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Research Paper

Aflatoxin B1 induced developmental nephrotoxicity in RIR egg

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Abstract

The aim of this study was to investigate the developmental nephrotoxic potential of aflatoxin B1. Fertile RIR eggs were intoxicated with 5ng of aflatoxin at day '0' incubation. Nephrotoxic activity of aflatoxin was assessed by biochemical parameters such as total protein, creatinine, acid phosphatase, N-acetylglucosaminidase, oxidative stress biomarkers and histopathology. *In vivo* administration of aflatoxin resulted in significant increases in ACP, creatinine and NAG levels and reduction in total protein content. The activity of catalase, glutathione peroxidase and reduced glutathione level decreased while the level of lipid peroxidation increased due to AFB1 intoxication. Histopathological lesion observed in kidney includes different degree of tubular necrosis, congestion, cellular hypertrophy and vacuolar degeneration. It was concluded that *in vivo* administered AFB1 adversely affected embryonic development of kidney and impaired its function.

Keywords: Aflatoxin B1, Nephrotoxicity, Oxidative stress, Histopathology.

Introduction

Mycotoxins are biological active substances which have adverse effect on human and animal health [1]. Among them are the aflatoxins (AF) which are synthesized by the fungi *Aspergillus flavus* and *A. parasiticus* [2]. *Aspergillus flavus* produces four different types of mycotoxins, which are B1, B2, G1, and G2 [3]. Aflatoxins are the most common contaminants of in the feed of domestic animals, including birds [2]. Aflatoxin B1 (AFB1) is one of the most toxic and potent carcinogen and has been directly correlated to adverse health effects, such as liver cancer, in many animal species[4]. AFB1 contamination normally found in poultry foods and foodstuffs thus toxicity of this mycotoxin is very common to poultry. AF causes several million dollars economic losses in the poultry industry [5,6].

Further pronounced problem is due to its residue found in fertilized egg which increases embryonic mortality and also organ malformations, depending on the residual level of AFB1 and their metabolites in the eggs [7]. In addition, AF intake can decrease productivity of egg due to hepatic [8,9], immunological [10] and renal function alteration [11].

A bean shaped organ, Kidney has regulatory role in body such as regulation of electrolytes, maintenance of acid–base balance and regulation of blood pressure [12]. During the processes of reabsorption and secretion, kidney is more exposed to AFB1, making this vital organ more susceptible to AFB1 insults than other organ [13]. If fertile eggs are contaminated by AFB1, this is resulted into developmental renal anomalies or alters the renal function. So, the purpose of the present study was to follow out the changes in some blood biochemical parameters specific for renal function, oxidative stress, as well as histopathological changes after *in ovo* AFB1 inoculation in *Rhode Island Red* (RIR) eggs at day '0', may reveal events associated with abnormal renal development.

Material and Methods

Experimental Protocol

Fertile RIR eggs were obtained from the department of livestock production & management, Anand agricultural university, Anand. All eggs were wiped with 70% ethanol and numbered. Eggs were grouped in three (control, vehicle control and treated), 25 in each. Protocol was approved by departmental ethical committee according to CPCSEA. The concentrations of 5ng/5µl 20%alcohol/egg of AFB1 in treatment group and 5µl 20%alcohol/egg were injected in vehicle control group in air sac of eggs with sterile syringe at '0' day of incubation. According to chick embryo toxicity screening test-I (CHEST-I), limits for AFB1 as 0.3 to 30 ng/egg to determine the embryo toxicity. Therefore, relatively moderate dose (5 ng AFB1/egg) was used in the study to observe toxic effect of AFB1. The eggs were placed into an incubator at 37.5 ^{0}C, 65% relative humidity and turned every 3 hours. Weight mobility of incubated eggs was followed every day, and the mortality estimated by candling of eggs. After 21 days of incubation, hatchlings were sacrificed after collecting blood through heart puncture and gross anatomical change of kidney was observed. Kidney was removed, washed in PBS then blotted and weighted. From a pair of kidney one was fixed in 10% formalin for histopathology and second was used to estimate biochemical parameters.

