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Experimental Pathological Studies on Ochratoxicosis in Broiler Chickens

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Abstract
Forty broiler chicks (Hubbard breed), two weeks old were used to study the pathological lesions and residues of ochratoxin A in some chicken organs. Experimental chicks were divided into two groups. First group (30 chicks) were fed on ration contaminated with 100ppb /kg ration, while second group (10 chicks) were kept as control and fed on ration free from OTA till the end of the experiment. Fifteen chicks from group 1and five chicks from group 2 were sacrificed 28 and 36 days post feeding (PF). The clinical signs, mortalities and lesions in addition to OTA residues were detected and recorded. The chicks in group 1 showed 20% mortalities beside diarrhea, dehydration and emaciation. The kidney lobules appeared pale with urates deposits due to intense nephrotic changes, nephritis, fibrosis and gout. The liver showed hepatic hemorrhages apoptosis, necrosis and hyperplastic bile ductules. Intense lesions in brain in the form of edema, meningeal lymphocytic cells aggregations, degenerated neurons and purkinje cells were recorded. GIT lesions (proventriculus and intestine) due to direct contact with OTA contaminated ration were also seen. OTA residue was higher in liver than kidneys while the lowest value was detected in skeletal muscle. It could be concluded that OTA induced neurotoxicity beside hepato-renal toxicity and GIT lesions due to its toxic effect and its adverse effect on growth performance.

Keywords: Ochratoxins, Residues, Carcinogen, Teratogen

Introduction

Ochratoxins is a general term describing a family of toxic compounds consisting of three members, A, B and C. They are structurally related and produced as secondary metabolites of several fungal species included in the genera Aspergillus. Both biliary excretion and glomerular filtration play important roles in the plasma clearance of ochratoxin A in rats [1].

Ochratoxin A causes significant losses in poultry industry due to its effects on performance and health. It causes a reduction in growth rate and feed consumption beside poorer feed conversion rate and increase mortality [2].

Ochratoxin A is considered as powerful nephrotoxin, carcinogen, teratogen, neurotoxin, mutagens and immunotoxins in rats, human and likely in poultry [3]. The effects of feeding one day old ducklings on 50 ppb OTA for 42 days once daily were emaciation, paleness in color of breast muscles, sub capsular ecchymosis and petechial hemorrhage on the liver and enlarged kidneys with linear hemorrhagic enteritis. Depletion of leg color with interpreted bluish pigmentation, atrophy of spleen, thymus and bursa of Fabricius were also recorded [4]. The effects of feeding of hatched broiler chicks on ration contaminated with 100 ppb ochratoxin A for 28 days were mild vacuolar degeneration of hepatocytes; biliary hyperplasia and periportal fibrosis were common in liver. Congestion, tubular epithelial degeneration and necrosis in kidneys, mucosal hyperplasia of the crop and glandular necrosis of the proventriculus were found. Defective keratin formation, mononuclear cell infiltration and periglandular fibrosis were noticed in the gizzard .Fusion and broadening of intestinal villi, congestion, mild lymphoid cell depletion, reticular cell hyperplasia were detected in intestine .Lymphoid cell necrosis of spleen together with lymphoid cell depletion and lymphocytolysis in the bursa of Fabricius and thymus were common, moreover lymphoid organs showed presence of apoptotic bodies which indicated immunosuppressive effect of OTA which evidenced by reduction in humoral and cell mediated immunity [5]. This work was planned to study pathological lesions and clinical signs due to ochratoxin (OTA) and its residues in different organs of broilers.

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Material and Methods

Experimental chicks

Forty healthy one-day-old commercial broiler chicks (Hubbard breed), were purchased from Alkahira Poultry Company and kept under standard hygienic conditions as described by Harrison and Harrison [6]. The chicks were vaccinated with recommended vaccination program and left two weeks for adaptation.

Mycotoxin (Ochratoxin A)

Pure ochratoxin A (OTA) was obtained from M/S Sigma Chemicals. European commission Recommendation 2006/J76/EC, suggests that maximum level of OTA in poultry feed should be set at 0.1 mg OTA/ kg ration. Crude OTA was added to ration at a dose of 100 ppb/kg feed for 36 days [5, 7].

