Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Ochratoxicosis in White Leghorn breeder hens: Production and breeding performance

Zahoor Ul Hassan*, Muhammad Zargham Khan, Ahrar Khan, Ijaz Javed[1], Umer Sadique[2] and Aisha Khatoon

Department of Pathology; [1]Department of Physiology and Pharmacology, University of Agriculture Faisalabad-38040; [2]Department of Animal Health, KPK Agricultural University, Peshawar-25120, Pakistan
*Corresponding author: zahoor82@gmail.com

ARTICLE HISTORY

Received: October 11, 2011
Revised: January 15, 2012
Accepted: April 13, 2012

Key words:
Breeding performance
Egg production
Histopathology
Ochratoxicosis
White Leghorn breeder hens

ABSTRACT

This study was designed to evaluate the effect of Ochratoxin A (OTA) upon production and breeding parameters in White Leghorn (WL) breeder hens. For this purpose, 84 WL breeder hens were divided into seven groups (A-G). The hens in these groups were maintained on feed contaminated with OTA @ 0.0 (control), 0.1, 0.5, 1.0, 3.0, 5.0 and 10.0 mg/Kg, respectively for 21 days. These hens were artificially inseminated with semen obtained from healthy roosters kept on OTA free feed. Egg production and their quality parameters were recorded. Fertile eggs obtained from each group were set for incubation on weekly basis. At the end of the experiment, hens in each group were killed to determined gross and microscopic lesions in different organs. OTA residue concentrations were determined in extracts of liver, kidneys and breast muscles by immunoaffinity column elution and HPLC-Fluorescent detection techniques. Feeing OTA contaminated diet resulted in a significant decrease in egg mass and egg quality parameters. Liver and kidneys showed characteristic lesions of ochratoxicosis. Residue concentration (ng/g) of OTA in the hens fed 10 mg/kg OTA, was the highest in liver (26.336±1.16) followed by kidney (8.223±0.85) and were least in breast muscles (1.235±0.21). Embryonic mortalites were higher, while hatachabilites of the chicks were lower in the groups fed higher doses of OTA. Feeding OTA contaminated diets to breeder hen resulted in residues accumulation in their tissues along with significantly reduced production and breeding performance.

To Cite This Article: Hassan ZU, MZ Khan, A Khan, I Javed, U Sadique and A Khatoon, 2012. Ochratoxicosis in White Leghorn breeder hens: Production and breeding performance. Pak Vet J, 32(4): 557-561.

INTRODUCTION

Mycotoxins, the secondary fungal metabolites, produced by some toxigenic species of Aspergillus and Penicillium, are unavoidable contaminants of animal and human food and feeds. In a long list of >300 known mycotoxins; aflatoxins, ochratoxins, zearalenone, fumonisin are important contaminants of poultry feed and its ingredients (Binder *et al.*, 2007; Ahsan *et al.*, 2010; Mukhtar *et al.*, 2010)

Ochratoxin A (OTA), among ochratoxins is most important for its nephrotoxic, hepatotoxic, immunotoxic, teratogenic and genotoxic effects (Hassan *et al.*, 2010, 2011; Zahoor-ul-Hassan *et al.*, 2012). The natural contamination of poultry feed and its ingredients with OTA have been reported in many countries of the world (Beg *et al.*, 2006; Nguyen *et al.*, 2007) including Pakistan (Hanif *et al.*, 2006; Saleemi *et al.*, 2009; Saleemi *et al.*, 2012). Poultry birds offered OTA contaminated feed resulted in decrease egg production, body weight, altered serum biochemical profiles, decrease FCR and decrease performance of the chicks hatched from the eggs laid by intoxicated hens (Koynarski *et al.*, 2007; Hassan *et al.*, 2010). In an experimental study, administration of OTA in the female rabbits resulted in the skeletal defects in the developing embryos (Wangikar *et al.*, 2004).