Biochemical Parameters

Blood samples were centrifuged and serum was separated. Kidney tissue was homogenized in PBS, centrifuged and supernatant was separated for biochemical assay. Protein content of serum was estimated by the Lowry method [14]. By Bessey *et al*. method [15], acid phosphatase (ACP) activity was measured. N-acetylglucosaminidase (NAG) activity was estimated using p-nitrophenyl N-acetyl glucosaminide as substrate by using Shibata and Yagi method [16]. Concentration of creatinine in serum was estimated by modified Jaffe method [17].

Malondialdehyde (MDA) was estimated by the method of Buege and Aust [18] based on the principle of thiobarbituric acid (TBA) reacts with MDA and forms red color. Catalase activity was assayed by described method of Sinha [19]. Dichromate in acetic acid is reduced to chromic acetate, when heated in presence of hydrogen peroxide with the formation of perchromic acid as an unstable intermediate. The chromic acetate formed is measured at 590nm. The method described by Rotruck et al. [20] was adopted for estimation of glutathione peroxidase (GPx) activity which was measured by following the increase in absorbance at 412nm using 5,5'- dithio-bis-2- nitrobenzoic (DTNB) as a substrate and the enzyme activity is expressed in terms of mM of GSH consumed/ mg tissue. The reduced glutathione level was estimated by the method of Beutler et al. [21]. This method was based on the development of yellow colour when thiol reagent, DTNB reacts with GSH present in tissue sample forming 5-thio nitrobenzoic acid (TNB) and GS-TNB, which can be measured at 412nm using UV/VIS Perkin–Elmer spectrophotometer.

Histopathological examination

The kidney samples were fixed in 10% buffered formalin, processed through routine histological preparation and stained by haematoxylin–eosin (H&E). Mounted slides were examined and photographed under a light microscope (Leica DM2500).

Statistical analysis

Data generated from the experiment were subjected to statistical analysis and presented as mean and standard error around mean. The statistical significance of the differences between the mean values of control and experimental groups was evaluated through one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Statistical analysis performed using GraphPad prism (version 6) software.

Results

In the present study, the AFB1 administered egg showed greater mortality compare to that of control. AFB1 intoxication resulted into decrease body weight but increased absolute and relative kidney weight of hatchling. Mean body weight obtained at the end of the incubation was significantly reduced whereas absolute and relative weight of kidney was not observed significantly increased in newly hatched chick of treatment group as compared with the control group (Table 1).

Table: 1 Effects of AFB1 on body weight, absolute and relative weight of kidney of newly hatched chick

Groups	Body weight of Embryo (gm/chick)	Absolute weight of Kidney (gm/100gm body weight)	Relative Weight of kidney (gm/100gm body weight)
Control	122±15.8	4.7 ±0.69	3.9 ± 0.47
Vehicle Control	125 ±16.1	4.6 ±0.70	3.6 ± 0.59
Treated	85 ±20.5$^{\alpha}$	4.9 ±0.89	5.8±0.93

n = 10, Values are in Mean ± SE, $^{\alpha}$ $P < 0.05$ significantly different from control

Mean (±SE) serum value of total protein is presented in the Table 2. There was a significant (P<0.05) difference in serum total protein value between the control and AFB1 intoxicated RIR eggs. Value of protein concentration was significantly declined in newly hatched chick after *in ovo* AFB1 exposure. *In ovo* AFB1 injection was resulted in significant increased in the level of creatinine in serum of newly hatched chick when compared with the control group (P<0.01, Table-2). Serum ACP activity showed a significant increase (P<0.05) in hatchling that underwent *in ovo* treatment of 5ng AFB1/egg. Likewise, the specific activity of NAG in tissue homogenate showed a significant increase (P<0.01) due to *in ovo* AFB1 intoxication at day '0'.

The level of MDA increased significantly in 5ng AFB1 treated renal tissue as compare to control (P<0.05). Catalase activity was decreased significantly due to *in ovo* AFB1 exposure (P<0.05, Table-3). As with catalase, the GPx activity and GSH level were significantly decreased in the renal extracts of the treated group as compared with the control group.

