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RESEARCH ARTICLE

Embryotoxic and Histopathological Investigations of In-Ovo Inoculation of Aflatoxigenic Fungal Extracts in Chicken Embryos

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A B S T R A C T

The present study was executed to investigate the embryotoxic effect of *in-ovo* inoculation of aflatoxigenic fungal extracts isolated from poultry feed and its ingredients. A total of 12 aflatoxigenic fungal isolates were randomly selected for determination of their embryo toxicity potential. The extract of each fungal isolate containing 10 and 100 ng/egg aflatoxin (AF), from each isolate were separately injected through chorioallantoic membrane (CAM) route into 12 embryos (96 hours old). The parameters studied included embryo mortality, hatchability, body weight, relative organ weights, gross and histopathological lesions. A variable degree of mortality and hatchability was observed in embryos administered same dose of AF produced by different isolates. However, calculations based upon the concentration of AFB1 in each dose revealed high mortality in embryos receiving higher amount of AFB1 compared with those receiving lower amount of AFB1. Embryonic mortality was 25.00 and 66.67% in embryos which received 0.00 and 9.83 ng/egg AFB1, respectively. In one case, however, 16.67% mortality was present in embryos which received 2.19 ng/egg AFB1. Similarly in AF 100 group, embryonic mortality reached 100% in embryos receiving above 90 ng/egg AFB1. For eggs receiving AFB1 between 78.83 and 84.64 ng/egg, morality was 50 and 83.3%, respectively. All the treatment groups had significantly higher relative weight of liver and kidney than control. Fatty change and individual cell necrosis were significantly higher in AF-100 compared with AF-10 group. In kidneys, congestion and tubular necrosis were significantly higher in AF-100 than that in AF-10 group. Microscopically, liver and kidneys showed severe changes in chicks produced from eggs of high dose groups compared with those of low dose groups suggesting a dose related response. In conclusion inoculation of aflatoxigenic fungal extracts leads to high embryonic mortality and embryonic toxicity.

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INTRODUCTION

Mycotoxins, the secondary fungal metabolites, are produced by several species of toxigenic fungi, belonging mainly to genus Aspergillus and Penicillium. The widespread occurrence of mycotoxin producing fungi and their ability to grow on the variety of economically important cereal crops have rendered them as unavoidable contaminants of animal and human food and feeds (Hassan *et al*., 2011). In the long list of more than 300 known mycotoxins, the most important for human and animal health are ochratoxins, aflatoxins, T-2 toxin, zearalenone, fumonisins, and deoxynivalenol (Devegowda *et al*., 1998; Binder *et al*., 2007).

Aflatoxins (AF) are a group of mycotoxins, which are classified aflatoxin B_1 (AFB_1), B_2, G_1 and G_2, depending on the nature of fluorescence they emit under UV light. Other metabolic derivatives include M_1 and M_2 (found in milk) and B_{2A}, G_{2A}, Q_1 and P_1 (Santini and Ritieni, 2013). These mycotoxins are mainly produced by toxigenic

strains of *Aspergillus* species, especially *Asp. flavus* and *Asp. parasiticus*.

In general, for the growth of fungi, high water activity, humidity and temperature (27-38°C) are needed. For AFs production by *Aspergillus*, environmental temperature of 33°C and water activity of 0.99 are considered as optimum conditions (Milani, 2013). These growth conditions are often available in Pakistan at various stages of cereals production and storage (Saleemi *et al*., 2010, 2012; Ahmad *et al*., 2012). The toxicities of AFB1 (most toxic among AFs) range from mild digestive illness to carcinogenesis. Based on experimental findings, International Agency for Research on Cancer (IARC) has placed this toxin as Group 1 human carcinogen. It is also considered as potent hepatotoxic, immunosuppressive, mutagenic, teratogenic and nephrotoxic agent (Hassan *et al*., 2012a; Khan *et al*., 2014a).

