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TOXICOPATHOLOGICAL AND SERUM BIOCHEMICAL ALTERATIONS INDUCED BY OCHRATOXIN A IN BROILER CHICKS AND THEIR AMELIORATION BY LOCALLY AVAILABLE BENTONITE CLAY

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Ochratoxin A (OTA), a potent carcinogen, poses a great threat to the lives of poultry, animals and humans. It causes nephrotoxicity, immunosuppression, mutagenicity and teratogenicity in birds. Bentonite clay, belonging to the subclass of aluminosilicates, has the ability to adsorb different molecules and can efficiently adsorb several mycotoxins too. The present study was designed to evaluate the ameliorative effects of locally available bentonite clay against OTA induced toxicopathological and serum biochemical alterations in broilers. For this, day old broilers were divided into 16 equal groups and were given different combinations of OTA (0.15, 0.3 and 1.0 mg/kg feed) and bentonite clay (5, 10 and 20 g/kg feed). Results indicated that feeding OTA alone caused a reduction in body weight gain, poor FCR, increased gross lesions, altered relative organ weights and serum biochemical values and all these changes occurred in a dose dependent manner. Feeding bentonite clay along with OTA did not ameliorate the OTA induced alterations in body weight, FCR, gross lesions, relative organ weights and serum biochemical parameters.
Keywords: Broilers chicks, Ochratoxin A, bentonite clay, toxicopathology, serum biochemical parameters, body weight, FCR, organ weights.

INTRODUCTION

Several toxigenic fungal strains of *Aspergillus* (Ghosh *et al*., 2015) and *Penicillium*, the most important being *Aspergillus ochraceus*, are being involved in the production of ochratoxin. Ochratoxin, particularly OTA is considered as a potent carcinogen and classified in Group 2B by the International Agency for Research on Cancer (IARC, 1993) for humans. Ochratoxins are considered to be the main reason for causing a serious anomaly namely Balkan Endemic Nephropathy (BEN) in humans exposed to its dietary exposure (Zahoor-ul-Hassan *et al*., 2012; Solcan *et al.*, 2015; Iftikhar *et al*., 2015; Ben Salah-Abbes *et al*., 2015).

Ochratoxin A (OTA) causes adverse effects in poultry and is considered to be the most toxic mycotoxin along with aflatoxins for poultry birds. OTA has several nephrotoxic, immunotoxic, mutagenic and teratogenic effects in poultry (O'Brien and Dietrich, 2005; Saleemi *et al*., 2015; Kumar *et al*., 2015). OTA is found in several grains, cereals, animal/poultry feeds, meats and human tissues all over the globe (Abidin *et al.*, 2011; Bilal *et al.*, 2014; Khan *et al.*, 2014a and 2014b; Valtchev *et al*., 2015).

Bentonite clay basically originates from volcanic ash in impure form containing montmorillonite as its major component (Pappas *et al.*, 2014). It is a phyllosilicates belonging to the subclass of aluminosilicate. Its crystalline structure, helps to adsorb different molecules within it (Grim and Guven, 1978; Jand *et al.*, 2005; Di Gregorio *et al.*, 2014). Several experiments have been conducted which has revealed the binding ability of bentonite against several mycotoxins (Smith and Rosa, 1991; Eralsan *et al.*, 2005). There are several natural reserves of bentonite clay in Pakistan, especially in Kashmir and Potohar plateau but limited use of bentonite clay as mycotoxin binder in feed is being practiced in this country.

A well pronounced effect of OTA in birds is growth suppression (reduced body weight, poor FCR and altered relative organ weights) which has been well documented by many scientists (Manning and Wyatt, 1984; Gibson *et al.*, 1989). Similarly, OTA altered serum biochemical changes in broilers is also a well-documented fact (Gentles *et al*., 1999; Stoev *et al.*, 2000; Elaroussi *et al.*, 2006; Abidin *et al.*, 2013). However, the available literature presents little to no data regarding ameliorative effects of bentonite clay against OTA induced alterations. Keeping this in view the present study was designed to evaluate the ameliorative effects of bentonite clay upon OTA induced toxicopathological and serum biochemical alterations in broilers.