A significant volume of information is available about the toxic effects of OTA in developing chicken embryos (Hassan *et al.*, 2011; 2012). However, a little is known about the minimum dietary OTA concentration that might effects the health of breeder hens and then its breeding performance in terms of fertility, embryonic mortality and hatchability.

Keeping in view the above mentioned concern this study was designed to evaluate what might be safe concentration of OTA in the diet of breeder hens that has no or minimum toxic effects in its production and breeding performance.

MATERIALS AND METHODS

Ochratoxin and feed: Ochratoxin A (OTA) was produced from *Aspergillus 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) and was quantified by high-performance liquid chromatography (HPLC) (Bayman *et al*., 2002).

Induction of ochratoxicosis in White Leghorn breeder hens: Basal feed (16% total protein and 2700 Kcal/kg metabolizable energy) was prepared as described previously (Hassan *et al*., 2011). Eighty four (84) White Leghorn (WL) breeder hens (40-week-old) were procured from a local breeder form and then kept in a poultry house at the Department of Pathology, University of Agriculture Faisalabad, Pakistan, under standard environmental conditions.

These hens were divided into seven groups (A–G), each consisting of 12 birds. Hens in Group A were kept on basal layer ration, while those in Groups B, C, D, E, F and G were kept on OTA-contaminated feed @ 0.1, 0.5, 1.0, 3.0, 5.0 and 10 mg OTA/Kg feed, respectively, for a period of 21 days. These hens were artificially inseminated by semen collected from healthy roosters of WL kept on OTA free basal feed. Hatching eggs were collected on daily basis and set for incubation on a weekly basis.

Parameters studied: Eggs production of the hens in different groups was recorded on daily basis and weekly average production was compared among all the groups. Eggs quality was assessed by determining their weight, shell thickness and Haugh unit score on daily basis (n=6/group).

At the end of OTA feeding period (day 21), all the hens in each group were killed by cervical dislocation. A subjective scoring of the gross lesions on different organs was performed based upon the absence, presence, extent and severity. A lesion was assigned a score from 0 to 3 (0 for absence, 1 for mild, 2 for moderate and 3 for severe alteration). The score of a particular lesion in each group was summed up. The liver and kidneys preserved in neutral buffered formalin were processed for histopathological studies. The tissues like liver, kidneys and breast muscles were stored at -20°C for OTA residues analysis by HPLC (Sizoo and Egmond, 2005).

Breeding performance: Eggs of each group set for incubation were examined for fertility and embryo development by candling at day 3 and 5. The fertility was calculated as percent of fertile eggs in total number of eggs set each week.

All eggs were candled at alternate days starting from day 4 of incubation (i.e. day 4, 6, 8, 10, 12, 14, 16 and 18) and dead embryos (if any) were examined under stereomicroscope to determine the stage of death according to HH scale (Hamburger and Hamilton, 1951). Embryonic mortalites were divided into early (HH scale 4-15), mid (HH scale 16-30) and late periods (HH scale 31-45). Hatchability of each group, on the fertile eggs basis, was calculated at the end of incubation period.

Statistical analysis: The data was 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 $P \leq 0.05$. Cumulative scores for gross lesions were compared with control group on arithmetical difference basis. The data showing fertility, embryonic mortality and hatchability was presented in percentages.

RESULTS

Production performance: In week 1 and 2, a significant decrease in egg production were noted in groups E, F and D than the hens in group A (Table 1). In week 3, a dose dependent decrease was found in the egg production of hens i.e. with increase in the dietary level of OTA, a decrease in eggs production was observed. All the groups maintained on OTA contaminated feed showed significantly lower values than group A (control).

A nonsignificant difference was observed in the quality parameters of the eggs layed by hens fed different levels of OTA, during the week 1 of experiment (Table 2). In week 2, a decrease in eggs shell thickness was observed in the groups fed OTA contaminated feed but this decrease was significant only in group G as compared to that of group A. Haugh unit score was significantly lower in groups D, E, F and G than that noted for the eggs in group A. In week 3, a significant decrease in the egg shell thickness and Haugh unit score was present in the groups F and G as compared to those observed in group A.