The presence of AFB1 in poultry feed and its ingredients originating from Pakistan has widely been reported. Ahsan *et al*. (2010) recorded aflatoxin contamination up to 62 µg/kg 85% maize samples. In another study, Shah *et al*. (2010) determined AFB1 (up to 30.92 µg/kg) and ochratoxin A (up to 7.32 µg/kg) in 83.33% and 77.78% in maize kernel samples respectively. Similarly, Khatoon *et al*. (2012) detected 14 mycotoxins, including AFs, ochratoxin and zearalenone in maize collected from 7 growing regions of Pakistan. Aflatoxins have been detected in other poultry feed ingredients, like rice (Hussain *et al*., 2011) and wheat (Iqbal *et al*., 2014).

In poultry industry aflatoxicosis is considered an important issue by virtue of its occurrence and toxicities. The transfer of AFs in poultry products (eggs and meat) not only poses the consumers at risk, but it also limits the trade of the country. In plasma, ~99% of the mycotoxins are bound to proteins, and a small proportion is free (Bow *et al*., 2006). This conjugation process takes place in liver and these proteins (with bound mycotoxins) are incorporated in the developing eggs. In this way, a reasonable quantity of AFB_1 is detected in eggs produced by hens kept on AF contaminated feed (Qureshi *et al*., 1998, Hassan *et al*., 2012b, Khan *et al*., 2014b). This residual transfer of AFB1, in the eggs leads to adverse effects in the developing embryos and hatching chicks (Hassan *et al*., 2012a).

The occurrence of toxigenic mycobiota, mycotoxins in poultry feed and its ingredients and their toxicological effects in the developing embryos and hatching eggs has led us to evaluate the toxicological potential of local isolates of fungi recovered from the poultry feed ingredients. Thus, the present study aimed at determining any toxicological consequences of fungal extracts, isolated from poultry feed and its ingredients, on the chicken embryos.

MATERIALS AND METHODS

Experimental design: Embryotoxicity of aflatoxigenic fungal extracts of isolates of Aspergillus fungi was evaluated through chick embryotoxicity screening test (Vesely *et al.*, 1983; Stoloff and Scott, 1984) using total of 12 aflatoxigenic fungal isolates. The extract of each fungal isolate containing known quantities of aflatoxins was dried under nitrogen stream in two glass vials and then dissolved in 30% ethanol to provide two dose levels of 10 and 100 ng/ 20µl aflatoxin. Two dose levels, 10 and 100 ng/egg AF, from each isolate were separately injected through chorioallantoic membrane (CAM) route into 12 embryos (96 hours old) of White Leghorn layer breeder hens (Hisex White) (Stoloff and Scott, 1984). Each group of inoculated embryos was marked by the identification number of the isolate. Before inoculation, external surface of each egg was disinfected with a swab of ethanol. Air cell boundary was marked on the egg shell surface with a lead pencil. A hole was made in the air cell membrane using standard widow opening technique of Vesela *et al*. (1983). Aflatoxigenic fungal extracts having known quantity of aflatoxin were injected, eggs were immediately sealed with melted paraffin wax and placed in a chick incubator at 37°C with 65% relative humidity.

Three controls including simple control, shamed control and 30% ethanol (Celik *et al.*, 2000) were also run in parallel. Following inoculation the candling of the eggs was performed at 48 hours (early), 168 hours (middle) and 288 hours (late). Dead embryos were removed and further examined for gross and microscopic lesions.

Mortality: Embryos died during the incubation period were noted and percentage mortality was calculated.

Body weights and relative organ weights: Body weight of each embryo was determined on hatching day, similarly visceral organs including liver, heart, and kidney were weighed and their relative weights were calculated as a function of body weight {(organ weight/bodyweight) X100}

Gross pathology and histopathology: Chicks hatched at completion of incubation period (21 days) were subjected to necropsy for the presence of gross lesions on different organs. Tissue samples of morbid organs were fixed in 10% neutral buffered formalin and processed for histo-pathological studies. Frozen sections of fixed liver tissue were also stained with Sudan Black B to confirm the presence of fats in the cytoplasmic vacuoles.