MATERIALS AND METHODS

***Production, extraction and quantification of OTA*:** Freshly prepared sterile slants of Potatoes Dextrose Agar (PDA) were used to sub-culture already preserved *Aspergillus ochraceus*, link: Fries. A (CECT 2948) and freshly grown slants of *A. ochraceus* spores were further used for the production of OTA by inoculating them on wheat grains following the method of Trenk *et al*. (1971). OTA thus produced on autoclaved wheat grains was extracted and quantified using HPLC according to the method described by Bayman *et al*. (2002). Bentonite clay (BC), procured locally from the contractors of bentonite mines of Potohar region was ground to fine powder for the ease of its mixing in feed.

***Experimental design*:** Four hundred and eighty, day old broiler chicks, procured from a local hatchery, were given basal diet and ad-libitum water under standard hygienic environmental conditions. Feed containing 22% total protein contents (Abu-Akkada and Awad, 2015) and 3100 Kcal/kg energy was offered to these birds without adding any toxin binder and/or antibody in it. The experimental study was approved by university scrutiny committee (UAF, PAK).

At day 3, these birds were divided into 16 equal groups having 30 birds in each group and offered different combinations of OTA (0.15, 0.3 and 1.0 mg/kg feed) and BC (5, 10 and 20 g/kg feed) the details of which has been presented in Table 1.

Table 1. Experimental groups along with their respective treatments.

Groups	Dietary Treatments	
	OTA (mg/kg BW)	Bentonite clay (g/kg BW)
Control	0	0
O1	0.15	0
O2	0.30	0
O3	1.00	0
BC1	0	5
BC2	0	10
BC3	0	20
O1BC1	0.15	5
O2BC1	0.30	5
O3BC1	1.00	5
O1BC2	0.15	10
O2BC2	0.30	10
O3BC2	1.00	10
O1BC3	0.15	20
O2BC3	0.30	20
O3BC3	1.00	20

Thirty birds were placed in each group.

Parameters studied:

***Mortality*:** Mortality in each group was recorded on daily basis and percentage mortality was calculated at the end of the experiment.

***Body weight and feed conversion ratio*:** Body weights and feed conversion ratio (FCR) of all groups was determined at the end of experiment.

***Gross lesions and gross lesion scoring*:** At the time of slaughtering of birds different visceral organs were observed for gross lesions and lesions were recorded by giving them a score ranging from 0-3.

Relative organ weights (liver, kidney, spleen, bursa and thymus) of birds were calculated by dividing the absolute organ weight with body weight of the bird and multiplying it with 100.

***Serum biochemical parameters*:** Serum was separated from the blood samples collected from birds of each group before slaughtering at 42nd day of experiment. The separated serum samples were used to evaluated the urea, creatinine and Alanine aminotransferase (ALT) levels through commercially available kits (Merck) while Biuret method was used to evaluate total protein levels and bromocresol green dye binding method for albumin levels (Anonymous, 1984) from these serum samples however; globulin levels were determined by subtracting serum albumin levels from that of serum total protein levels.

***Statistical analysis*:** Data thus obtained was subjected to statistical analysis using factorial test and means were compared through Duncan's multiple range test (DMR).

RESULTS

***Mortality*:** No mortality was observed in birds of control, BC1 and BC2 groups. Highest mortality was observed in group O3 (23.33%) followed by O3BC2 (20.00%) and O3BC3 (20.00%). The mortality was 6.67% in groups O1 and O1BC2 while it was 3.33% in groups BC3 and O1BC.

***Body weight*:** Body weights of all groups calculated at the end of the experiment have been presented in Figure 1. The highest body weight was presented by the birds of control group while body weights of OTA treated groups (O1, O2 and O3) were significantly lower than control. The body weights of groups BC1 and BC2 were non-significant however group BC3 had significantly lower body weight than control. Body weights of all the combination groups were significantly lower as compared to that of control.

