic effects, as well as its capacity to impart growth suppressive effects in different avian and mammalian species (Kumar et el., 2004; Hassan *et al*., 2010, 2011).

In general, the toxicological effects of mycotoxins are attributed to a decrease in feed intake, reduced nutrient absorption (along with concomitant altered metabolism), endocrine disturbances, and immunosuppression

(Whitlow and Hagler, 2002). Though the specific cellular and molecular mechanisms underlying the pathological effects of OTA are not known, inhibition of protein, DNA, and RNA synthesis, as well as degenerative/ apoptotic changes in visceral organs due to ochratoxicosis provide significant clues to determine how OTA mediates its effects (Pfohl-Leszkowicz *et al*., 1998). In broiler birds feeding OTA at 0.4 and 0.8 mg/kg feed for 1-5 week of age resulted significant decrease in feed intake and body weight gain along with increase in relative gizzard weight (Elaroussi *et al*., 2006). In an experiment feeding OTA contaminated diet at 2 mg/kg to broiler chicks for 35 days, Kumar *et al*. (2004) found severe degenerative and necrotic changes in liver and kidneys. A significant increase in the serum concentration of ALT, urea and creatinine, while a decrease in levels of total protein and albumin was noted in OTA treated laying hens in one of our previous study (Hassan *et al*., 2010) and also in broiler chicks by Stoev *et al*. (2004).

While information is available in the literature about the pathological effects of OTA (at high doses) in various animal species (Mobashar *et al*., 2010) including the WL chicken (adults as well as young), little is known about the effects of low doses of this toxin on chicks. It is important to note that earlier studies reported sex-specific responses to OTA intoxication in chicks (Stoev, 2010), where males were more sensitive to OTA when compared to females. This difference in response - at least in rats - was attributed to the differences in expression and/or activity of select drug-metabolizing enzymes (i.e., cytochrome P450s [CYPs] isoforms) that convert OTA into reactive intermediates that form DNA-adducts and lead to carcinogenic outcomes in the hosts (Pfohl-Leszkowicz *et al*., 1998). Thus, keeping in mind all the above-mentioned factors, the present study was designed to evaluate some pathological responses of male (being presumptively more sensitive than female counterparts) WL chicks that are maintained (for 3 wk) on a diet contaminated with various levels of OTA to assess what might be a 'safe' level for potential exposure of cockerels to this mycotoxin. Addressing this issue has tremendous ramifications for the poultry industry not only in Pakistan, but all over the world where mycotoxin contamination of feed-stocks is an ongoing challenge.

MATERIALS AND METHODS

Ochratoxin and feed: OTA was produced from *A. ochraceus* (CECT 2948, Culture Collection Center, University De Valencia, Valencia, Spain) by culturing on wheat grain using a modified method of Trenk *et al*. (1971) as described by Hassan *et al*. (2010).

Basal feed (19% total protein and 2750 Kcal/kg metabolizable energy) was prepared without addition of toxin binder, vitamins, minerals, and antibiotics. Prior to use, each batch of the basal feed was analyzed for ochratoxin, aflatoxin, and zearalenone to ensure that the levels of each were < 1.0 µg/kg. OTA-contaminated feeds were prepared by incorporation of known quantities of OTA. For this purpose, fermented wheat grains were extracted by soaking in a 3-fold quantity of chloroform (1:3) overnight and then filtered through cotton cloth. All the chloroform was then evaporated and the concentrated residues re-suspended into polyethylene glycol (PEG). This suspension was then evenly mixed in the required quantity of basal feed to prepare the experimental feeds containing each desired concentration of OTA. Prior to being used for feeding, the OTA concentration in each experimental diet was determined by HPLC.