Gross lesions: Hens in group A did not show any gross deviation from the normal pattern of different organs. Liver was normal in size, shape, consistency and color. In group B, C and D the liver was slightly enlarged, hemorrhagic, pale and friable in consistency. In the hens fed higher doses of OTA (groups E, F and G), more severe changes were observed in the gross lesions of livers (Table 3).

Kidneys of the hens from group A (control) were normal in size and color. In group B, C and D, kidneys were slightly enlarged and lighter in color along with petechial hemorrhages at some places. A dose dependent increase in the lesions score was noted and more severe changes were observed in the hens from groups E, F and G with a highest cumulative score of lesions in group G compared to all other groups.

Histopathological observations: Hens in group A did not show deviation from the normal histological structure of liver. However, the liver of the hens in group B showed a mild degree of cellular infiltration around the blood vessels. The histological structures of liver in groups C, D, E, F and G showed fatty change of mild to severe degree depending upon the dietary levels of OTA (Fig. 1). A similar dose dependent trend was present in individual hepatocyte necrosis and cellular infiltration in the OTA fed hens.

Table 1: Egg production (%) of White Leghorn breeder hens fed different levels of OTA for 3 weeks

Group (OTA mg/Kg)	Week 1	Week 2	Week 3
A (0)	80.95±4.07^{a}	90.48±8.91^{a}	104.77±18.54^{a}
B (0.1)	76.19±11.21^{a}	82.14±5.75^{a}	82.14±7.50^{b}
C (0.5)	71.43±10.60^{a}	80.95±9.27^{a}	72.62±20.25^{bc}
D (1.0)	71.43±4.45^{a}	78.57±15.85^{a}	61.91±4.45^{cd}
E (3.0)	53.57±10.60^{b}	55.95±9.27^{b}	50.00±9.62^{d}
F (5.0)	41.67±6.80^{c}	45.24±10.60^{bc}	50.00±12.73^{d}
G (10.0)	45.24±9.45^{bc}	42.86±13.11^{c}	33.33±19.84^{e}

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

Table 2: Egg quality parameters of White Leghorn breeder hens fed different levels of OTA for 3 weeks

Groups (mg OTA/Kg feed)	Egg weight (g)	Egg shell thickness (mm)	Haugh unit score
	Week 1		
A (0)	59.70±3.41	0.37±0.03	89.26±1.93
B (0.1)	61.52±3.91	0.34±0.06	92.63±5.77
C (0.5)	60.80±3.98	0.36±0.06	92.46±3.33
D (1.0)	59.07±3.77	0.35±0.03	92.05±3.14
E (3.0)	57.82±7.05	0.39±0.04	91.32±5.42
F (5.0)	57.25±5.09	0.35±0.08	88.14±3.43
G (10.0)	58.63±4.33	0.37±0.04	88.00±2.89
	Week 2		
A (0)	59.98±3.98	0.40±0.04^{a}	93.45±3.07^{a}
B (0.1)	58.88±6.24	0.40±0.08^{a}	91.08±0 .8^{ab}
C (0.5)	56.44±3.94	0.42±0.05^{a}	91.71±3.57^{ab}
D (1.0)	58.48±3.58	0.42±0.05^{a}	86.40±5.85^{bc}
E (3.0)	57.21±1.17	0.32±0.04^{b}	86.08±9.25^{bc}
F (5.0)	59.44±2.88	0.37±0.07^{ab}	86.61±4.75^{bc}
G (10.0)	54.92±2.88	0.38±0.04^{ab}	83.61±3.45^{c}
	Week 3		
A (0)	59.29±3.16	0.40±0.02^{a}	92.10±3.71^{a}
B (0.1)	59.70±4.55	0.36±0.05^{ab}	92.28±2.88^{a}
C (0.5)	57.12±3.42	0.38±0.06^{ab}	88.94±4.73^{ab}
D (1.0)	60.04±3.30	0.38±0.03^{ab}	88.17±3.72^{ab}
E (3.0)	60.01±4.39	0.38±0.04^{ab}	85.79±4.17^{b}
F (5.0)	57.61±4.12	0.33±0.04^{b}	87.02±4.41^{b}
G (10.0)	58.50±3.65	0.34±0.03^{b}	87.14±2.85^{b}

Values (mean±SD) in each row followed by different letters are significantly different (P≤0.05).