***Feed conversion ratio (FCR)*:** Feed conversion ratio (FCR) of the birds fed OTA, bentonite clay (BC) and/or their combinations have been presented in Figure 2. Poor FCR was presented by groups fed different levels of OTA alone (O1, O2 and O3) in a dose-dependent manner while groups BC1, BC2 and BC3 had FCR values close to that of control group. The groups fed different combinations of OTA and BC had higher values than that of control.

***Gross lesions*:** No gross lesion or morphological alteration was observed on any of the visceral organs of the birds of control group at the time of slaughtering. Liver had a normal size, shape and texture having fine edges and no unusual marking or spots were observed on liver surface. Kidneys were also normal in appearance and were lying within their bony sockets. No swelling was observed on kidneys. Similarly, no lesion or visual abnormality was observed on other visceral organs like bursa of Fabricius, thymus and spleen. Similar gross morphology was also exhibited by different visceral organs of groups BC1, BC2 and BC3.

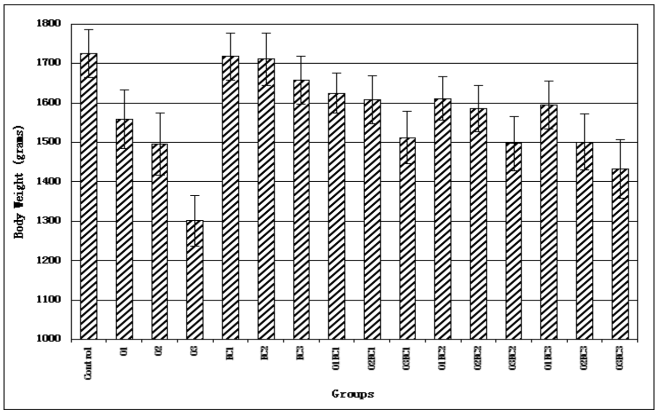

Figure 1. Body weight (g) of broiler chicks given different dietary levels of OTA and bentonite clay. Description of abbreviations: O1 = 0.15 mg OTA/kg feed, O2= 0.3 mg OTA/kg feed, O3= 1.0 mg OTA/kg feed, BC1=5 g Bentonite Clay/kg feed, BC2=10 g Bentonite Clay/kg feed, BC3=20 g Bentonite Clay/kg feed.

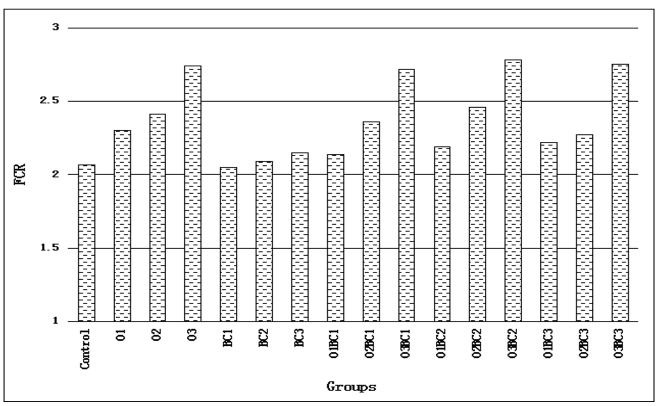

Figure 2. Feed conversion ratio of broiler chicks given different dietary levels of OTA and bentonite clay. Description of abbreviations remains the same as that footnote of Figure 1.

Groups fed OTA alone (O1, O2 and O3) had light colored, friable and enlarged liver with some petechial hemorrhages present on their surfaces (Figure 3). Kidneys were bulging out of their bony sockets and were also enlarged (Figure 3). Other visceral organs like bursa of Fabricius, thymus and spleen also presented mild to moderate degree petechial hemorrhages on their surfaces. These gross lesions increased with increasing doses of OTA and maximum intensity of these lesions was observed in group O3.

