EXPERIMENTALLY INDUCED CHRONIC AFLATOXICOSIS IN LAYING CHICKS

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المستخلص

اجريت هذه التجربة لدراسة ووصف الاثار السريرية المرضية للتسمم المزمن التجريبي بمادة الافلاتوكسين في كتاكيت البياض من سلالة بوفان و لتقييم فعالية مضادات السموم الفطرية والثوم في تقليل الاثار السمية لمادة الافلاتوكسين لاول مرة بالمختبر في السودان.

استخدم في هذه الدراسة خمس مجموعات ، كل مجموعة مكونة من عشرين كتكوت عمر يوم . المجموعة (أ) اعطيت عليقة تحتوي علي ٩٠ جزء للبليون من مادة الافلاتوكسين ، المجموعة (ب) غذيت على عليقة تحتوي علي ٩٠ جزء للبليون من مادة الافلاتوكسين و ١٠ ملجرام من الثوم المسحون لكل ملليتر من المادة يوميا ، المجموعة (ج) اعطيت عليقة تحتوي علي ٩٠ جزء للبليون من مادة الافلاتوكسين مخلوط بمادة هيدريتد صوديم كالسيوم الامينوسيليكين (هسكس) بتركيز ١ ملجرام (هسكس) لكل كيلوجرام من العليقة ، المجموعة (د) تم اعطاؤها عليقة تحتوي علي ١٠٣ جزء للبليون من مادة الافلاتوكسين ، بينما المجموعة (و)تم اعطاؤها عليقة تحتوي علي ٢٤.٦ جزء للبليون من مادة الافلاتوكسين وتم معاملتها كمجموعة ضابطة.

كل المجموعات كانت توزن اسبوعيا طوال فترة التجربة (١٠٠ يوم).

اظهرت المجموعة (ا)و(ب)و(ج)و(د)هزال ونقصان في الوزن. اما الخمول والترنج و شلل الارجل والاجنحة وصعوبة التنفس والاستلقاء التام لوحظت في

المجموعات (أ)و(د). كانت عينات كبد المجموعات (أ) و(ب) و(د) شاحبة وهشة ومحتقنة وفيها نزيف. كما لوحظ التنكس المائي، ارتشاح الخلايا البيضاء خاصة الليمفاوية ،زيادة نسيج بالقنوات الصفراء ،تجمع العقيدات الليمفاوية، نزف متني، نزف تحت المحفظة ، توسع الوريد البابي ، ارتشاح الخلايا البيضاء في المنطقة البابية ونخر في المجوعات (أ)و(د) ، اما انتشار خلايا كوفر لوحظ في المجموعات (ب)و(ج). لوحظ تليف حول الوريد البابي في المجموعة (د) فقط.

المجموعة (د) اظهرت اعلي نسبة ايجابية لتركيز الافلاتوكسين في الكبد (٥٨.٨٢٤%) ، بينما المجموعة (أ) كانت (٢٣.٥٢٩%) ، المجموعة (ب) كانت (١٧.٦٤٧%) ، المجموعة (ج) كانت (٥.٨٨٢%) ، المجموعة (و) لم تظهر وجود لمادة الافلاتوكسين (٠%). هذه الدراسة اوضحت عدم فعالية الهسكس التجاري في تقليل الاثار السامة لمادة الافلاتوكسين كما اظهرت ايضا ان الثوم بجرعة ١٠ ملجرام/مل يوميا لا تقي من فقدان الوزن الحي الناتج عن التسمم بمادة الافلاتوكسين.

المجموعة (د) اوضحت اختلافا واضحا عن المجموعة الضابطة لكن في العموم كل المجموعات اظهرت قيم متوسطة اعلي من المجموعة الضابطة ، وان كل المجموعاتاختلفت تختلف اختلافا واضحا من بعضها البعض ٍ

Abstract

This study was designed to examine and describe the clinicopathological aspects of experimentally-induced chronic aflatoxicosis in laying one-day-old-Bovan chicks and to assess the efficacy of antimycotoxins and garlic (*Allium sativum*) in amelioration of its toxic effects. Five groups (A, B, C, D and E) of 20 one-day-old chicks were used in this experiment. Group A received a ration containing 90 ppb aflatoxins, group B received the same ration plus 10 mg ground garlic/ml daily, group C received the same ration plus Hydrated Sodium Calcium Alaminosilicate (HSCAS) at 1gm/kg, group D received a ration with 103 ppb aflatoxins, group E received a ration with 24.6 ppb aflatoxins and served as control. The experiment continued for 100 days,

during which, the chicks were weighed weekly. At the end of this priod the chicks were killed for further investigations. Chicks in group A, B, C and D showed emaciation and pronounce loss of weight. Dullness, ataxia, paralysis of the legs and wings, gasping and complete recumbence were evident in group A and D. At necropsy, the livers of group A, B and D were fragile, pale with areas of haemorrhages and congestion. Histopathologically, group A and D displayed hepatocytes hydropic degeneration, infiltration of mononuclear cells predominantly lymphocytes which occasionally formed lymphoid follicles, bile duct proliferation and haemorrhage which was more prominent in sub-capsular areas. Kupffer cells proliferation was evident only in group B and C. whereas, periportal fibrosis was shown in group D only. Group D had the highest aflatoxins residues in the livers (58.824%), followed by group A, B and C respectively. Group E showed no aflatoxins residues in the livers. The study demonstrated the inefficiency of garlic and the commercial Hydrated Sodium Calcium Alaminosilicate (HSCAS) in ameliorating the toxic effects of aflatoxins.