Induction of ochratoxicosis in White Leghorn chicks: All animal experiments were conducted according to the rules and regulations of the Animal Ethics Committee, Faculty of Veterinary Science, at the University of Agriculture, Faisalabad (Pakistan). Male White Leghorn (WL) chicks (n=350, one-day-old) were procured from a local hatchery and then kept in a poultry house at the Department of Pathology in the University of Agriculture Faisalabad, Pakistan, under standard environmental conditions. Animal rooms were kept at ~33°C for the first week and then at ~32°C for the remaining period of the study, a 60% relative humidity, and with a 12-hr/12-hr light-dark cycle; all chicks had access to fresh water *ad libitum*.

These chicks were divided into five groups (A–E), each consisting of 70 birds. All the groups were reared in a separate metallic cage of 5´x4´. Chicks in Group A were kept on basal chick starter ration (verified not to contain OTA or aflatoxin-B_1 (AFB_1) contents at a level > 1.0 µg/kg feed), while those in Groups B, C, D, and E, were provided OTA-contaminated feed at 0.1, 0.5, 1.0, or 1.5 mg OTA/kg feed, respectively, for a period of up to 21 d.

Parameters studied

Clinical parameters: Feed intake of each group was daily determined, while body weight of birds was determined at the end of each week. Clinical signs of ochratoxicosis were recorded on daily basis. A subjective evaluation of the clinical signs was performed based upon the absence, presence, extent and severity and each sign was assigned a score from 0 to 3. The score of a particular sign in each group was summed up at the end of trial.

Necropsy of the birds: At Days 14- and 21-of-age, twelve chicks from each group were euthanized by cervical dislocation. Different organs including liver, kidneys and gizzard were harvested. These organs were weighed and their relative weight (as percentage of total body weight) was calculated. The liver and kidneys collected from the chicks fed OTA for 21 days were scored for gross lesions (as described in clinical parameters section) and were preserved in neutral buffer formalin for histopathological studies as described by Jalees *et al.* (2011). Formalin fixed tissue of the liver of birds showing cytoplasmic vacuoles of hepatocytes were also stained with Sudan IV stain to confirm the presence of fat vacuoles as described by Lillie and Fullmer (1976). Prior to killing, at day 21 of age, blood was collected from the wing vein of each bird and allowed to clot for serum separation. Serum collected from each bird was used for determination of biochemical parameters.

Serum biochemical parameters: Serum samples collected from birds of each group at the end of the experiment were used to determine Alanin-Aminotransferase (ALT), creatinine, urea, total proteins

and albumin concentrations using commercially available kits (Diasys Diagnostic system GmbH, Germany).

Statistical analysis: All data were subjected to analysis of variance tests. Means of the different groups were compared by Duncan's Multiple Range test using MSTATC statistical package. Data were considered significantly different from one another at a P-value ≤ 0.05. Cumulative scores for clinical signs and gross lesions were compared with control group on arithmetical difference basis.

RESULTS

Feed intake: A non-significant difference was noted in the feed intake of the chicks among all the groups, during week 1 of the experiment (Table 1). However, during week 2 and 3 feed intakes of chicks were significantly lower in the groups maintained on OTA contaminated ration.

Table 1: Feed intake (g) of male White Leghorn chicks kept on OTA contaminated feed (mean±SD).

Group	Age of chicks (Weeks)		
(mg OTA / Kg diet)	1	2	3
A (0)	9.10±2.60	20.62±2.71[a]	30.14±3.42[a]
B (0.1)	7.50±1.70	15.85±2.05[b]	27.88±2.14[a]
C (0.5)	7.67±1.39	13.57±3.26[b]	23.40±1.75[b]
D (1.0)	7.02±1.28	13.14±2.05[b]	22.73±2.01b[c]
E (1.5)	6.81±0.97	12.73±2.97[b]	18.45±3.39[c]

Values in each column followed by different letters are significantly different (P≤0.05).

Body weight: A non-significant difference was found in the body weights of chicks (during week 1 and 2) kept on OTA contaminated feed than the chicks fed basal feed (Table 2). However, feeding OTA for the period of 3 weeks resulted in the significant decrease in BW of the chicks fed higher dose of OTA i.e. in Group C, D and E, as compared to the values shown by their counterpart chicks in group A.