Histopathological lesion scoring: During the microscopic examination of slides of different groups, each histopathological lesion was subjectively assigned a score from 0-3, depending upon the absence, presence and severity. Score values given for a particular lesion in liver or kidney tissues of all birds examined were added to obtain an individual lesion score which in turn was divided by total number of sections examined to obtain a mean of score for liver and kidney of an experimental group.

Statistical analysis: Different parameters of experimental groups were compared by "t" test. A statistical software MSTATC® was used for statistical analysis. The mortality in different groups was determined as total number and percentage mortality of each group.

RESULTS

Chick Embryotoxicity screening test (CHEST)

Mortality: Mean values for mortality and hatchability of 96 hours old chick embryos have been presented in Table

1. Mortality was lowest and hatchability was highest in control embryos. A variable degree of mortality and hatchability was observed in embryos administered same dose of AF produced by different isolates. However, calculations based upon the concentration of AFB1 in each dose revealed a high mortality in embryos receiving higher dose of AFB1 compared with those receiving lower dose of AFB1. Embryonic mortality was 25.00 and 66.67% in embryos which received 0.00 and 9.83 ng/egg AFB1, respectively. In one case, however, 16.67% mortality was present in embryos which received 2.19 ng/egg AFB1.

Mortality and hatchability of chick embryos administered 100 ng/egg aflatoxin has been presented in Table 2. Similar pattern of mortality and hatchability was observed as in AF 10. Mortality was lowest and hatchability was highest in embryos kept as controls. However, this pattern was not constant and some embryo groups which received low amount of AFB1 showed a high mortality and vice versa. Embryo mortality reached 100 percent in embryos receiving above 90 ng/egg AFB1. While in another group administration of 64 ng/egg AFB1 resulted in 100 percent embryonic mortality. Eggs receiving AFB_1 78.83 to 84.64 ng/egg showed morality varying from 50 to 83.30%.

Relative organ weights of chicks: Body weight and relative organ weights of chicks hatched from the embryos administered 10 and 100 ng/egg AF have been presented in Tables 3 and 4. All the treatment groups had significantly lower bodyweights as compared with control. Birds in sham and vehicle treated control groups did not differ from the control group in terms of relative bodyweights of different organs. All the treatment groups had significantly higher relative weight of liver than control. In AF 10 relative weight of heart in treatment groups 59, 171, 181, 219, 225, 281, 283, 292, 381 and 400 was significantly higher than that of control group. Relative weight of kidneys of all the treatment groups was significantly higher than that of control chicks. Groups including AF 10, 28, 171, 219, 225, 283 and 292 had significantly lower relative weight of bursa while all other groups had non-significantly different bursa weight compared with control.

The relative organ weights of chicks hatched from the embryos administered 100 ng/egg AF have been presented in Table 4. All the groups had non-significantly different relative heart weight from control except AF 10 which showed significantly higher heart weight. Relative weight of kidneys and bursa of Fabricius in all treatment groups was significantly higher than control birds

Gross lesions: Grossly liver was more swollen and hemorrhagic in appearance in the groups administered AF100 ng/egg in comparison with low dose AF 10ng/egg, where these changes were less in severity. Hemorrhages were seen on epicardium in few groups. Intestinal mucosa was congested and thick with excessive exudates. The kidneys were swollen, congested and bulging out from bony socket. Such changes were not seen in all three control groups. No gross changes were observed in bursa of Fabricius. Similarly no changes were observed in spleen.