Residual detection

Samples

Residues of OTA were determined in kidneys, liver and muscles of the experimental chickens at the end of the toxin-feeding period (36 days PF) after collection of fresh specimens and kept at -20^{0}C until its preparation for extraction.

Sample preparation and extraction

Twenty grams aliquot of fresh chicken tissues samples (liver, kidney and muscle) was homogenized with 6 ml of 1 ml phosphoric acid in an ultra Turrax T25 homogenizer for 5 min. 2.5 gms aliquot of the homogenate was transferred into a pyrex centrifuge tube. Then, it was extracted twice with 5 ml of ethyl acetate and centrifugated for 5 min. The organic phases were combined, reduced to approximately 3 ml and back-extracted with 3 ml of 0.5 µ NaHCO3 (pH 8.4). The aqueous extract was loaded onto an ochre Test WB column. After washing with 10 ml of PBS and 10 ml of water, the mycotoxin was clouted with 1.5 ml of methanol. We added 1.5 ml of water to all samples before injecting onto high performance liquid chromatography (HPLC) to make the solvent for the standard and samples similar to the mobile phase [8,9].

Immunoaffinity column clean-up

Extracted samples were passed through the immunoaffinity clean-up of 1-2 drops [10] under gentile pressure using a vacuum clean-up assembly after washing the column with 10 ml of water methanol. The column was dried under nitrogen gas (N_2) for 5 minutes and toxin was eluted from the column by passing 2.0 ml of methanol.

High performance liquid chromatography for the determination of mycotoxin contents, 20 µl of sample was loaded onto the HPLC system (Prominence TM, Shimadzu® Tokyo, Japan) coupled with spectroflorometeric detector RF-10 AXL® (Shinalzu). Acetonitrile methanol and double distilled deionized water used as mobile phase in the ratio of 49.5:49.5:1 for OTA. The temperature of the column oven was set at 40ºC for OTA.

The chickens were divided into 2 groups, first group contained 30 chickens and second group which kept as a control group and enclosed 10 birds.

Group (1): chicks were given ration contaminated with 100 ppb OTA/kg ration from two weeks age till the end of the experiment (36 days)

Group (2): control group which was given the recommended ration, free from mycotoxins, from two weeks age till the end of the experiment. Fifteen chickens from group (1) and five chickens from group (2) were sacrificed on the 28th day and 36th days from the beginning of the experiment (Table 1).

The clinical signs, mortalities and postmortem lesions in both dead and sacrificed chickens during the experimental period were recorded.

Table 1: Experimental design of ochratoxin 100ppb/ Kg ration given to broiler chickens

Group	No. of chicks	Treatments	dead * and Sacrificed chicks/ day			
		Ochratoxin100 ppb /kg ration	25 day*	28 day	Day 33*	36 day
1	30	+	2*	13	4*	1
2	10	-	5	-	5	-

***Days of scarifying of birds**

Histopathological investigation

Tissue specimens from kidneys, liver, proventricular, intestine, brain, skeletal muscles, were collected and fixed in 10% buffered neutral formalin. Paraffin sections 5 micron thick were prepared and stained with hematoxylin and eosin stain [11] and examined microscopically.

Results

Clinical signs and mortality

Loss of vitality, dullness, ruffled feather and restlessness in the majority of the experimental chickens were noticed. Anorexia, few cases showed diarrhea followed by emaciation, with paleness of comb and wattles were also observed 28 and 36 days post feeding. Two chicks were died during the first period (at day 25) and four extra birds were died in the same group (at day 33) that's mean 20% mortalities in total among birds of this group was detected.