Table 2: Biochemical parameter after *in ovo* AFB1 exposure to hatchling

Groups	Protein (mg/dL)	Creatinine (mg/dL)	ACP (pNP released /mg tissue)	NAG (µmole / gm tissue)
Control	4.89 ±0.22	0.48 ± 0.02	7.2± 1.1	2.08±0.17
Vehicle Control	4.91 ±0.23	0.51 ± 0.09	8.9± 1.4	2.24± 0.22
Treated	4.32 ±0.31*	0.97 ± 0.13**	12.8± 1.7*	5.57± 1.31**

n = 10, Values are in Mean ± SE, $^{*}P < 0.01$, $^{**}P < 0.001$ significantly different from control

Table: 3 ROS parameter after *in ovo* AFB1 exposure in renal tissue of hatchling

Groups	LPO (nM of MDA released /gm of tissue)	Catalase (Unit/gm of tissue)	GSH (µg/gm of tissue)	GPx (mM of GSH consumed/ mg tissue)
Control	4.98± 0.46	19.9 ± 2.41	17.3 ± 1.1	16.7 ± 0.67
Vehicle control	5.04± 0.62	18.6 ± 2.47	16.1 ± 1.8	15.9 ± 0.82
Treated	7.66± 1.16*	12.1 ± 3.13*	12.6 ± 2.4*	7.42 ± 0.92**

n = 10, Values are in Mean ± SE, $^{*}P < 0.01$, $^{**}P < 0.001$ significantly different from control

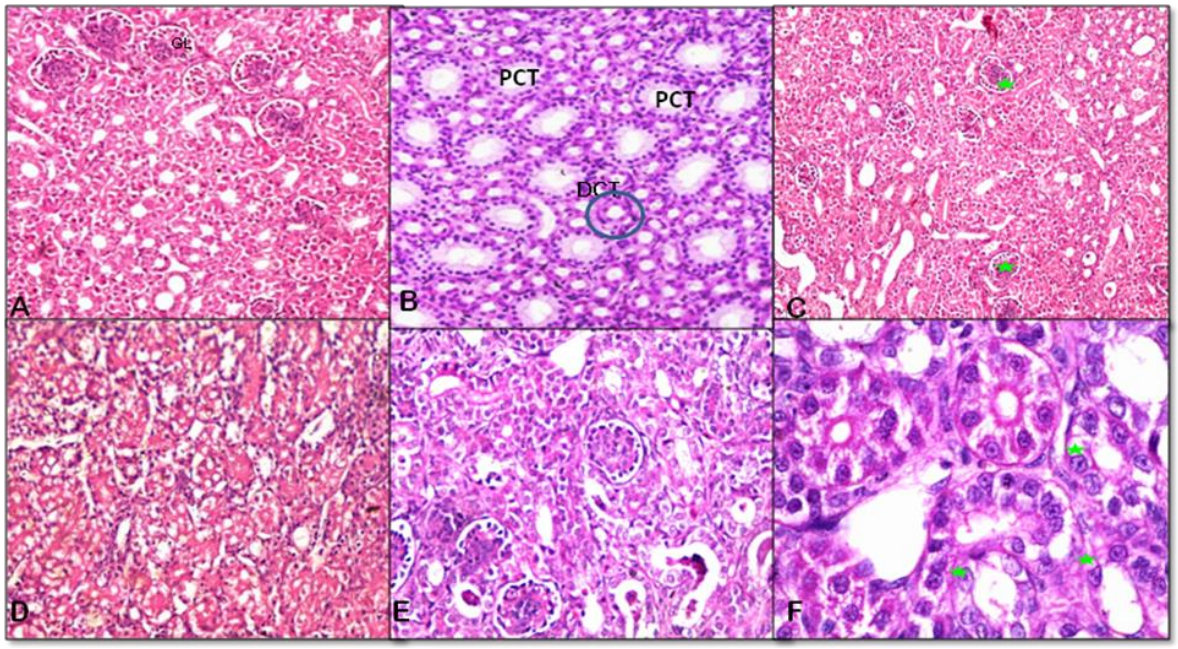
Microscopically, examination of untreated hatchling kidney tissue was observed with normal histoarchitectural structure. Kidney of control chicken shows intact Bowman's capsule, distal tubules with intact cuboidal cell and proximal tubules with columnar epithelial cells (Figure 1). Examined section showed well vascularize glomeruli with intact podocyte. In treated group of hatchling, AFB1 induced degenerative lesion was observed in kidney. Exposure of AFB1 resulted in reduction of nephron number and cellular hypertrophy as a compensatory effect of weight enlargement of tissue. Examined kidney section showed tubular epithelial vacuolization and degenerative changes (Figure 1). Cellular swelling with vacuolization in tubular epithelial cells leading to occlusion of tubules was