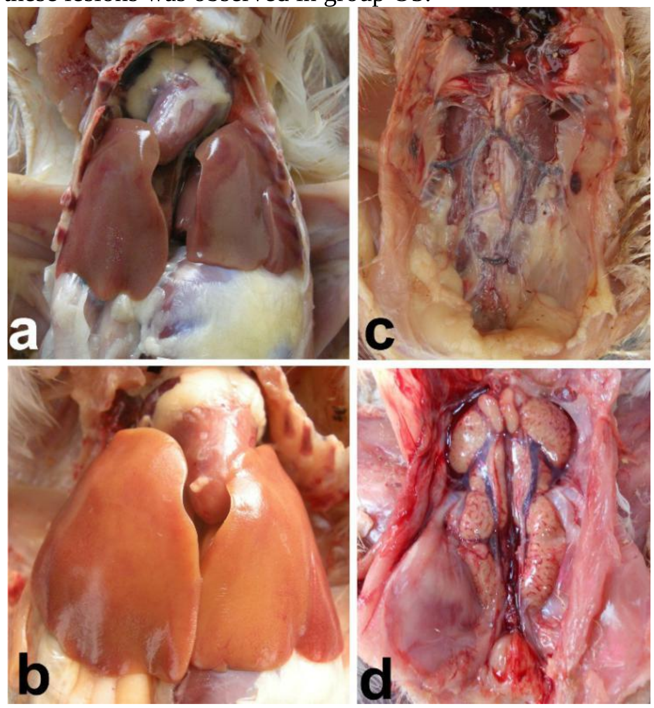

Figure 3. a) Liver of control group with normal color and appearance, b) Light yellow colored, friable and enlarged liver in OTA (O3 group), c) Kidneys of control group with normal appearance and d) swollen kidneys bulging out of their bony sockets in OTA (O3 group).

Regarding combination groups, groups receiving O1 with all BC dietary levels mild to moderate gross lesions were observed on different visceral organs when compared to O1 group while moderate to severe gross lesions were observed on different visceral organs of groups fed O2 and O3 along with all dietary levels of BC used. There was a reduction in the size of Bursa of Fabricius while the morphology of spleen and thymus remained unchanged. The subjective gross lesions scoring of liver and kidneys on the basis of visual evaluation has been presented in Table 2.

Relative organ weights**:** The relative organ weights of all the groups fed different combinations of OTA and BC have been presented in Table 3.

Liver and kidney**:** The relative weights of liver and kidney of groups fed OTA alone (O1, O2 and O3) was significantly higher while that of groups BC1 and BC2 was non-significantly different from control. However, the relative weights of liver and kidney of group BC3 along with all combination groups were significantly higher than that of control.

Table 2. Lesions scoring of organs of birds fed different levels of ochratoxin A and bentonite clay.

Organ	Liver				Cumulative score liver	Kidney		Cumulative score kidney	Total score Liver+Kidney
Lesion	Enlarged	Paleness	Friable	Hemorrhages		Enlargement	Dark Discoloration		
Max. possible score (Range 0-3)	27	27	27	27	108	27	27	54	162
Groups: Control	0	0	0	0	0	0	0	0	0
O1	4	6	5	2	17	9	9	18	35
O2	7	9	7	4	28	18	15	33	61
O3	12	14	15	6	47	24	26	50	97
BC1	0	0	0	0	0	0	0	0	0
BC2	0	1	1	0	2	1	0	1	3
BC3	2	2	1	0	5	2	1	3	8
O1BC1	8	6	5	3	24	5	7	12	36
O2BC1	6	8	6	3	23	8	8	16	39
O3BC1	15	10	16	6	47	16	12	28	75
O1BC2	4	6	7	3	20	7	5	12	32
O2BC2	14	15	15	8	52	14	12	26	78
O3BC2	13	13	12	7	45	41	13	54	99
O1BC3	10	6	6	3	25	10	5	15	40
O2BC3	16	9	10	10	47	15	10	25	72
O3BC3	20	16	16	6	58	18	18	36	94

Description of abbreviations: O1 = 0.15 mg OTA/kg feed, O2= 0.3 mg OTA/kg feed, O3= 1.0 mg OTA/kg feed, BC1=5 g Bentonite Clay/kg feed, BC2=10 g Bentonite Clay/kg feed, BC3=20 g Bentonite Clay/kg feed.