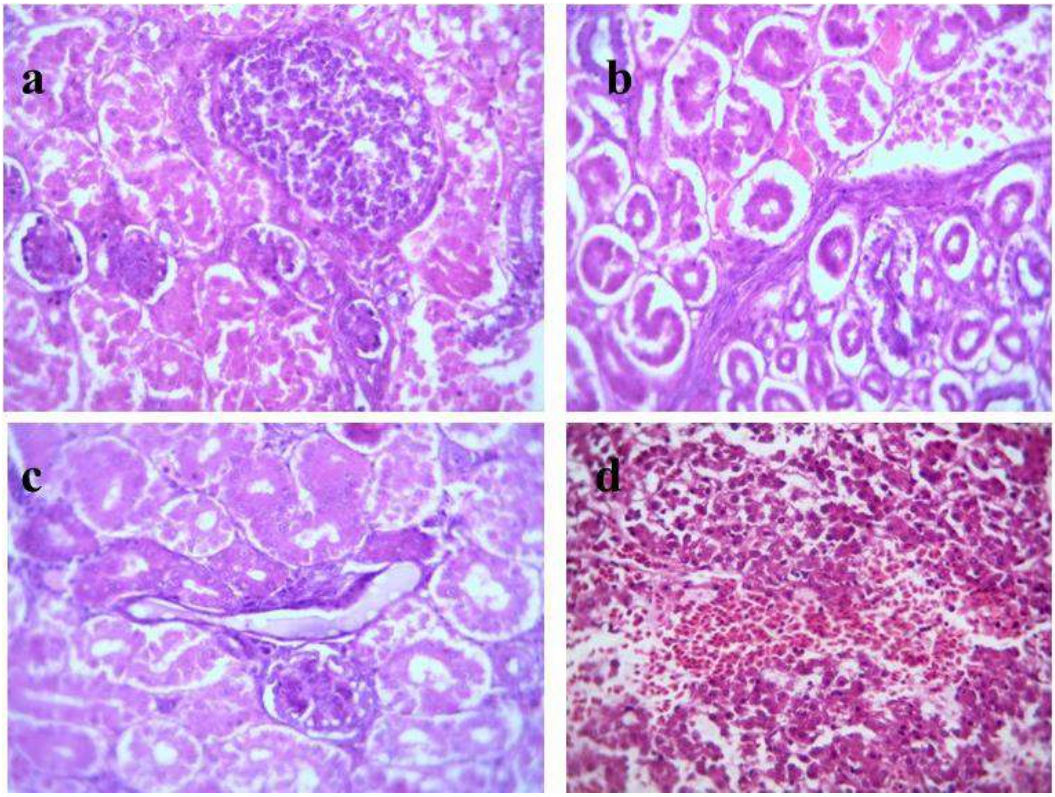

Figure 1: (a) photomicrograph of kidney, group1 - 28 days PF showing replacement of the renal parenchyma with focal lymphocytic aggregation in renal cortex and necrosis of renal tubules (H&E×1200). (b): photomicrograph of kidney, group1-, 36 days PF showing atrophy of the renal tubules with disassociated tubular epithelia and interstitial fibrosis (H&E ×1200). (c): photomicrograph of kidney, group1- 36 days PF showing deposition of urates in some renal tubules beside necrosis or degeneration of the majority of the renal tubular epithelium (H&E ×300). (d): photomicrograph of liver, group1-, 28 days PF OTA, showing diffuse interstitial hemorrhage replacing the hepatic parenchyma (H&E ×1200).

Pathological findings

Macroscopically

Both sacrificed chickens 28 days post feeding (PF) and died carcasses were dehydrated, emaciated with pale and swollen kidneys. Other carcasses showed congested kidneys with ureters distended with urates. The liver was enlarged, congested with blood oozing from its cut surface, while proventricular mucosa appeared normal in most cases. The majority of chickens showed congestion and thickening of the intestinal mucosa. The skeletal muscles were apparently normal or pale in color moreover; slight to moderate congestion of meningeal blood