examined with gross necrosis and congestion. Treated kidney showed increased glomerular cellularity and hyalinization of glomeruli. Damaged glomeruli were observed with degenerative podocyte.

Discussion

Kidney is the major excretory organs and about 20–25% of the total amount of circulating toxins, including mycotoxins and its metabolites, excreted through kidneys which are competent to inflate renal damage [22,23]. Increased mortality was observed in current study which is also observed in broiler egg by Cravens et al. [24] after AFB1 exposure. Body weight of newly hatched chickens was depressed by *in ovo* AFB1 injection in current study. Similar results of weight reduction due to aflatoxicosis have been reported by Quezada et al. [2] and Magnoli et al. [25]. The increased absolute and relative weight of kidney observed in hatchling those were exposed with AFB1 at day '0' of development. Quezada et al., [2] have reported increase weight of target organ due to AFB1 treatment in developing broiler chicken. Renal enlargement might be related to a compensatory functional effect due to AFB1 exposure which may lead renal hypertrophy.

Figure 1: Histopathology of *in ovo* AFB1 treated kidney tissue of RIR egg hatchling



A&B- Control kidney histology, C to F- AFB1 treated kidney. (A) Control kidney showing cortical glomeruli (10X), (B) Control kidney showing renal tubules (PCT- Proximal convoluted tubules, DCT- Distal convoluted tubules, 40X), (C) Tubular Necrosis and hyalinisation of glomeruli progressing towards destruction of glomeruli (10X), (D)Vacuolar degeneration in renal tissue (4X), (E) Tubular degenerative changes and increased glomerular cellularity (40X), (F) Cellular swelling with vacuolization in tubular epithelial cells leading to occlusion of tubules (100X).

Significant reductions in serum concentrations of total protein probably can be attributed to a suppression of protein biosynthesis, as a result of structural damage of renal tissue due to AFB1 toxicity during development of embryo. Decrease in concentration of total protein has been reported by Quezada et al. [2]. Renal tissue damage causing outflow of ACP enzyme into blood stream, this is a possible way to increased serum ACP activity of enzyme by embryonic AFB1 intoxication. Increased activity of enzymes like creatine kinase, acid phosphatase and alkaline phosphatase was reported by Edrington et al. [26].

Creatinine is the final metabolite of creatine conversion and a major marker molecule to observe kidney function. In birds creatinine is converted into creatine to provide energy to muscle or it is rapidly excreted by kidney. Increased blood serum creatinine in AFB1–treated birds indicates enhanced conversion of muscle phosphocreatine to creatinine which is not excreted out due to renal damage. Increased urea and creatinine as indices of impaired renal function in aflatoxicosis were

reported in chickens [12, 27-29]. It is assumed that higher urea and creatinine levels are due to disturbed transportation function of epithelial cells in collecting tubules and diffuse impaired function of proximal tubules function [23,30,31]. These data are associated with a marked increase activity of N-acetylglucosaminidase which corresponds to renal proximal tubular alterations of kidney. NAG is a lysosomal enzyme produced by renal proximal tubular cells and has been widely used as a marker for renal tubular damage. Information about NAG is limited in birds.