Table 2: Body weights (g) of male White Leghorn chicks kept on OTA contaminated feed (mean±SD).

Group	Age of chicks (Weeks)		
(mg OTA / Kg diet)	1	2	3
A (0)	67.00±6.63	122.80±13.01	250.80±26.47[a]
B (0.1)	66.40±5.41	110.00±20.75	269.00±41.98[a]
C (0.5)	63.00±2.12	109.00±7.71	174.00±35.31[b]
D (1.0)	69.60±7.77	106.20±25.53	170.00±46.22[b]
E (1.5)	66.40±10.01	113.00±11.58	158.20±47.64[b]

Values in each column followed by different letters are significantly different (P≤0.05) (n = 20/group).

Clinical signs: Scoring of clinical signs exhibited by the male WL chicks, kept on OTA contaminated diet have been presented in the Table 3. Chicks in group A were alert throughout the length of the experiment and responded well upon tapering the cages. Feeding of OTA led to depression in chicks which increased with increasing the dose of toxin. Few birds in the group B showed mild depression at the last week of toxin feeding. Feeding OTA at higher doses resulted severe depression in the group D and E. Attraction toward the feed was normal in the chicks of control groups but feeding of OTA resulted in decreased interest in feed which increased with increased dietary OTA concentration. Chicks in the group G showed maximum interest in water than all other groups while control group showed normal interest in water. Addition of OTA in the feed affected the consistency of fecal material from semisolid to watery in the dose dependent manner. Group E showed sever watery diarrhea throughout the length of experiment. Feathers of chicks of group A were shiny and well formed throughout the length of experiment while the chicks fed OTA resulted in the ruffled feathers which showed dose dependent increase.

Gross lesions: The subjective scoring of gross lesions as observed by visual examination of the chicks noted at postmortem examination has been presented in Table 4. Livers of the chicks of group A showed normal size, color and consistency. Chicks fed OTA contaminated feed showed enlargement, pale discoloration, friable in consistency and hemorrhages on the surfaces. All the changes increased in severity in dose dependent manner. Kidneys of the chicks of control group were normal in size and color while those of experimental groups were bulging out of sockets and hemorrhagic. Chicks from group D and E showed severe hemorrhagic and enlarged kidneys.

Relative organs weights: The relative weight of liver and kidneys (determined at day 14 and 21 of age) was significantly higher in all OTA fed groups as compared to values noted in the chicks from Group A (Table 5). The values of relative weight of the gizzard of the chicks fed OTA for 14 days were significantly higher in all OTA treated groups than the values noted in the chicks belonging to control group, while in the chicks fed OTA for 21, significantly higher value was noted in chicks from group E than the chicks in group A.

Histopathology

Liver: Histologically livers of the chicks from group A were normal and did not show any vascular disturbances. No fatty change or cellular infiltration was found. There was mild congestion accompanied by fatty change of milder degree in the liver of group B. In group C, there was moderate to severe fatty change with normal sinusoidal spaces. The chicks in group D showed moderate to severe fatty change along with congestion in the parenchyma. Dilated sinusoidal spaces and infiltration mononuclear cells around blood vessels were found. The sections from group E, liver showed fatty change of severe degree (Fig. 1[confirmed by Sudan IV staining using frozen sections Fig. 2]) along with pyknotic nuclei at some places. Infiltration of inflammatory cells around blood vessels and increased sinusoidal spaces were also noted in most of the section in this group.

Kidneys: The chicks in group A showed normal histological structures of kidneys with no degenerative changes or cellular infiltration. In group B, some sections showed mild to moderate degree of congestion, and foci of pyknotic nuclei. The sections of kidneys of chicks belonging to group C showed moderate degree of congestion and pyknotic nuclei at some places. In group D, detachments of tubular epithelial cells from basement membrane along with degenerative changes were noted in some sections of kidneys. Reduction in the urinary spaces due to proliferation of glomerular cells was also noted. In

Table 3: Score of clinical signs and behavior of male White Leghorn chicks fed different levels of OTA.