Histopathology: In AF 100 groups, hepatocytes showed one to multiple vacuoles in their cytoplasm. Sinusoidal spaces were not prominent due to cell swelling (Fig. 1). Cellular infiltration around the blood vessels was present at few places. Congestion and necrotic foci in the parenchyma, individual cell necrosis, inflammatory cells and connective tissue proliferation was present. Vacuolar degeneration was confirmed as fatty changes with Sudan Black B staining (Fig. 2). In kidneys, areas of necrosis of epithelial cells of proximal convoluted tubules (Tubular necrosis) and congestion of the parenchyma was consistent feature (Fig. 3). Similar changes at low intensity were observed in AF 10 group.

Histopathological lesion scoring: Histopathological scoring of liver and kidney tissues in chicks of AF-10 and AF-100 groups has been presented in Table 5. Fatty change and individual cell necrosis in liver were significantly higher in AF 100 compared with AF 10 group. Other microscopic changes such as congestion, dilated sinusoidal spaces, and cell swelling and cellular infiltrations did not differ between two groups. In kidneys congestion and tubular necrosis was significantly higher in AF 100 than that in AF 10 group. However the difference in glomerulopathy and cellular infiltration between both the groups was not significant.

Table 1: Mortality and hatchability of 4 days-old chick embryos administered aflatoxin (10 ng/egg) produced by fungi isolated from different poultry feeds and feed ingredients

Sample ID	AFB1 ng/egg	Mortality number (%)					Hatchability
		48 hrs. PI.	168 hrs. PI.	288 hrs. PI.	504 hrs. PI.	Cumulative	No (%)
Control	0.00	0(00.0)	0(00.0)	0(00.0)	2(16.7)	2(16.7)	10(83.3)
Shamed Control	0.00	2(16.7)	0(00.0)	0(00.0)	0(00.0	2(16.7)	10(83.3)
Vehicle control	0.00	2(16.7)	0(00.0)	0(00.0)	1(8.3)	3(25.0)	9(75.0)
Positive control (10 ng/egg AF)	10.00	2(16.7)	2(16.7)	0(00.0)	2(16.7)	6(50.0)	6(50.0)
28	9.73	2(16.7)	3(25.0)	0(00.0)	2(16.7)	7(58.3)	5(41.7)
59	7.88	2(16.7)	0(00.0)	2(16.7)	0(00.0)	4(33.3)	8(66.7)
171	8.20	2(16.7)	0(00.0)	0(00.0)	2(16.7)	6(50.0)	6(50.0)
181	8.20	2(16.7)	0(00.0)	0(00.0)	2(16.7)	4(33.3)	8(66.7)
190	9.76	4(33.3)	0(00.0)	0(00.0)	2(16.7)	6(50.0)	6(50.0)
219	8.46	2(16.7)	0(00.0)	1(8.3)	0(00.0)	3(25.0)	9(75.0)
225	9.90	4(33.3)	0(00.0)	0(00.0)	2(16.7)	6(50.0)	6(50.0)
281	2.19	0(00.0)	0(00.0)	2(16.7)	0(00.0)	2(16.7)	10(83.3)
283	9.83	2(16.7)	0(00.0)	2(16.7)	4(33.3)	8(66.7)	4(33.3)
292	8.25	2(16.7)	2(16.7)	0(00.0)	0(00.0)	4(33.3)	8(66.7)
381	4.14	2(16.7)	0(00.0)	0(00.0)	1(8.3)	3(25.0)	9(75.0)
400	8.35	2(16.7)	0(00.0)	2(16.7)	0(00.0)	4(33.3)	8(66.7)

Table 2: Mortality and hatchability of 4 days-old chick embryos administered aflatoxin (100 ng/egg) produced by fungi isolated from different poultry feeds and feed ingredients