Our result show marked increase MDA level and decrease activity of its related antioxidant in AFB1 treated hatchling. This is an indication of cellular damage caused by AFB1. Enhanced activity of LPO, increases oxygen derives free radicals which cause DNA damage. Therefore the reduction in protein content may be due to either direct effect of AFB1 on protein synthesis or indirectly through DNA damage. This result is in coinciding with those reported by Verma and Mathuria [32] in mice and those of Gokhan et al. [33] in broiler chickens.

A significant decrease in catalase and GPx activity were noted in aflatoxin treatment group. The H_2O_2 produce can then be decomposed enzymatically by catalase and GPx. GPx not only decomposes H_2O_2 but can also interact with lipid peroxidation and provides protection [34]. Thus significant reduction in these enzyme activities could be responsible for increased lipid peroxidation observed in AFB1 exposed renal tissue. Verma, and Nair [35] in mice testis and Gokhan et al. [33] in broiler chickens, reported decrease activity of catalase and Gpx enzymes. Due to cellular renal damage activity of these enzymes could be depleted as a result of elevated lipid peroxidation.

Glutathione content decreased significantly in kidney of *in ovo* AFB1 injected hatchling, suggesting its rapid oxidation. Glutathione has a beneficial effect by virtue of possessing –SH groups. It helps to protect biological membranes, which are readily susceptible to injury by peroxidation [31,35]. Thus, significantly lower GSH level would further aggravate the toxic effects of AFB1. The observed results of histopathology, indicated *in ovo* AFB1 toxicity in kidney of chicken. Current examined result is correlated with other investigators which have shown AFB1 potency as a nephrotoxic compound. Motawe et al. [11] reports walk with same line of our investigation. He reported vacuolar degeneration and necrosis in kidney of broiler chicken. Several histopathological changes were observed by Matri [37] in kidney and other organ due to aflatoxicosis. These findings are in agreement with previous reports, in which the kidney was a target organ by AFB1 myotoxin [38-40].

Conclusion

This study has shown that *in ovo* AFB1 intoxication adversely affected embryonic development via oxidative stress, biochemical changes and alter histoarchiture of kidney. AFB1 induces toxic effects on biochemical functions which correlate well with the histopathological changes in the kidney of hatchling. Lipid peroxidation is more pronounced in treated hatchlings whereas level of antioxidant enzyme is observed low after *in ovo* AFB1 treatment. Aflatoxin B1 modifies renal functions by either inducing nephron-glomeruli lesions or altering biochemical parameter in damaged renal cells. A better understanding of AFB1 induced biochemical change, ROS-mediated damage and their impact on embryonic development is important to ensure optimal outcomes.

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References

1. Goldman R. and Shields P. G., Food mutagens, J. Nutr., 133, 965S **(2003)**

2. Quezada T., Cuellar H., Jaramillo-Juarez F., Valdivia A.G. and Reyes J.L., Effects of aflatoxin B1 on the liver and kidney of broiler chickens during development, Comp. Biochem. Physiol. Part C., 125, 265 **(2000)**

3. Yin Y., Yan L., Jiang J. and Ma Z., Biological control of aflatoxin contamination of crops, J. Zhejiang Univ. Sci. B., 9, 787 **(2008)**

4. Martins M.L., Martins H.M. and Bernardo F., Aflatoxins in spices marketed in Portugal, Food Addit. Contam., 18, 31 (**2001)**

5. Requena F., Saume E., and Leon A., Micotoxinas: Riesgosy prevencion, Zoot. Trop., 23, 393 **(2005)**

6. Fraga M.E., Curvello F., Gatti M.J., Cavaglieri L.R., Dalcero A.M. and Darocha Rosa C.A., Protemtial aflatoxin and ochratoxin A production by *Aspergillus* species in poultry feed processing, Vet. Res. Communications, 31, 345 (**2007)**