Clinical sign and behavior	Score range	Group (mg OTA / Kg diet)				
		A(0)	B(0.1)	C(0.5)	D(1.0)	E(1.5)
Alertness Normal – depressed	0-3	0	3	13	17	31
Attraction to feed Normal – less interest	0-3	0	5	19	23	42
Attraction to water Normal – more interest	0-3	0	11	17	21	32
Feces consistency Normal Formed – watery	0-3	0	9	12	31	39
Feather Normal Shiny – ruffled & Broken	0-3	0	0	11	14	31
Cumulative score		0	28	72	106	175

Table 4: Scores of gross lesions on different organs of male White Leghorn chicks fed OTA contaminated feed.

Organ	Lesion	Max. possible score	Group (mg OTA / Kg diet)				
			A (0)	B (0.1)	C (0.5)	D (1.0)	E (3.0)
Liver	Enlargement	36	0	12	17	21	28
	Pale discoloration	36	0	6	13	16	22
	Friable	36	0	0	5	8	10
	Hemorrhage	36	0	4	11	18	21
Total score liver		144	0	22	46	63	81
Kidneys	Enlargement	36	0	7	11	19	25
	Hemorrhage	36	0	3	9	11	17
Total score kidney		72	0	10	20	30	42
Cumulative score Liver+Kidney		216	0	32	66	93	123

Table 5: Relative organs weights (% of body weight) of male White Leghorn chicks kept on OTA contaminated feed (mean±SD)

Group (mg OTA / Kg diet)	A (0)	B(0.1)	C(0.5)	D(1.0)	E(1.5)
At 14 days of age					
Liver	3.53±0.13^{d}	3.96±0.03^{c}	4.40±0.05^{b}	4.51±0.04^{b}	5.30±0.13^{a}
Kidney	0.92±0.05^{d}	1.01±0.04^{d}	1.17±0.04^{c}	1.41±0.08^{b}	1.56±0.05^{a}
Gizzard	7.58±0.09^{c}	8.58±0.09^{b}	8.39±0.17^{b}	8.62±0.18^{b}	9.58±0.28^{a}
At 21 days of age					
Liver	3.59±0.07^{d}	3.81±0.08^{c}	3.88±0.05^{c}	4.46±0.09^{b}	4.83±0.06^{a}
Kidney	0.84±0.04^{d}	1.07±0.03^{c}	1.16±0.02^{b}	1.19±0.04^{b}	1.42±0.05^{a}
Gizzard	7.09±0.25^{b}	6.19±0.77^{b}	7.38±0.50^{b}	7.26±0.07^{b}	9.40±1.12^{a}

In rows, values followed by different letters are significantly different from each other at P≤0.05 (n = 10/group).

Table 6: Serum biochemical profile of chicks kept on OTA contaminated feed (mean±SD)

Group (mg OTA / Kg diet)	ALT (U/l)	Urea (mg/dl)	Creatinine (µmol/l)	Total protein (g/dl)	Albumin (g/dl)
A (0)	21.00±4.36^{c}	12.67±3.06^{c}	26.00±2.00^{c}	5.50±0.78^{a}	4.31±0.22^{a}
B (0.1)	29.33±2.08^{bc}	20.33±1.53^{bc}	28.33±3.21^{c}	4.68±0.80^{ab}	3.58±0.60^{ab}
C (0.5)	35.00±2.65^{ab}	27.00±6.24^{ab}	38.67±1.53^{b}	3.87±0.54^{bc}	3.14±0.12^{bc}
D (1.0)	39.33±5.51^{a}	31.67±3.06^{a}	54.79±5.57^{a}	3.64±0.33^{bc}	2.80±0.20^{bc}
E (1.5)	40.67±3.06^{a}	35.67±3.21^{a}	55.65±5.55^{a}	3.28±0.02^{c}	2.63±0.55^{c}

Values in each column followed by different letters are significantly different (P≤0.05).

group E, more severe degenerative changes in the tubular epithelial cells were noted along with detachment of cells from the basement membrane. Congestion in parenchyma, pyknotic nuclei (Fig. 3) and vacuolation in tubular epithelial cells were also observed in some section of kidneys.