vessels was detected. After 36 days PF. The kidney's lobules became paler and contained whitish urates deposits inside it, while liver became slightly enlarged and pale in color and sometimes dark red or greyish areas could be seen on the hepatic surface. The proventricular mucosa became thickened and edematous in some chickens. Partial necrosis of their mucosa and others showed thickening in the intestinal wall and excessive amount of mucus. While, pale colored breast muscle fibers could be seen, other cases exhibited petechial hemorrhage on thigh and breast muscles. The majority of chickens' brains were apparently normal. Microscopically, 28 days PF OTA kidneys showed focal replacement of the renal parenchyma with extravasated erythrocytes. Focal lymphocytic aggregation in renal cortex with necrosis of the renal tubules was common (Fig. 1-a). Moreover, infiltration of heterophils within the renal parenchyma was detected in most cases. Some cases showed regenerative attempts with necrosis in the adjacent area. After 36 days PF OTA, the lesions became more pronounced and represented by radiating basophilic crystals inside the lumina of some renal tubules beside destruction of renal epithelium which was seen in some cases (Gout). Some cases showed degenerative changes in the collecting renal tubules. Moreover, most cases revealed extensive focal lymphocytic cells infiltration replacing the renal parenchyma especially the renal cortex. Other cases exhibiting atrophy of the renal tubules with disassociated tubular epithelia and interstitial fibrosis (Fig. 1-b).

Hyperplasia of the epithelial lining of the collecting ducts beside periductular fibrosis also seen in some cases. A few cases showed urates deposition in some renal tubules beside necrosis or degeneration of the majority of the renal tubular epithelium (Fig.1-c). Liver, 28 days PF OTA, revealed diffuses interstitial hemorrhage replacing the hepatic parenchyma in the most examined cases (Fig.1-d), in addition to intense portal heterophilic infiltration. Some cases showed edema in the wall of portal vein. Moreover, focal lymphocytic cells aggregation was the main lesion seen in all examined tissue in this stage. Mild congestion appeared in most cases 36 days PF with perivascular leukocytic cells infiltration. Most cases showed dilated hepatic sinusoids with atrophied hepatic cords. Some hepatocytes showed also apoptosis intermingled with necrosis involving the parenchyma (Fig.2-a). The proventriculus 28 days PF OTA, revealed focal lymphocytic cells aggregation inside the submucosal compound glands, while necrotic debris in the lumen of proventricular gland was also seen in other cases (Fig. 2-b). Mucosal edema with inflammatory cells infiltration and partial desquamation were also appeared in some examined tissue samples. After 36 days PF of OTA in addition to hyperplasia of the proventricular epithelium of the compound glands, there was arboreal growth from the mucosa (Fig. 2-c). The compound glands of some chickens showed coagulative necrosis while few cases showed desquamated epithelial lining within the lumen. At 28 days PF OTA, the intestine revealed fusion of some intestinal villi tips and partial desquamation of villus enterocytes (Fig. 2-d) while villous hemorrhage was seen, in other cases, after 36 days PF OTA.