Kidneys of hens in group A and B showed normal histological pattern with clear glomerular spaces and well preserved tubular structure, however a mild degree of congestion was noted in the sections from group C. The necrosis of tubular epithelial cells (Fig. 2), proliferation of cells in the glomeruli and parenchymal congestion were more severe in groups fed higher doses of OTA with the most severe changes in group G.

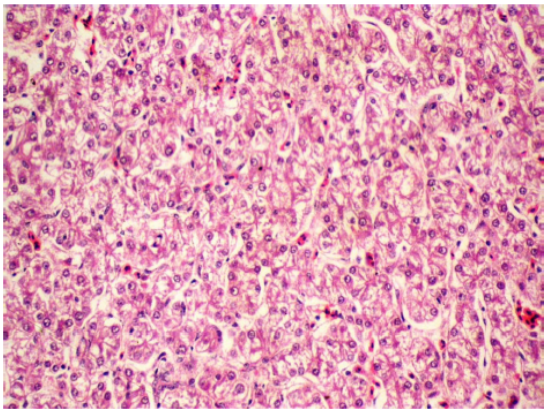

Fig. 1: Photomicrograph of the liver of hen in group F, fed OTA for 21 days, showing extensive vacuolation in hepatocytes (H and E stain 200X).

OTA residues in tissues: No OTA contents were detected in the tissues of hens from group A. However, OTA residues were detected in the liver, kidney and breast muscles of the hens from all intoxicated groups. A significantly higher concentration of OTA was detected in liver, kidneys and muscle tissues of hens from group G as compared to all other groups (Table 4). A dose depend increase in OTA concentration (ng/g) was found in all the tissues with highest in liver (26.336±1.16) followed by kidney (8.223±0.85) and lowest in breast muscles (1.235±0.21) of hens in groups C to G.

Breeding performance: Fertility (%), embryonic mortality (%) and hatchability (%) of hens fed different levels of OTA for 3 weeks has been presented in Table 5. The embryonic mortalities were noted in the eggs obtained from the hens kept on OTA contaminated diet, with higher mortalities in early developmental stages as compared to mid and late stages. An increase in mortalities was also noted in the eggs obtained from the hens fed OTA for longer duration.

Hatchability of chicks calculated on the basis of fertile eggs set for incubations in each group, showed dose and duration dependent relationship i.e. with increasing the dose and/or duration of OTA in the diet of hens, a decrease in the embryonic hatchability was noted.

Table 3: Scores of gross lesions of different organs of breeder hens fed OTA contaminated feed

Organ	Lesion	Max. possible score	Groups (OTA mg/kg feed) A (0)	B (0.1)	C (0.5)	D (1.0)	E (3.)	F (5.0)	G (10)
Liver	Enlargement	36	0	6	10	12	14	18	20
	Pale discoloration	36	0	8	12	16	22	22	24
	Friable	36	0	6	12	10	12	16	22
	Hemorrhage	36	0	1	6	8	10	10	14
Total score liver		144	0	22	40	46	58	66	80
Kidneys	Enlargement	36	0	4	6	14	28	32	32
	Hemorrhage	36	0	6	6	12	20	22	22
Total score kidney		72	0	10	12	26	48	54	54
Cumulative score Liver+Kidney)		216	0	32	52	72	106	120	134

DISCUSSION

A significant decrease in eggs production by the hens maintained on ochratoxin A (OTA) contaminated feeds, suggested OTA-mediated toxicity. This decrease in the