Serum biochemical parameters: Serum ALT, urea and creatinine concentration were significantly higher in Group C, D and E, as compared to the values shown by their counterpart chicks in Group A [Table 6]. The values of serum total protein and albumin were significantly higher in group A as compared to the values in chicks from group C, D and E.

DISCUSSION

A significant decrease in the feed intake of the male WL chicks was noted during week 2 and 3 of experiment, in groups fed OTA contaminated ration. This decrease in feed intake of the chicks may be due to the metabolic disturbances due to ochratoxicosis as have been previous reported (though not in male WL chicks) in broilers (Stoev *et al*., 2002) and layers (Sawale *et al*., 2009). Feeding OTA contaminated diet resulted in decrease in body weight gain of chicks as noted in the week 3 of experiment. Among the various mechanisms of pathogenesis of ochratoxin A, the impairment of protein synthesis is considered to be most important one. This decrease/blockage of protein synthesis in ochratoxin A treated birds may be involved in growth depression of the chicks, as it has been proposed earlier (Mohiudin *et al*., 1993). Also, this decrease in weight gain of the chicks may be co-related to decrease feed intake or poor health status due to ochratoxicosis. A decrease in body weight gain of chicks has previously been reported by Elaroussi *et al*. (2006) in broiler chicks.

The increase in fecal moisture contents, dullness, depression, increased water intake and ruffled feather were prominent signs exhibited by the chicks in OTA fed groups. Similar clinical signs have been reported in OTA fed broiler chicks (Koynarski *et al*., 2007). Intramuscular injection of OTA to the White Leghorn pullet at the rate of 0.25 to 0.50 mg/kg body weight resulted increase water intake and increase fecal moisture contents (Glahn *et al*., 1981).The macroscopic changes observed in the liver and kidneys of OTA fed chicks included enlargement and hemorrhages on the surfaces of these organs. The enlargement of liver and kidneys may be the consequence of inflammatory reactions occurring here due to route of elimination of OTA via kidneys and partially by liver due to enterohepatic recirculation and hepatobiliary excretion

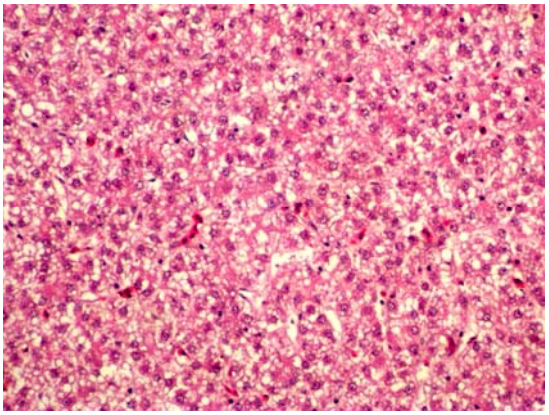

Fig. 1: Photomicrograph of liver of White Leghorn chick fed OTA @ 1.5 mg/kg feed. Fatty change is found in hepatocytes. (H & E stain 200X)

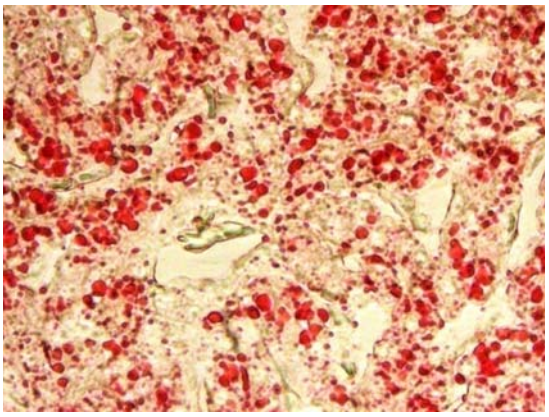

Fig. 2: Photomicrograph of liver stained with Sudan -IV for the confirmation of vaculation as fatty change. Fat vacuoles are pink stained (400X)