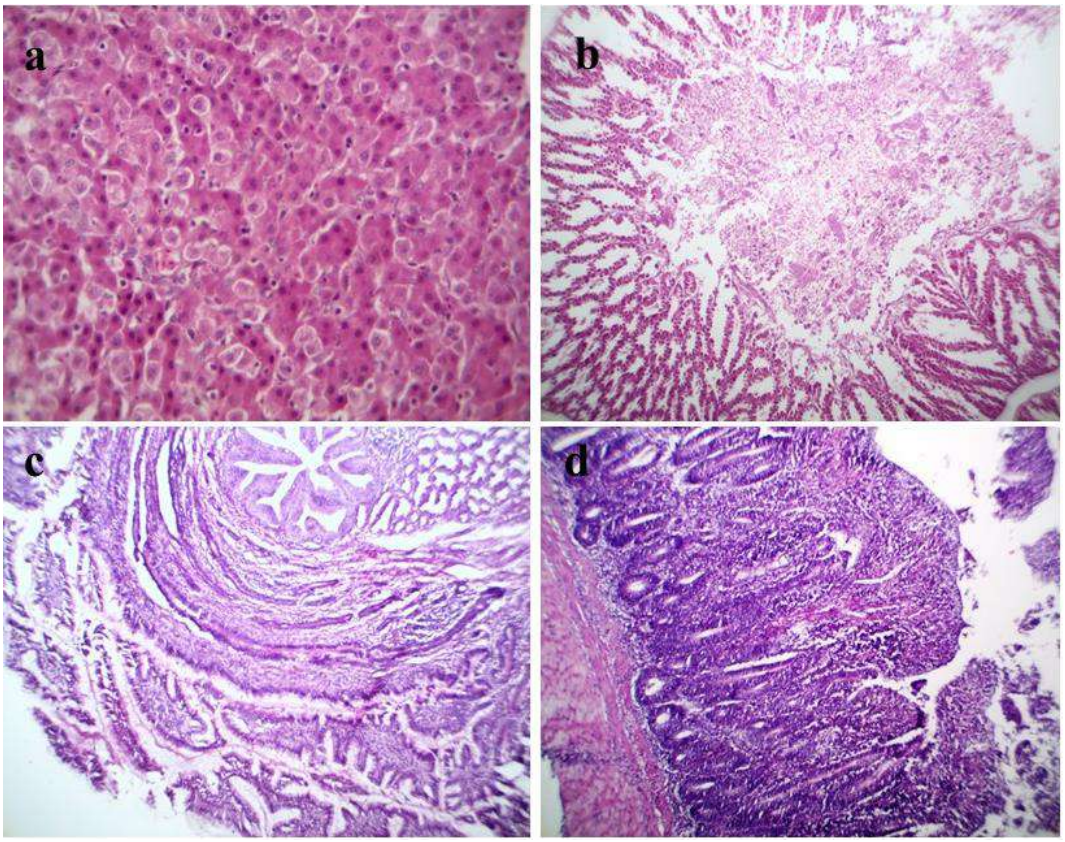

Figure 2: (a): photomicrograph of liver, group (1), 36 days PFOTA showing apoptosis intermingled with necrosis (H&E ×1200). (b): photomicrograph of the proventriculus, group (1), 28 days PF OTA showing necrotic debris inside the submucosal compound glands (H&E×1200). (c): photomicrograph of the proventriculus, group (1), 36 days PF OTA showing hyperplasia of the proventricular epithelium of the compound glands with arboreal growth from the mucosa (H&E ×300). (d): photomicrograph of intestine, group (1), 28 days PF OTA showing fusion of some intestinal villi tips and partial desquamation of villus enterocytes (H&E*300).

Necrohemorrhagic enteritis which was the main pathognomonic lesion encountered in the majority of examined tissue at that stage (Fig. 3-a). Hyaline degeneration of the muscular layer was also observed in some cases. The skeletal muscles, 28 days PF OTA showed intensive lymphocytic cells infiltration, partial hyalinization of muscle fibers with interstitial edema (Fig. 3-b). Brain, 28 days PF OTA showed vasogenic edema in Virchow rubin space (Fig. 3-d). Degeneration of some neurons with neuronophagia beside perineuronal edema were also observed in other cases in addition to proliferation of chroid plexus capillaries. At 36 days PF OTA, focal submeningeal lymphocytic cells aggregation was noticed (Fig.3-d) beside degeneration of some Purkinje cells of cerebellum.

Regarding to OTA residues, higher values were detected in liver than in kidney. Residues of OTA in liver and kidneys were higher than that in skeletal muscles. The mean amounts of OTA residues in liver, kidneys and skeletal muscles of broilers after 36 days were 422.635-287.224 and164.360 respectively.