Introduction

Among the most common toxigenic fungi affecting human and animal food chains are Aspergillus spp., infecting major agricultural commodities such as corn, sorghum, peanuts, cotton, other oil-seed sources, rice, cassava, nuts, chilies, and spices (Calvo et al, 2004; Qazi and Fayyaz, 2006).

Aflatoxins (AF) are difuranocoumarin derivatives produced by a polyketide pathway by many strains of Aspergillus flavus and Aspergillus parasiticus. In particular, Aspergillus flavus which is a common contaminant in agriculture. Aspergillus bombycis, Aspergillus ochraceoroseus, Aspergillus nomius and Aspergillus pseudotamari are also aflatoxin-producing species, but they are encountered less frequently (Bennett and Klich, 2003). These fungi are ubiquitous and can affect many of the developing-country dietary staples (Qazi and Fayyaz, 2006).

Aflatoxicosis represents one of the serious diseases of poultry, livestock and other animals. The cause of this disease in poultry and other food-producing animals has been attributed to the ingestion of various feeds contaminated with A. flavus.

Avian species, especially chicks, gosslings, ducklings and turkey poults are more susceptible to AF toxicity. The toxic effect of AF is mainly localized in the liver and is manifested by hepatic necrosis, bile duct proliferation, icterus and hemorrhage. Chronic toxicity in those avian species is characterized by loss of weight, decline in feed efficiency, drop in egg production and increased susceptibility to infections (Dalvi, 1986).

Garlic-diallyl sulfide (DAS) affects AF metabolism and DNA binding by inhibiting phase I enzymes and may therefore be considered as potential cancer chemopreventive agents (Tadi et al, 1991). On the other hand HSCAS can antagonize the ability of AF to cause DNA damage that leads to the formation of sister chromatid exchanges (Türkez, 2007).

This study aims to clarify the chronic aflatoxicosis in layer chicks using two different concentrations of aflatoxins (90 ppb and 103 ppb) for 100 days, and to assess the effects of 10 mg garlic/ml/day and commercial HSCAS in amelioration of its toxicity in layer chicks.

Materials and Methods:

Preparation of the Experimental Rations:

Aspergillus flavus was isolated from a groundnut ration used in a former project which was conducted in the Department of Mycology-Central Veterinary Research Laboratories Centre (CVRLC), Soba, Khartoum. This fungus was inoculated into rice as described by Shotwell *et al*, (1966). 500 ml water were added to one kilogram of rice in a 5 litters-flask and shacked continuously for 2 hours, then sterilized in an autoclave. Two ml of Aspergillus flavus's spore suspension was inoculated into the rice medium and incubated at room temperature for 10 days. Then the inoculated rice was mixed with 50 kg of groundnut cake, 5 litres of water were added to the mixture, and incubated at room

temperature for 5 days. After that, white-cottony with some yellowish green areas were seen which has a distinctive odor.

The groundnut cake inoculated with *Aspergillus flavus* was mixed with a finished ration containing 58 ppb aflatoxins and designated as the experimental ration (1). Its concentration was determined using HPLC as 90 ppb. The experimental ration (1) was further mixed with old sesame cake having a concentration of 130 ppb, at the rate of 1:9 and designated as experimental ration (2). The aflatoxin concentration of which was at 103 ppb.

Control Ration:

It was unavoidable to find a fresh ration which is completely free from aflatoxins to feed the chicks in the untreated control group. Accordingly five recently purchased growing layer rations composed of the same constituents as the afore mentioned finished ration were analyzed. Selection was made for the ration containing the lowest concentration of aflatoxins (24.6 ppb) to feed the birds in the control group.

Experimental Chicks:

Hundred one-day-old female Bovan laying chicks purchased from Coral Company, were housed in cages at the Central Veterinary Research Laboratories Centre at Soba. The chicks were allowed a 3-day acclimatization period, during which, they were given doses of Oxytetracycline (Oxytetra 200, Pantex, Holand) for the control of bacterial infection. Sulpha (Trisul 80/400 wsp, Kepro B.V.- Holland) was used as coccidiostatic and coccidiocidal, and vitamins (Introvit, Interchemie, The Netherlands) were given as a feed supplement. All chicks were vaccinated orally against infectious bursal disease (IBD) using D78 vaccine (Intervet International, The Netherlands) at 23 and 36 days old, and Newcastle disease vaccine (Komarov, CVRLC, Khartoum, Sudan) intranasaly at 32 days old. The chicks were divided into five groups of 20 chicks each .