7. Celik I.H., Oguz O., Demet M., Boydak H.H., Donmez E.S. and Nizamlioglu F., Embryotoxicity assay of aflatoxin produced by *Aspergillus parasiticus* NRRL 2999, Br. Poult. Sci., 41, 401 **(2008)**

8. Jansen van Rensburg C., Van Rensburg C.E.J., Van Ryssen J.B.J., Casey N.H. and Rottinghaus, G.E., *In vitro* and *in vivo* assessment of humic acid as an aflatoxin binder in broiler chickens, Poult. Sci., 85, 1576 **(2006)**

9. Farah N., Saleem J. and Abdin M.Z., Hepatoprotective effect of ethanolic extract of Phyllanthus amarus Schum. Et Thonn. on aflatoxin B1 induced liver damage in mice, J. Ethnopharmacol., 113, 503 **(2007)**

10. Tessari E.N., Oliveira C.A., Cardoso A.L., Ledoux D.R., and Rottinghaus G.E., Effects of aflatoxin B1 and fumonisin B1 on body weight, antibody titres and histology of broiler chicks, Br. Poult. Sci,. 47, 357 **(2006)**

11. Glahn R.P., Beers K.W., Bottje W.G., Wideman Jr. R.F. and Huff W.E., Altered renal function in broilers during aflatoxicosis, Poult. Sci. 69, 1796 **(1990)**

12. Motawe H.F.A., Abdel Salam A.F. and El Meleigy Kh. M., Reducing the toxicity of aflatoxin in broiler chickens' diet by using probiotic and Yeast, Int. J. Poul.Sci., 13, 397 **(2014)**

13. O'Brien E. and Dietrich D. R., Mycotoxins affecting the Kidney, CRC Press, Florida, 895 **(2004)**

14. Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R. J., Protein measurement with Folin Phenol Reagent, J.Biol.Chem. 193, 265 **(1951)**

15. Bessey O.A., Lowry O.H. and Brick N.T., A method for rapid determination of acid and alkaline phosphatase 5cu mm, of serum, J. Bio. Chem., 164, 321 **(1946)**

16. Shibata H. and Yagi T., Rate assay of N-acetyl-beta-D-hexosaminidase with 4-nitrophenyl N-acetyl-beta-D-glucosaminide as an artificial substrate, Clin. Chim. Acta., 251, 53 **(1996)**

17. Jendrassik L. and Grof P., Vereinfachte photometrische methode zar bestimmung des blutbilirubins, Biochem Z., 297, 81 **(1938)**

18. Buege J.A. and Aust S.D., Microsomal lipid peroxidation, Methods Enzymol., 52, 302 **(1978)**

19. Sinha A.K., Colorimetric assay of catalase, Analytical Biochem., 47, 389 **(1972)**

20. Rotruck J.T., Pope A.L., Ganther H.E., Swanson A.B., Hafeman D.G., Hoekstra, W.G., Selenium: Biochemical role as a component of glutathione peroxidase, Science, 179, 588-590 **(1973)**

21. Beutler E., Duron O. and Kelly B.M., Improved method for the determination of blood glutathione, J. Lab. Clin. Med., 61, 882 **(1963)**

22. Harriet A.M., Is indoor mold contamination a threat to health? Part 2, Journal of Environmental Health, 62, 47 **(2003)**

23. Valchev I., Kanakov D., Hristov T.S., Lazarov I., Binev R., Grozeva N. and Nikolov Y. Effects of experimental aflatoxicosis on renal function in broiler chickens, BJVM., 17, 314 **(2014)**

24. Cravens R.L., Goss G.R., Chi F., De Boer E.D., Davis S.W., Hendrix S.M., Richardson J.A. and Johnston S.L., The effects of necrotic enteritis, aflatoxin B1, and virginiamycin on growth performance, necrotic enteritis lesion scores, and mortality in young broilers, Poult Sci*.,* 92, 1997 **(2013)**

25. Magnoli A.P., Texeira M., Rosa C.A.R., Miazzo R.D., Cavaglieri L.R., Magnoli C.E., Dalcero A. M. and Chiacchiera S.M., Sodium bentonite and monensin under chronic aflatoxicosis in broiler chickens, Poult. Sci., 90, 352 **(2011)**