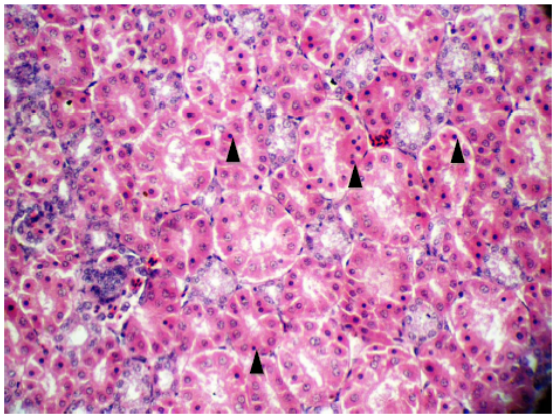

Fig. 2: Photomicrograph of kidney of hen in group F, fed OTA for 21 days, showing Pyknotic nuclei (arrow heads) in the tubuler epithelial cells. (H and E stain 400X).

egg production might be due to decrease in feed intake, poor health and decrease in protein synthesis, especially albumin. This hypothesis is supported by previous studies (Prior and Sisodia 1978; Hassan *et al*., 2010) in which a decrease in the protein synthesis and degenerative changes were noted in histological sections of livers of WL hens kept on OTA contaminated diet.

Table 4: Residues of OTA (ng/g) in tissues of breeder hens fed OTA for 3 weeks

Groups (mg OTA /Kg feed)	Liver	Kidneys	Muscles
A (0)	ND	ND	ND
B (0.1)	0.276±0.03e	0.431±0.09d	0.046±0.03e
C (0.5)	1.260±0.22e	0.439±0.17d	0.291±0.07d
D (1.0)	6.125±0.80d	2.294±0.50c	0.435±0.07cd
E (3.0)	12.839±1.51c	4.343±1.13b	0.547±0.08bc
F (5.0)	23.849±1.51b	4.306±0.89b	0.664±0.02b
G (10.0)	26.336±1.16a	8.223±0.85a	1.235±0.21a

ND = Not detected; Values (mean±SD) in each column followed by different letters are significantly different (P≤0.05).

Table 5: Breeding performance (embryonic mortalities, fertility and hatchability) of the hens kept on ochratoxin A contaminated diet

OTA feeding period (Days)	Group (mg OTA/Kg)						
	A	B	C	D	E	F	G
Early embryonic deaths (%)							
7	0.00	0.00	2.23	0.00	4.56	5.23	7.41
14	0.00	0.00	0.00	5.00	5.23	10.00	10.34
21	2.45	3.56	4.76	6.45	12.12	15.00	18.18
Mid embryonic deaths (%)							
7	0.00	0.00	0.00	2.38	4.17	4.53	5.26
14	0.00	0.00	0.00	0.00	9.09	5.23	9.09
21	0.00	0.00	0.00	5.13	5.00	6.90	10.34
Late embryonic deaths (%)							
7	0.00	0.00	0.00	0.00	2.00	1.00	3.00
14	0.00	0.00	0.00	0.00	1.00	2.26	3.25
21	0.00	0.00	0.00	0.00	0.00	2.00	4.00
Fertility (%)							
7	89.3	85.3	88.9	92.2	93.5	85.7	87.1
14	83.3	90.5	80.0	82.4	88.9	93.8	100
21	93.9	100	100	93.2	94.3	96.9	100
Hatchability (%)							
7	100	100	87.5	97.6	83.3	89.5	81.8
14	100	100	80.0	78.6	78.6	73.3	70.3
21	91.7	87.5	88.6	85.3	80.9	72.7	65.5

The decrease in the quality of eggs such as, egg shell thickness and egg weight also indicated the effects of OTA in the different part of female reproductive system including shell glands. The altered calcium metabolism, either poor absorption and/or its excretion via kidney due to nephrotoxic effects of OTA in the hens might also be the possible factor for the poor egg quality.