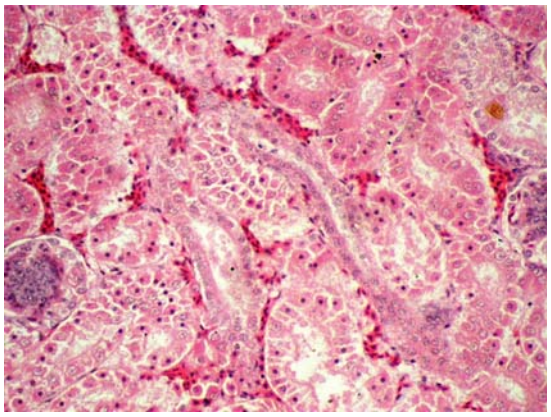

Fig. 3: Photomicrograph of Kidney of White leghorn chick fed OTA contaminated feed @ 1.5 mg/kg. Congestion and pyknotic nuclei are shown. (H & E stain 600X)

(Stoev *et al*., 2000). The hemorrhagic changes on the surfaces of these organs may be due to vascular disturbances in ochratoxicosis as it has been previously observed in chicks fed OTA contaminated ration at 5 mg/kg feed (Stoev *et al*., 2002). Moreover, the disturbances of blood clotting due to decrease in fibrinogen and increased prothrombin time observed in ochratoxicosis (Prior and Sisodia, 1978) could also contribute to hemorrhages seen in these organs.

The increase in the relative weight of liver and kidneys in the OTA fed chicks may be attributed to infiltration of mononuclear cells in the parenchyma of these organs as it has been observed in the histological sections of these organs. Stoev *et al.* (2002) suggested that this increase in the weight is due to proliferation of connective tissues in these organs as result of regenerative attempt to OTA mediated damage. However, no such activity was found in the histological structures of these organs in present study. A significant increase in the weight of gizzard noted in present study in OTA fed chicks have previously been reported in the broiler chicks (Elaroussi *et al*., 2006) and breeder hens (Hassan *et al*., 2010). This increase in the weight of gizzard may be attributed to the affinity of OTA for the gastrointestinal tract affecting its physiological systems (Raju and Devegowda, 2000) and/or due to irritative properties when it is in direct contact (Huff *et al*., 1988).

Microscopically, the livers of the WL chicks showed congestion, fatty change and infiltration of mononuclear cells and at some places hepatocytes showed pyknotic nuclei. Also degenerative changes were noted in the sections of kidney, especially in the tubular epithelial cells from OTA treated groups. These changes are attributed to the direct effects of OTA in its execratory route i.e. primarily in kidney and partly via enterohepatic recirculation and hepatobiliary excretion. A significant increase in the serum ALT concentration and then decrease in the values of serum total protein and albumins are co-related to direct toxic effects of OTA in liver. The damage to hepatocytes are observed in histological sections enhances our understanding regarding the increased ALT and decrease protein concentration. An increase in serum urea and creatinine concentration shows that kidney functions were severely impaired in all OTA treated groups. These finding are in line with our previous studies in which different levels OTA were fed to breeder hens for 21 days (Hassan *et al*., 2010). The histological changes in these organs are also suggestive of their corresponding serum biochemical alteration in this study.

In conclusion, feeding of OTA contaminated feed to male White Leghorn chicks from one day of age resulted in diarrhea, decrease feed intake, increase water intake and decreased body weight. Liver and kidneys were grossly enlarged, lighter in color and had petechial hemorrhages on the surfaces. Microscopically liver had fatty change and kidneys showed tubular degeneration and necrosis. An increase was observed in serum concentration of ALT, urea, creatinine while decreased concentration of serum total protein and albumin was present. All the pathological findings showed dose dependent increase in its severity.

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