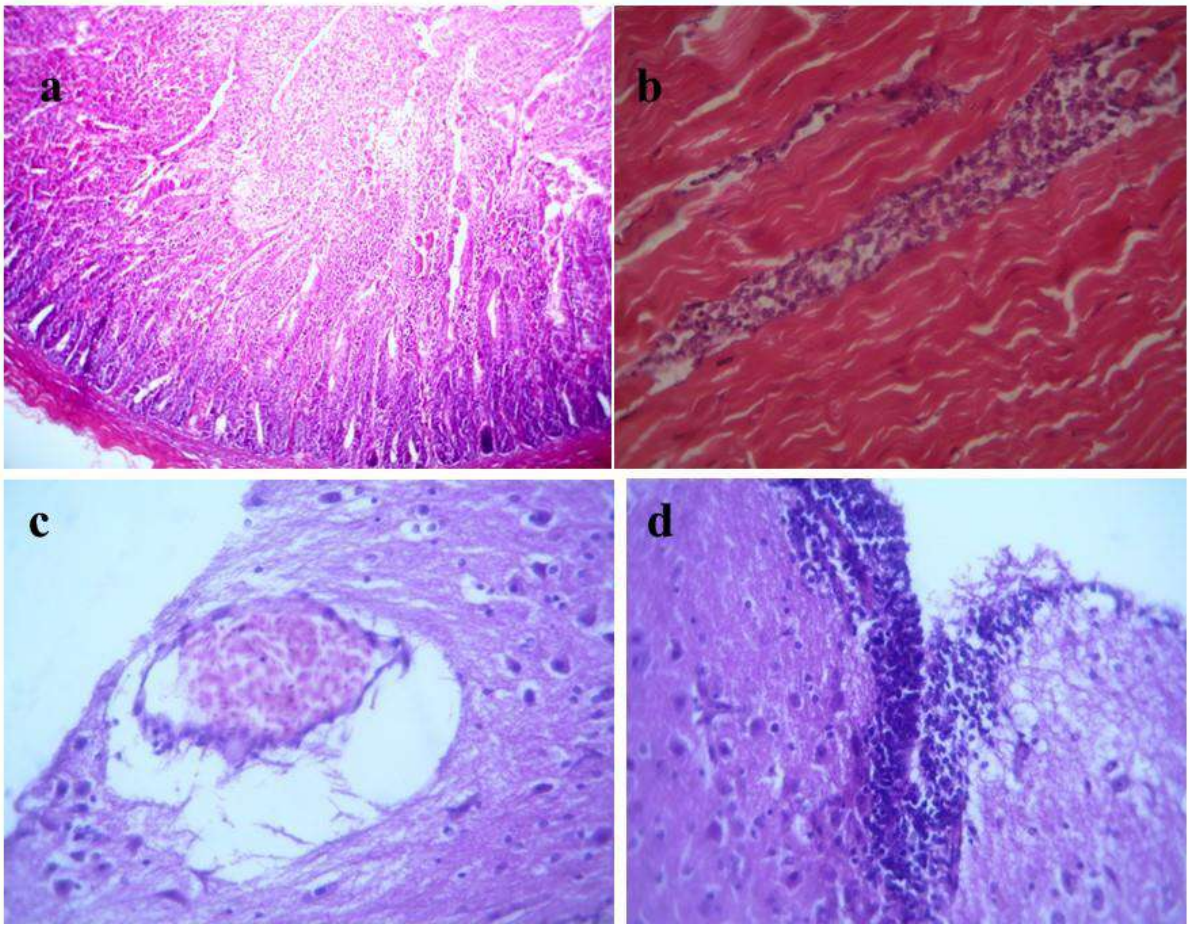

Figure 3: (a): photomicrograph of intestine, 36 days PF showing, necrohemorrhagic enteritis (H&E ×300). (b): photomicrograph of skeletal muscles, 28 days PF OTA, showing intense lymphocytic cells infiltration between muscle fibers with interstitial edema (H&E×1200). (c): photomicrograph of brain, 28 days PF OTA showing vasogenic edema in Virchow rubin space (H&E ×1200). (d): photomicrograph of brain, 36 days PF OTA showing focal submeningeal lymphocytic cells aggregation (H&E × 300).

Discussion

The current study results declared that avian mycotoxicosis (ochratoxicosis) is the most irritable problem facing poultry industry, as it causes, restlessness of the majority of the experimental chickens in group (1) followed by frequent degree of anorexia, diarrhea followed by emaciation, ruffled feather with paleness of comb and wattles. Later on these signs may be due to the toxic immunosuppressive effect of OTA which acting as stress factor [12]. Emaciation may be due to decrease in feed absorption from intestinal tract and lead to intestinal lesions [13].

Clinical signs forms in the intoxicated chickens may be attributed to the amount of consumed OTA contaminated diet and immunological status of each bird. These signs were in agreement with those obtained by other researchers [14,15,16] and these findings were in partial agreement with Zahoor *et al.* [17] who observed dullness, diarrhea, increased water intake, ruffled feathers, paleness of comb and wattles and decrease in egg mass in breeder hens fed on 0.1, 0.5, 1, 3, 5 and 10 mg OTA/kg feed, respectively.