Sample ID	AFB1 ng/egg	Mortality number (%)					Hatchability
		48 hrs. Pl.	168 hrs. Pl.	288 hrs. Pl.	504 hrs. Pl.	Cumulative	No (%)
Control	00.00	0(00.0)	0(00.0)	0(00.0)	2(16.7)	2(16.7)	10(83.3)
Shamed Control	00.00	2(16.7)	0(00.0)	0(00.0)	0(00.0)	2(16.7)	10(83.3)
Vehicle control	00.00	2(16.7)	0(00.0)	0(00.0)	1(8.3)	3(25.0)	9(75.0)
Positive control (100 ng/egg AF)	100.00	6(50.0)	2(16.7)	2(16.7)	0(00.0)	10(83.3)	2(16.7)
28	97.30	10(83.3)	0(00.0)	0(00.0)	2(16.7)	12(100.0)	0(00.0)
59	78.83	4(33.3)	4(33.3)	0(00.0)	0(00.0)	8(66.7)	4(33.3)
171	82.04	4(33.3)	0(00.0)	4(33.3)	2(16.7)	10(83.3)	2(16.7)
181	82.05	6(50.0)	0(00.0)	0(00.0)	2(16.7)	8(66.7)	4(33.3)
190	97.62	10(83.3)	0(00.0)	0(00.0)	2(16.7)	12(100.0)	0(00.0)
219	84.64	4(33.3)	0(00.0)	2(16.7)	0(00.0)	6(50.0)	6(50.0)
225	99.039	6(50.0)	0(00.0)	2(16.7)	2(16.7)	10(83.3)	2(16.7)
281	21.95	3(25.0)	0(00.0)	0(00.0)	0(00.0)	3(25.0)	9(75.0)
283	98.33	8(66.7)	2(16.7)	2(16.7)	0(00.0)	12(100.0)	0(00.0)
292	82.50	2(16.7)	2(16.7)	1(8.3)	2(16.7)	7(58.3)	5(41.7)
381	41.40	2(16.7)	2(16.7)	1(8.3)	0(00.0)	5(41.7)	7(58.3)
400	83.49	4(33.3)	2(16.7)	0(00.0)	0(00.0)	6(50.0)	6(50.0)

Table 3: Bodyweights and relative organ weights of day-old chicks hatched from embryos administered aflatoxin (10 ng/egg) in chorioallantoic membrane

Group	Bodyweights (gm)	Relative organ weights (% of body weight)			
		Liver	Heart	Kidney	Bursa
Control	40.91±1.50	2.86±0.051	0.68±0.025	0.95±0.029	0.32±0.015
Shamed Control	40.43±1.78	2.87±0.058	0.70±0.020	0.97±0.024	0.32±0.010
Vehicle control	40.88±1.55	2.87±0.064	0.71±0.021	0.96±0.021	0.31±0.013
Positive control (10 ng/egg AF)	38.00±0.70*	3.20±0.044*	0.72±0.012*	1.05±0.015*	0.30±0.013*
28	37.62±0.95*	3.18±0.063*	0.70±0.020	1.05±0.016*	0.30±0.013*
59	38.02±0.33*	3.06±0.023*	0.72±0.025*	1.04±0.027*	0.33±0.015
171	37.85±0.95*	3.17±0.035*	0.71±0.017*	1.02±0.021*	0.30±0.017*
181	37.39±0.97*	3.19±0.045*	0.72±0.018*	1.01±0.031*	0.31±0.014
190	37.71±0.79*	3.17±0.046*	0.71±0.019	1.04±0.021*	0.31±0.018
219	37.98±0.51*	3.14±0.028*	0.72±0.020*	1.03±0.017*	0.29±0.018*
225	38.02±0.83*	3.16±0.049*	0.72±0.014*	1.01±0.025*	0.30±0.016*
281	37.64±0.51*	3.09±0.040*	0.73±0.022*	1.01±0.024*	0.33±0.015
283	38.05±0.96*	3.17±0.044*	0.70±0.030*	1.05±0.028*	0.30±0.010*
292	38.07±0.92*	3.15±0.055*	0.72±0.022*	1.04±0.037*	0.30±0.017*
381	38.00±0.92*	3.06±0.057*	0.73±0.024*	1.03±0.025*	0.34±0.020
400	37.91±0.66*	3.16±0.036*	0.72±0.022*	1.05±0.036	0.31±0.022

Values (Mean±SD) bearing asterisk in a column differ significantly (P≤0.05) than control.