The gross pathological changes in liver of hens fed OTA contaminated feeds including an increase in the size, pale coloration, hemorrhages on the surfaces and friable in consistency have also well reported by many researchers (Sawale *et al.*, 2009; Hassan *et al.*, 2012). The nephrotoxic effects of OTA were indicated by increase in the size of kidneys that were bulging out from urinary sockets and lighter in coloration. The increase in the size of both these organs might be due to inflammatory responses accompanied by accumulation of inflammatory cells like leukocytes (Stoev *et al*., 2002). Also in the histological sections of these organs, a severe inflammatory response was noted at many places. At the early stage of inflammatory process there is accumulation of inflammatory cells and then enlargement in the size of organs, which later on subsides with the initiation of degenerative process. Due to shorter exposure period a similar response was noted in the histological pattern of these organs.

The OTA residues were detected in liver, kidneys and breast muscle, with significantly higher concentration in liver than kidneys and muscles. The findings of earlier researchers (Niemiec *et al*., 1994; Biro *et al*., 2002) are in line with the present study, but some researcher (Prior and Sisodia 1978) reported higher OTA residues in kidneys than in the liver.

Feeding OTA-contaminated diet to breeder hens did not have any adverse effect upon fertility, but embryonic mortality was significantly increased, contributing to decrease in the hatchability. Prior and Sisoda (1976) in the similar way reported no adverse effect of OTA feeding upon fertility of hens. The increase in the embryonic mortalites in the early developmental stages i.e., HH scale 5-15, showed that embryos are more sensitive to OTA exposure at early stages of their development. Similar findings were observed in one of our previous study (Hassan *et al*., 2011) in which OTA at different concentrations was placed in the air cell membrane of developing embryos. The cytotoxic effects of OTA in the developing chicken embryos as reported by Wei and Sulik (1996), supported the finding of our study. The decreases in the hatachabilites noted in the preset study were mainly due to i) increased in the embryonic deaths, and ii) increased in the skeletal defects in the fully developed embryos noted at day 22 of incubation. A similar finding was noted in an earlier study (Hassan *et al*., 2011) when inoculation of OTA in the fertile eggs resulted in the maxillary and mandibular retrognathism in the fully developed chick which were, though alive, unable to hatch at the completion of incubation period.

Conclusion: On the basis of findings of this study it can be concluded that feeding OTA contaminated diet to breeder hens significantly affects its health and performance. The transmission of OTA in the eggs placed by intoxicated hen results in decreased hatachabilites due to embryonic mortalites and developmental defects.

Acknowledgment: The authors highly acknowledge the financial support of Higher Education Commission of Pakistan.

REFERENCES

Ahsan S, IA Bhatti, MR Asi, HN Bhatti and MA Sheikh, 2010. Occurrence of aflatoxins in maize grains from central areas of Punjab, Pakistan. Int J Agric Biol, 12: 571–575.

Bayman P, JL Baker, MA Doster, TJ Michailides and NE Mahoney, 2002. Ochratoxin production by *Aspergillus ochraceus* and *Aspergillus Alliaceus*. Appl Environ Microbiol, 68: 2326-2329.

Beg MU, M Al-Mutairi, KR Beg, HM Al-Mazeedi, LN Ali and T Saeed, 2006. Mycotoxins in poultry feed in Kuwait. Arch Environ Conta Tox, 50: 594-602.

Binder EM, LM Tan, LJ Chin, J Handle and J Richard, 2007. Worldwide occurrence of mycotoxins in commodities, feeds, and feed ingredients. Anim Feed Sci Tech, 137: 265-282.

Biro K, L Solti, IB Vetro, G Bago, R Glavits, E Szabo and JF Gremmels, 2002. Tissue distribution of ochratoxin A as determined by HPLC and ELISA and histopathological effects in chickens. Avian Pathol, 31: 141-148.

Hamburger V and HL Hamilton, 1951. A series of normal stages in the development of the chick embryo. J Morphol, 88: 49–92.

Hanif NQ, M Naseem, S Khatoon and N Malik, 2006. Prevalence of mycotoxins in poultry rations. Pak J Sci Indust Res, 49: 120-124.

Hassan ZU, MZ Khan, A Khan, and I Javed, 2010. Pathological responses of White Leghorn breeder hens kept on ochratoxin a contaminated feed. Pak Vet J, 30: 118-123.