Mortality rate in the same group was 20% that could be attributed to both direct and indirect toxic effect of OTA on chickens of group (1) as reported previously [15], Mortality rate in this experiment was less than that obtained formerly [18,19].

The pathological changes due to 100 ppb OTA/kg diet ochratoxin (OTA) were described

in this work after two weeks adaptation from day one till the end of the experiment.

The pathological findings revealed severe and variable renal lesions in experimental chicks as the kidneys have been considered as the key target organ of OTA toxicity [20]. The macroscopical lesions of kidneys were swelling and pale colored. These lesions were nearly similar to those obtained by Hameed *et al*. [21] who recorded in broiler chicks which fed 0.5 mg/kg OTA for 21 and 35 days where they showed enlargement, pale discoloration, friable consistency and hemorrhages in kidneys. Microscopically, 28 days PF OTA kidneys revealed focal hemorrhage replacing renal parenchyma and congestion of renal blood vessels, necrosis and degeneration of some renal tubules and lymphocytic cells aggregation with regenerative changes. Gout produced due to tubular epithelial necrosis, which inhibited normal renal uric acid excretion. Furthermore, OTA decrease concentration of protein, cholesterol, calcium, phosphorous and potassium followed by an increase in the level of uric acid and creatinine and a decrease in glomerular filtration [22]. OTA increase lipid peroxidation and decrease in activity of enzymatic and non enzymatic antioxidant in the kidneys which explain nephrotoxic changes in tubular epithelium [23] The enlargement of kidneys and liver of group (1) could be attributed to the involvement of these organs in detoxification and elimination. Ochratoxin inhibits protein synthesis, produced acute proximal tubular epithelial necrosis in kidneys and inhibits normal renal uric acid excretion followed by its deposition(Gout) [22]. Renal results in both 28 and 36 days PF were in partial agreement with those obtained in earlier studies [4,14,19,21,22,24]. While the liver revealed enlargement and congestion with oozing blood from its cut surface with distended gall bladder. These lesions could be attributed to the hepatotoxic effect of OTA. The previous gross lesions were in partial agreement with Abo El-Fetouh [19]. Microscopically, variable hepatic lesions with variable severity were noticed, 28 days PF as, intense portal heterophilic infiltration, diffuse interstitial hemorrhage and edema in the wall of portal vein. After 36 days PF mild congestion with perivascular leucocytic cell infiltration and dilated hepatic sinusoids with atrophied hepatic cords and focal lymphocytic cells aggregation were in partial agreement with other researchers [4,14 ,19]). The degenerative changes in liver and kidney may be due to the route of elimination of OTA through the kidneys and liver through hepatobiliary route of excretion of OTA and to direct toxic action of OTA on these cells [25]. Proventriculus, macroscopically 28 days PF revealed thickening of proventricular mucosa and necrosis. The later could be attributed to the direct exposure of these parts of the organ to the toxic OTA particles. These lesions were disagreed with those obtained by Belal [26] who noticed hemorrhage of proventricular mucosa, the differences could be attributed to differences in dose and time of exposure. Microscopically, in 28 days PF organs; proventriculus revealed focal lymphocytic cells aggregation inside the submucosal compound glands, necrotic debris in the lumen of proventricular gland. Mucosal edema with inflammatory cell infiltration, while hyperplasia of the proventricular epithelium and coagulative necrosis of compound glands and desquamation of the epithelial lining were noticed 36, days PF. The hyperplasia could explain the enlargement and increasing proventricular weight which was in as mentioned before [27, 28].