Table 4: Bodyweights and Relative organ weights (Mean±SD) of day-old chicks hatched from embryos administered aflatoxin (100 ng/egg) in chorioallantoic membrane

Group	Bodyweights (g) 100	Relative organ weights (% of BW)			
		Liver	Heart	Kidney	Bursa
Control	40.91±1.50	2.86±0.051	0.68±0.025	0.95±0.029	0.32±0.015
Shamed Control	40.43±1.78	2.87±0.058	0.70±0.020	0.97±0.024	0.32±0.010
Vehicle control	40.88±1.55	2.87±0.064	0.71±0.021	0.96±0.021	0.31±0.013
Positive control (100 ng/egg AF)	37.85±1.34*	3.17±0.038*	0.73±0.007*	1.08±0.001*	0.32±0.007*
59	36.8±0.56*	3.15±0.053*	0.71±0.025	1.13±0.006*	0.32±0.009*
171	37.05±0.21*	3.21±0.020*	0.70±0.004	1.07±0.013*	0.31±0.017*
181	37.18±1.41*	3.24±0.083*	0.71±0.011	1.08±0.025*	0.32±0.030*
219	37.73±0.9*	3.19±0.019*	0.70±0.018	1.05±0.024*	0.32±0.015*
225	36.56±0.78*	3.27±0.011*	0.70±0.004	1.11±0.004*	0.31±0.013*
281	38.3±0.57 *	3.11±0.032*	0.71±0.017	1.04±0.026*	0.31±0.016*
292	37.94±1.42*	3.24±0.048*	0.71±0.016	1.08±0.02*	0.32±0.032*
381	37.56±0.59*	3.23±0.038*	0.70±0.11	1.08±0.022*	0.34±0.016
400	37.52±0.58*	3.22±0.025*	0.71±0.012	1.07±0.02*	0.31±0.013*

*Significantly different from control in each column (P≤ 0.05).

DISCUSSION

Embryonic mortality: Aflatoxin induced embryonic mortalities can be categorized as: i) early embryonic deaths usually due to direct cytotoxic effects (Celik *et al*., 2000) of high dose of toxins and ii) late deaths, which are associated with AF metabolism, in the embryonic liver, leading to generation of highly toxic metabolites. According to Van Vleet *et al*. (2006) AFB_1 is metabolized to more toxic form AFB1-2,3 epoxy by cytochrome P450 dependent mixed function oxidase in the presence of molecular oxygen and NADPH. For this mechanism of embryonic mortality, liver of the exposed embryos must be functional at the time of exposure, which happens at the later stages (day 5 to 6) of development. In the present study, AFB_1 fungal extract was inoculated at day 4 of incubation, so the embryonic mortalities seems to be due to metabolic conversion of AFB_1 to AFB_1-2,3 epoxy. The pattern and intensity of mortalities of isolates varied widely and ranged from 16.7 to 66.7% in the eggs treated with 10 ng AFB1/egg. However, Celik *et al*. (2000) recorded 74.5% mortality in chick embryos administered 10 ng/egg AFB_1 prior to start of incubation. Considering AFB_1 level (ng/egg) a trend of higher embryonic mortality

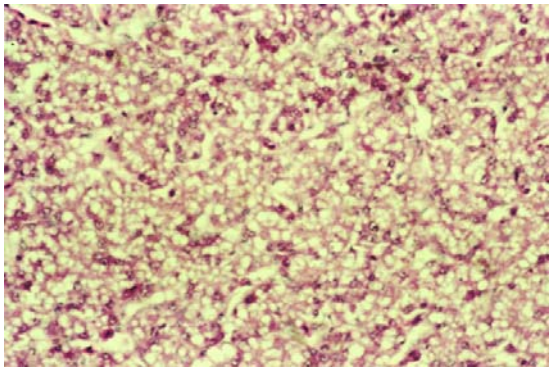

Fig. 1: Photomicrograph of liver of chicks hatched from embryos treated/inoculated with aflatoxigenic fungal extracts no 190 (97.62ng/g AFB1) showing vacuoles in the cytoplasm of hepatocytes. (H & E; 200X).