Table 3. The relative weight of different organs (percent of the body weight) at 42 days of age of birds fed different levels of ochratoxin A and bentonite clay.

Groups	Liver	Kidneys	Spleen	Bursa	Thymus
Control	2.32±0.14	0.68±0.05	0.17±0.02	0.17±0.01	0.38±0.04
O1	3.13±0.37*	1.02±0.04*	0.14±0.02	0.15±0.01*	0.31±0.02*
O2	4.02±0.21*	1.35±0.03*	0.15±0.03	0.13±0.01*	0.30±0.02*
O3	4.63±0.09*	1.80±0.05*	0.14±0.03	0.07±0.02*	0.25±0.02*
BC1	2.35±0.11	0.68±0.03	0.15±0.02	0.15±0.02	0.35±0.03
BC2	2.36±0.11	0.70±0.03	0.17±0.02	0.14±0.01	0.33±0.03
BC3	2.72±0.12*	0.76±0.04*	0.15±0.02	0.14±0.01*	0.34±0.02
O1BC1	2.89±0.11*	0.79±0.02*	0.15±0.02	0.13±0.01*	0.29±0.02*
O2BC1	3.33±0.16*	1.08±0.05*	0.16±0.02	0.14±0.01*	0.29±0.02*
O3BC1	3.52±0.16*	1.50±0.04*	0.14±0.02	0.10±0.02*	0.28±0.03*
O1BC2	2.96±0.10*	0.88±0.04*	0.16±0.01	0.12±0.02*	0.28±0.02*
O2BC2	3.61±0.20*	1.20±0.03*	0.17±0.01	0.12±0.01*	0.29±0.03*
O3BC2	3.71±0.17*	1.56±0.04*	0.15±0.01	0.10±0.01*	0.30±0.04*
O1BC3	3.04±0.18*	0.96±0.02*	0.14±0.01	0.12±0.02*	0.27±0.03*
O2BC3	3.81±0.19*	1.24±0.03*	0.15±0.02	0.12±0.01*	0.27±0.03*
O3BC3	3.89±0.21*	1.66±0.04*	0.14±0.02	0.10±0.02*	0.23±0.04*

Values (Mean±SD) bearing asterisk in a column are statistically different from control ($p \leq 0.05$); Description of abbreviations remains the same as that footnote of Table 2.

***Spleen*:** The relative weight of spleen of all the groups was non-significant as compared to the relative weight of spleen of control group.

***Bursa of Fabricius*:** Highest relative bursal weight was observed in control group. The bursal weight of groups fed OTA alone (O1, O2 and O3) was significantly lower while that of groups BC1 and BC2 was non-significantly different from control. The bursa weight of group BC3 along with all combination groups was significantly lower than control.

***Thymus*:** Like bursa of Fabricius highest relative thymus weight was noticed in the control group at 42 days of age. The relative weight of thymus of groups O1, O2 and O3 along with all combination groups was significantly lower while that of groups BC1, BC2 and BC3 was non-significantly different as compared with control.

***Serum biochemical parameters*:** Serum biochemical parameters of all the groups fed different combinations of OTA and BC have been presented in Table 4.

Table 4. Serum Biochemical Parameters of broiler chicks administered different levels of ochratoxin A and bentonite clay concurrently.