Hassan ZU, MZ Khan, A Khan, I Javed and MK Saleemi, 2011. Immunological status of progeny of breeder hens kept on ochratoxin A (OTA)-contaminated feed. J Immunotoxicol, 8: 122-130.

Hassan ZU, MZ Khan, A Khan, I Javed, U Sadique and MR Hameed, 2012. Effect of ochratoxin a (OTA)-contaminated feed on several health and economic parameters in white leghorn cockerels. Pak Vet J, 32: 35-40.

Koynarski V, S Stoev, N Grozeva, T Mirtcheva, H Daskalov, J Mitev and P Mantle, 2007. Experimental coccidiosis provoked by *Eimeria acervulina* in chicks simultaneously fed on ochratoxin A contaminated diet. Res Vet Sci, 82: 225–231.

Mukhtar A, AH Gilani and N Bhatty, 2010. Some nutritional and microbiological aspects of apples of common varieties available for household consumption. J Anim Plant Sci, 20: 253-257.

Nguyen MT, M Tozlovanu, TL Tran and A Pohl-Leszkowicz, 2007. Occurrence of aflatoxin B1, citrinin and ochratoxin A in rice in five provinces of the central region of Vietnam. Food Chem, 105: 42-47.

Niemiec J, W Borzemska, P Goliński, E Karpińska, P Szeleszczuk and T Celeda, 1994. The effect of ochratoxin A on egg quality, development of embryos and the level of toxin in eggs and tissues of hens and chicks. J Anim Feed Sci, 3: 309-316.

Prior MG and CS Sisodia, 1976. Ochratoxicosis in White Leghorn hens. Poult Sci, 55: 786-90.

Prior MG and CS Sisodia, 1978. Ochratoxicosis in White Leghorn hens. Poult Sci, 57: 619-623.

Saleemi MK, MZ Khan, A Khan and I Javed , 2009. Mycoflora of poultry feeds and mycotoxins producing potential of aspergillus species. Pak J Botany, 42: 427-434.

Saleemi MK, MZ Khan, A Khan, I Javed, ZU Hassan, MR Hameed, S Hameed and MA Mehmood, 2012. Occurrence of toxigenic fungi in maize and maize-gluten meal from Pakistan. Phytopathol Medit, 51: 219-224.

Sawale GK, RC Gosh, K Ravikanth, S Maini and DS Rekhe, 2009. Experimental mycotoxicosis in layer induced by ochratoxin A and its amelioration with herbomineral toxin binder 'Toxiroak'. Int J Poult Sci, 8: 798-803.

Sizoo EA and HV Egmond, 2005. Analysis of duplicate 24-hour diet samples for aflatoxin B_1, aflatoxin M_1 and ochratoxin A. Food Addit Conta, 22: 163-172.

Stoev SD, H Daskalov, B Radic, A Domijan and M Peraica, 2002. Spontaneous mycotoxic nephropathy in Bulgarian chickens with unclarified mycotoxin aetiology. Vet Res, 33: 83–93.

Trenk HL, ME Butz and FS Chu, 1971. Production of ochratoxins in different cereal products by *Aspergillus ochraceus*. Appl Microbiol 21: 1032-1035.

Wangikar PB, P Dwivedi and N Sinha, 2004. Effects in rats of simultaneous prenatal exposure to ochratoxin A and aflatoxin B1. I. maternal toxicity and fetal malformations. Birth defects Res (part B), 71: 343-351.

Wei X and KK Sulik, 1996. Pathogensis of caudal dygensis/sirenomelia induced by ochratoxin A in chick embryos. Teratology, 53: 378-391.

Zahoor-ul-Hassan, MZ Khan, MK Saleemi, A Khan, I Javed and SA Bhatti, 2012. Toxico-pathological effects of *in-ovo* inoculation of ochratoxin A (OTA) in chick embryos and subsequently in hatched chicks. Toxicol Pathol, 40: 33-39.