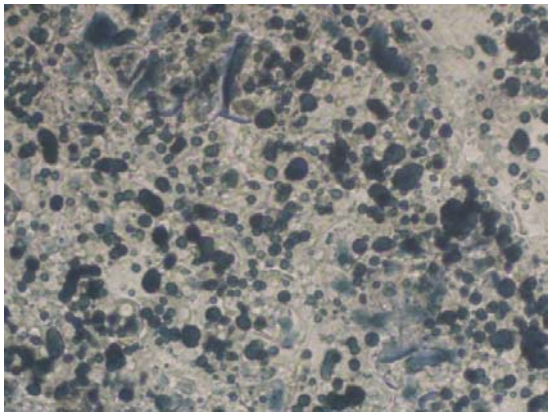

Fig. 2: Sudan Black staining of liver tissue of chicks (Aflatoxin administered group) for the confirmation of fatty change, dark blue color vacuoles are visible in microphotograph. (Sudan Staining; 400X).

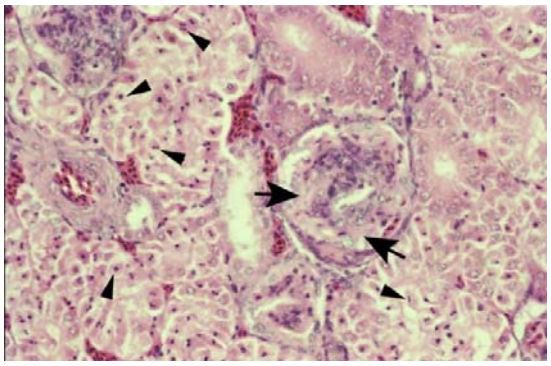

Fig. 3: Photomicrograph of kidneys of chicks hatched from embryos inoculated with aflatoxigenic (100 ng/egg) fungal extracts showing pyknotic nuclei in tubular epithelial cells and congestion (arrow heads) in renal parenchyma (H & E; 400X).

was observed with higher AFB_1 level. Essentially similar pattern was observed when 100 ng/egg AF was administered in embryos from the same isolates. However, the mortality values increased to 100% with higher AFB_1 levels, suggesting a dose related effect. Increase in mortality with increase in AFB_1 dose levels also reflected that AFB_1 was a major factor in embryotoxicity of aflatoxigenic fungi extracts. Khan *et al.* (2014) also ranked AFB_1 as most toxic aflatoxin for embryotoxicity and embryo lethality. Embryonic mortality reported by Celik *et al*. (2000) following administration of 10 ng/g AFB_1 was higher (75.5%) than that observed in the present study (up to 66.7%), this difference might be due to the age of embryo at the time of exposure as Celik *et al*. (2000) inoculated AFB_1 prior to incubation and while did it at day 4 of incubation. It also suggests that an age related development of resistance to AFB_1. This hypothesis is supported by Neldon-Ortiz and Qureshi (1992) and Celik *et al.* (2000), who reported that chick embryos were more sensitive to AFB_1 at day 1 than at day 7 of age. Age related increase in the resistance of embryos to AFB_1 was linked to the activation of detoxification mechanism at day 5-6, by the time liver and kidney are functional (Khan *et al.*, 2014b).