Groups	Urea (mg/100ml)	Creatinine (mg/100ml)	ALT (IU/µL)	Total Protein (g/100ml)	Albumin (g/100ml)	Globulin (g/100ml)
Control	15.03±2.04	28.36±2.69	19.43±1.72	4.65±0.06	3.04±0.05	1.60±0.06
O1	21.24±3.26*	34.20±2.60*	23.63±2.43*	3.92±0.10*	2.66±0.03*	1.25±0.03*
O2	29.47±2.49*	40.88±1.36*	31.90±3.18*	3.46±0.04*	2.56±0.03*	0.90±0.04*
O3	33.89±2.21*	51.19±1.86*	42.23±3.99*	3.30±0.02*	2.63±0.04*	0.68±0.04*
BC1	14.90±1.06	28.99±1.86	19.48±1.47	4.69±0.06	3.08±0.06	1.61±0.01
BC2	15.11±1.79	29.16±2.20	21.33±2.38	4.67±0.05	3.08±0.04	1.59±0.03
BC3	15.70±0.91	29.91±1.98	21.83±1.89	4.66±0.05	3.07±0.05	1.59±0.04
O1BC1	20.11±3.51*	32.60±1.88*	19.10±1.40	4.16±0.04*	2.90±0.05*	1.26±0.02*
O2BC1	25.73±1.11*	35.61±1.79*	27.48±2.13*	3.84±0.05*	2.84±0.07*	1.00±0.10*
O3BC1	30.36±2.04*	46.44±1.59*	36.09±3.29*	3.71±0.06*	2.74±0.02*	0.97±0.05*
O1BC2	20.96±2.46*	33.93±2.98*	24.95±2.09*	4.08±0.05*	2.80±0.13*	1.28±0.13*
O2BC2	25.16±2.26*	38.47±2.95*	28.61±2.56*	3.79±0.03*	2.79±0.10*	1.00±0.11*
O3BC2	29.18±2.48*	48.55±2.21*	40.51±2.70*	3.49±0.10*	2.75±0.06*	0.74±0.16*
O1BC3	24.21±2.16*	37.51±2.69*	27.91±3.00*	3.82±0.09*	2.80±0.10*	1.01±0.08*
O2BC3	28.15±2.89*	42.78±1.83*	35.63±3.32*	3.54±0.10*	2.73±0.07*	0.81±0.15*
O3BC3	31.23±4.15*	49.20±2.55*	38.45±3.26*	3.37±0.10*	2.59±0.08*	0.78±0.13*

Values (Mean±SD) bearing asterisk in a column are statistically different from control ($p \leq 0.05$); Description of abbreviations remains the same as that footnote of Table 2.

***Alanine aminotranferase (ALT)*:** Alanine aminotransferase (ALT) of the groups BC1, BC2 and BC3 was non-significant as compared to that of control. Except for the group O1BC1, the ALT values of all other groups were significantly higher except for group O1BC1 whose ALT value was non-significantly different than control

***Urea and creatinine*:** Groups BC1, BC2 and BC3 had non-significant while groups O1, O2 and O3 had significantly higher serum urea and creatinine values as compared with control. However, all combination groups had significantly higher serum urea and creatinine values when compared with that of control.

***Total protein*:** Groups O1, O2 and O3 presented significantly lower serum total protein values while these values were non-significantly different in groups BC1, BC2 and BC3 when compared with control. The total protein values of all combination groups were significantly lower as compared to control.

***Albumin and globulin*:** Serum albumin and globulin values of groups BC1, BC2 and BC3 had non-significant differences while these values in groups O1, O2 and O3 along with all combination groups were significantly lower as compared that of control.

DISCUSSION

The mitogen, ochratoxin A (OTA) was used at the rates of 0.15, 0.3 and 1.0mg/kg in this study for a period of 42 days and the selection of these levels was made on the basis of different available studies in which OTA was used in the diet at the rates ranging from 0.13 to 8.0 mg/kg feed in birds (Santin *et al*., 2002; Elaroussi *et al.*, 2006; Khatoon *et al.*, 2013; Abidin *et al.,* 2013; Marin and Taranu. 2015). Bentonite clay (BC) was used at the rates of 5, 10 and 20 g/kg feed to evaluate its ameliorative effects against different toxicopathological and serum biochemical alterations induced by OTA.