26. Edrington T.S., Kubena L.F., Harvey R.B. and Rottinghau G.E., Influence of a super activated charcoal on the toxic effects of aflatoxin or T-2 toxin in growing Broilers, Poult Sci., 76, 1205 **(1997)**

27. Yildirim E.I., Yalchinkaya M., Kanbur M. and Oruc E., Effects of yeast glucomannan on performance, some bio-chemical parameters and pathological changes in experimental aflatoxicosis in broiler chickens, Rev. Med. Vet., 162, 413 **(2011)**

28. Hassan Z.U., Khan M.Z., Khan A., Javed I., and Hussain Z., Effects of individual and combined administration o ochratoxin A and aflatoxin B1 in tissues and eggs of White Leghorn breeder hens, J. Sci. Food Agr., 92, 1540 **(2012)**

29. Pattar J., Shridhar N.B., Jagadeesh S.S., Satyanarayana M.L., Upendra H.A., and Suhasini K., Evaluating the potential toxic effect of aflatoxin and ochratoxin on serum biochemical parameters in coloured (Raja Ii) broilers, Vet Sci., 8, 215 **(2014)**

30. Ortatatli M., Oguz H., Hatipoglu F. and Karaman M., Evaluation of pathological changes in broilers during chronic aflatoxin (50 and 100 ppb) and clinoptilolite exposure, Res. Vet. Sci., 78, 61 **(2005)**

31. Umar S., Arshad A., Ahmad B. and Arshad M., Clinico biochemical and hematological changes in broilers induced by concurrent exposure to aflatoxin B1 and ochratoxin, A.J. Public Health Biol. Sci*.,* 1, 79 **(2012)**

32. Verma R.J., and Mathuria N., Curcumin ameliorates aflatoxin-induced lipid-peroxidation in liver and kidney of mice, Acta Pol. Pharm*.,* 65, 195 **(2008)**

33. Gokhan E., Mehmet A., Ender Y., Fatma, S. and Dinc EsSiZ L. A., The effects of aflatoxins on oxidative stress in broiler chickens, Turk. J. Vet. Anim. Sci., 29, 701 **(2005)**

34. Mates J.M., Perez-Gomez C. and Nunez de Castro I., Antioxidant enzymes and human diseases, Clin. Biochem*.,* 32, 595 **(1999)**

35. Verma R.J. and Nair. A., Vitamin E ameliorates aflatoxin-induced biochemical changes in the testis of mice, Asian J. Androl., 3, 305 **(2001)**

36. Devendran G. and Balasubramanian U., Biochemical and histopathological analysis of aflatoxin induced toxicity in liver and kidney of rat, Asian J. Plant Sci. Res.,1, 61 **(2011)**

37. Matri A., Identification of fungal contamination and determination of mycotoxigenic molds by micellar electrokinetic capillary chromatography in smoked paprika, J. Food Prot., 68, 815 **(2001)**

38. Valdivia A.G., Martiriez A., Damitrn F.J., Quezada T., Ortiz R., Martirlez C., Llwmas J., Rodriguez M.L., Yamamoto L., Jaramillo F., Loarca-Pina M.G., and Reyes J.L., Efficacy of N-acetylcysteine to reduce the effects of aflatoxin B1 intoxication in broiler chickens, Poult. Sci., 80, 727 **(2001)**

39. Del Bianchi M., Oliveira C.A.F., Albuquerque R., Guerra J.L. and Correa B., Effects of prolonged oral administration of Aflatoxin B1 and Fumonisin B1 in broiler chickens, Poul Sci*.,* 84, 1835 **(2005)**

40. Martinez-de-Anda A., Valdivia A.G., Jaramillo-Juarez F., Reyes J.L., Ortiz R., Quezada T., de Luna M.C. and Rodriguez M.L., Effects of aflatoxin chronic intoxication in renal function of laying hens, Poultry Sci*.,* 89, 1622 **(2010)**