Table 5: Histopathological lesions scoring (Mean±SD) of different tissues of chicks hatched from eggs injected with extracts of different aflatoxigenic fungi

Organ/ Tissue	Conditions	AF-10 (10 ng/egg)	AF-100 (100 ng/egg)
Liver	Fatty change	1.833±0.718	2.727±0.467*
	Congestion	0.25 0±0.452	0.273±0.467
	Dilated sinusoidal spaces	0.167±0.389	0.454±0.820
	Cell swelling	0.333±0.492	0.909±1.044
	Cellular infiltration	0.083±0.289	0.272±0.467
	Individual cell necrosis	0.750±0.622	1.636±0.504*
Kidney	Congestion	1.000±0.426	1.727±0.410*
	Tubular necrosis	1.083±0.469	1.818±0.404*
	Glomerulopathy	0.667±0.492	1.182±0.845
	Cellular infiltration	0.167±0.389	0.182±0.404

*Significant difference in a row: AF-10 vs AF100 (P≤0.05)

Effect of aflatoxins on egg hatchability: In present the study all the chicks hatched out from AF administered eggs showed significantly lower bodyweight and higher relative weight of liver than chicks hatched from untreated eggs. Kidney weight of all the groups was non-significantly different in AF 10 group but was significantly higher in AF 100 group compared with control. Similar decrease in bodyweight has been reported in rat embryos from the dams treated with AFB_1 alone or in combination with OTA (Wangikar *et al.*, 2004). Growth retardation following AF exposure has been reported in chick (Khan *et al.*, 2014a) and rodent embryos (Roll *et al.*, 1990). The decrease in the body weight of the chicks hatched from the eggs inoculated with AFB_1 might be due to hepatotoxic effects of the toxin. The decrease in protein synthesis and increase in the maker enzymes of hepatotoxicity, i.e., ALT, AST upon natural and experimental exposure to AF have widely been reported (Sridhar *et al*., 2014). The altered liver function affects the protein metabolism and health of the individual, which lead to decrease in the weight gain. There is an increase in the accumulation of inflammatory cells in the organs, leading to cell swelling and increased organ weight. These changes have been proved at least in part, by the presence of inflammatory cells in the histological sections of liver and kidneys.

Pathology: Severe histological changes in the kidneys and liver of the chicks hatched from the AFB1 contaminated eggs were observed. Liver is potentially target organ of the AFB1 while kidneys, as in case with other mycotoxins like OTA are major excretory organ. As major component of AFB_1, is bound to plasma proteins, it can't pass through with glomerular filtration. This bound

AFB_1 is only execrated via organic Anion Transporter (OAT) route, which prones the proximal tubular epithelial cells to damage through depletion of indigenous dicarboxylic acid (glutarate, ketoglutarate) on expense of AFB_1 internalization (Sekine *et al*., 2006). Although several other families of OAT including recently discovered organic anion-transporting polypeptides (OATPs), oligopeptide transporters (PEPTs), and ATP-binding cassette (ABC) transporters, such as MRP2 and BCRP are involved in the molecular transport, but all these transporter families play a significant role in the development of AFB_1-mediated nephrotoxicity (Anzai *et al*., 2010).

In published accessible literature, no work regarding the histological profile of the chicks, hatched from the AFB_1 intoxicated eggs can be found. However, similar lesions have been reported in young broiler chicks experimentally intoxicated with aflatoxins (Hussain *et al*., 2008). This comparison suggests that the lesions are similar in chicks exposed to AFB_1, either during postnatal life or embryonic stage. It also suggested that the mechanism of toxicity in chick embryos might be similar to that of broiler chicks in postnatal life. A comparison of the microscopic lesion scores in liver and kidneys revealed more severe changes in chicks produced in high dose groups compared with those of low dose groups suggesting a dose related response.

The induction of pathological lesion in the chick embryos and hatched chicks, by the extracts of local isolates of fungi harvested from the poultry feed ingredients seems to be the first report of its nature in Pakistan. Also pathological alterations in liver and kidneys of chicks hatched from AF inoculated embryos have never been reported earlier. Moreover there is no report of quantitative or semi quantitative comparison of histopathological alterations in these organs. The findings of the present study highlight the need for screening of the local fungal isolates for their toxigenic profile both at cellular and subcellular levels.

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