The previously mentioned lesions were in partial agreement with those obtained by Belal, [26]. Macroscopically, the intestine showed congestion of intestinal mucosa (28 days PF) and thickening of intestinal mucosa (36 days PF) that also attributed to the direct contact of contaminated to intestinal. Microscopically, 28 days PF intestine revealed fusion of some villi, villous hemorrhage and, necrohemorrhagic enteritis. The severity of this lesion was dose and time dependent and was differ than that obtained bsome authors [4,14,26,29,] who noticed catarrhal enteritis. Skeletal muscles macroscopically showed pale colored and petechial hemorrhage in thigh and breast muscles of some cases, comparable lesions were recorded [4,14,30] including, petechial hemorrhage on thigh muscle only. While, microscopically intense lymphocytic cells infiltration, partial hyalinization of the muscle fiber with interstitial edema were encountered, the aforementioned lesions could be attributed

to the toxic action of OTA particles residue in the muscle. Brain macroscopically was normal except in few cases that showed congestion of meningeal blood vessels. Microscopically, degenerated neurons, edema and meningeal lymphocytic aggregation beside degeneration of purkinje cells in cerebrum were the most common lesions which could be attributed to the neurotoxic effect of OTA particles. Paradells *et al*. [31] mentioned that OTA has been proven a potent neurotoxic effect. These previous lesions were in complete accordance with those obtained by Jamell [32].

Residues of OTA metabolites particles appeared in liver, kidney and muscle. Higher residues values were found in liver than kidney and finally muscle. This result disagrees with [33] who mentioned that higher residue was found in kidney than liver and muscle and agree with [34, 35] who reported higher residues in liver then kidneys.

Conclusion

It could be concluded that contaminated ration with OTA induced adverse effect on the experimental birds according to lesion and residues of toxins in different organs of experimental chickens.

Conflict of interest

The authors have no any conflict of interest.

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الملخص العربى

دراسات باثولوجيه تجريبيه على التسمم بالاوكراتوكسين بدجاج التسمين

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اجريت هذه الدراسه على عدد اربعون طائر تسمين هابرد عمر خمسه عشر يوما بعد فتره اسبوعين من التاقلم حيث قسمت الى مجموعتين الاولى ضابطه للتجربه دون تلقى اى معاملات يوميه خاصه بينما تم اضافه مركب الاوكراتوكسين بجرعه ١٠٠جزء فى البليون لكل كجم بالعليقه للمجموعه الثانيه وذلك لدراسه التغيرات الباثولوجيه والاعراض الاكلينيكيه المصاحبه هذا وقد تم ذبح الطيور بعد ٢٨ و٣٦ يوما من بدايه التجربه هذا وقد اظهرت نتائج التجربه بالمجموعه الثانيه بعض الاعراض السالبه كالخمول مع درجات متفاوته من عدم الاقبال على الطعام مع ظهور بعض حالات الاسهال يتبعه هزال وخشونه بالريش كما اظهرت التغيرات الباثولوجيه ارتشاح للخلايا الليمفاويه وخلايا والهتيروفيل مع وجود مناطق نزفيه وحدوث تنكرز بالانابيب الكلويه عند اليوم ٢٨ من بدايه التجربه بينما ظهر تلف وضمور لبعض هذه الانابيب مع زياده الطبقه الطلائيه المبطنه لها بينما ظهر الكبد عند اليوم ٢٨ من بدايه التجربه بنزيف بين نسيجى وتورم لجدار الوريد البابى وارتشاح لخلايا الهتيروفيل بينما ظهر تنكرز تخثرى بسيط عند اليوم ٣٦بينما ظهرت المعدة الهاضمة تحوى بقايا متنكرزه بتجويفها الغدى وذلك عند اليوم ٢٨ بينماحدث انقسامات عديده بالغشاء الطلائى المبطن للغدد المركبه فبدا وكانه تفرع شجيرى وذلك عند اليوم ٣٦هذا وقد اظهرت الامعاء التصاق لبعض الخملات عند اليوم ٢٨ والتهاب معوى نزفى تنكرزى عند اليوم ٣٦بينما بدت العضلات الهيكليه باضمحلال زجاجى مع ورم بين نسيجى عند اليوم ٢٨ وارتشاح مكثف لخلايا الليمفاويه بين الليفات العضليه واخيرا ظهر المخ بتورم وعائى بفضاء فيرشاو روبن عند اليوم ٢٨وارشاح محلى لخلايا الليمفاويه تحت الاغشيه السحائيه من بدايه التجربه كما اظهرت نتائج قياسات المتبقيات السميه لمركب الاوكراتوكسين زياده فى قيم هذه المتبقيات فى الكبد يليه الكلى ثم العضلات الهيكليه