Feeding OTA to birds caused an adverse effect on the growth of the birds which could be well attributed by a decreased body weight and poor FCR as observed in this study in OTA treated birds and this reduced performance became more adverse in a dose dependent manner. Decreased body weights and poor FCR in OTA intoxicated birds have been reported by many scientists (Kubena *et al.*, 1988; Stoev *et al.*, 2000; Verma *et al.*, 2004; Koynarski *et al.*, 2007). The reduction in feed intake along with poor FCR could be a possible reason for reduced weight gain in birds (Elaroussi *et al.*, 2006). Supplementing BC did not cause amelioration of OTA induced poor FCR and reduced body weight gain which could be well attributed by significantly different values in the combination groups when comparison was made with control. These findings are in agreement with Santin *et al.* (2002) and Watts *et al.* (2003) who also observed a reduced body weight gain and poor FCR in birds concurrently fed OTA along with bentonite at the rates of 2 and 0.5 ppm respectively. However, contrary to our studies, Che *et al*. (2011) reported amelioration of these effects by 0.2% HSCAS which could possibly occurred due to lower dose of OTA (68.4 µg/kg) used by these researchers as compared to those levels used in this study.

A significant increase in relative kidney and liver weights in OTA treated birds as observed in this study has also been reported by different scientists (Santin *et al.*, 2002; Elaroussi *et al.*, 2006). As liver and kidney are the primary organs being involved in the elimination of toxic materials from the body so the increased sizes of these organs might occur due to the damages caused during the elimination of OTA from the body through kidney and liver (Fuchs *et al*., 1988). Feeding bentonite clay did not cause any amelioration in OTA induced alterations in relative liver and kidney weights. Reduced size of thymus and bursa in OTA treated birds has also been related by many authors (Stoev *et al.*, 2000; Kumar *et al.*, 2004; Elaroussi *et al.*, 2006). Immunosuppression, a major alteration associated with ochratoxicosis, might occur due to reduced sizes of immunological organs as observed in this study after feeding OTA to birds. OTA disturbs the functions of proximal tubules resulting in the reduction of a primary renal organic anion, the para-aminohippuric acid (PAH), transport leading to glucosuria and enzymuria (Gekle and Silbernagl, 1994).

Gross lesion on different visceral organs as observed in this study has also been observed by many scientists (Elaroussi *et al.*, 2006; Hanif *et al.*, 2008). These gross lesions became severe in a dose dependent manner. BC, when given along with OTA did not ameliorate the OTA associated gross lesions on different organs. Santin *et al.* (2002) reported similar observations when birds were given 2 mg/kg OTA along with 0.25% HSCAS in feed. Similarly, Nedeljkovic-Trailovic *et al.* (2015) presented similar results in birds given OTA along with 2 g/kg modified zeolite.

Regarding serum biochemical alterations, significant increase in creatinine, ALT and urea levels in OTA intoxicated birds has also been reported by many workers (Kubena *et al*., 1988; Bailey *et al*., 1989; Gentles *et al.*, 1999; Stoev *et al.*, 2002). Similarly, reduction in serum total proteins, albumin and globulin concentrations in OTA treated birds has also been well reported previously (Bailey *et al.*, 1989; Gentles *et al.*, 1999; Stoev *et al.*, 2002; Garcia *et al*., 2003; Koynarski *et al.*, 2007). Feeding BC did not result in the amelioration of OTA induced alterations in serum biochemical parameters. Similar to our findings, Watts *et al.* (2003) and Garcia *et al.* (2003) also reported that dietary bentonite did not ameliorate the OTA associated changes in serum biochemical parameters. One possible reason in this regard might be the non-polar nature of ochratoxin and secondly, up till now no single binder has been proved efficient against all the mycotoxins present in field (Denli and Perez, 2010; Sirhan *et al.*, 2012).

***Conclusions*:** From this study it can be well concluded that feeding bentonite clay (5, 10 and 20 g/kg feed) did not ameliorate ochratoxin A (0.15, 0.3 and 1.0 mg/kg feed) induced toxicopathological and serum biochemical alterations in broilers.

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