Experimental Design and Treatment:

Group A was fed with the experimental ration (1), while group B received the experimental ration (1) and fresh garlic water given at 10 mg ground garlic/ml/day. Group C received the experimental ration (1) mixed with HSCAS at a dose of 1gm/kg ration as an antimycotoxin substrate. Group D was fed the experimental ration (2). While group E was fed the control fresh ration. The experiment continued for 100 days after which the chicks were killed. All the chicks were weighed weekly till day 100 of age.

Determination of Aflatoxins Concentration:

High Performance Liquid Chromatography (HPLC) was used to determine the concentrations of aflatoxins in the feed rations. The aflatoxins were extracted and measured according to Elzupir *et al*, (2009.)

ELISA Assay was also used to determine aflatoxins in the livers. Using ELISA kits (Veratox Quantitative Aflatoxin Test, GIPSA FGIS 2005- 102, Neogen Corporation, 2007, USA/ Canada) were used. For logistical and technical reasons this method was performed instead of HPLC to determine the concentration of aflatoxins in livers. The aflatoxins have been extracted from the livers and measured as described by Mursal, (2009).

Histopathological Techniques:

Necropsies were performed immediately after humane slaughter, gross lesions were identified and recorded, a portion of each liver was fixed in 10% formalin, processed in tissue processor, embedded in paraffin wax, sectioned at 5 µm thick sections and stained with haematoxylin and eosin (H & E).

Statistical Analyses:

The significance of differences between groups was compared at each time point using Independent Sample T-test, and P-value was obtained using One-Way-ANOVA test.

Results

Clinical Signs:

Low body weight gains were observed in all treated groups as compared to the chicks in the control group (E) (Fig 1)., Group A and C gained an average of 5.97 gm/day, and group B showed a gain of 5.54 gm/day. The lowest average weight gain/day was noticed in group D. The highest daily weight gain of 8.80 gm/day was encountered in the control group (E). At the end of the experiment, the birds in the control group were 800 gm heavier than at the outset. Those in other groups did not exceed 400 gm (Fig, 2 &3). Dullness, ataxia, paralysis of the legs and wings, gasping and complete recumbence were manifested by chicks in group A and D,

Gross Pathological findings:

At necropsy, the most prominent lesion in chicks of group A, B, C and D were pale fragile livers with areas of congestion and haemorrhages. In addition chicks of group A presented enlarged bursa of fabricious whereas chicks of group B displayed enlarged kidney. No observable gross lesions were seen in the organs of control chicks (Table 1).

Fig 1. 52-day-old Chicks from group D and E. The left one from group D and the right one from group E

Fig 3. Average daily weight gain (gm)

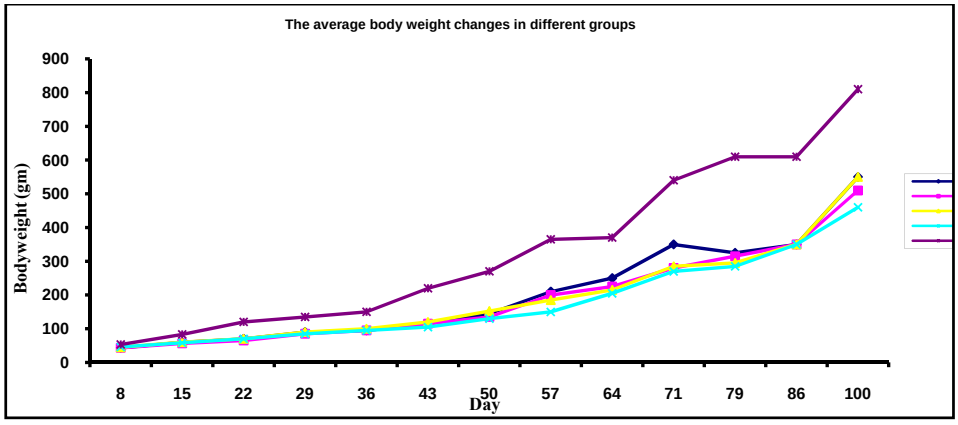

Fig 2. Post-treatment average bodyweight changes in different groups (A,B, C, D & E)

Table 1: Necropsy findings in chicken of group A, B, C and D

Organs	Gross lesions	Groups				
		A	B	C	D	E
Liver	Pale	++	+	+	++	_
	Congestion	++	++	+	++	_
	Haemorrhage	++	++	±	++	
	Fragile	++	±	±	++	_
Kidney	Enlarged	_	+	_	_	_
	Congestion	_	+	_	_	_
Bursa	Enlarged	+	_	_	_	_

Histopathological findings:

The histopathological changes observed are presented in Table 2.The most prominent changes observed in livers of all treated groups (A, B, C and D) included, hepatocyte necrosis, lymphoid cells infiltration with occasional formation of lymphoid follicles (Fig. 4), subcapsular haemorrhages, dilatation of the central vein, leucocytic infiltration in the portal area, collapsed hepatocyte and dilatated sinusoids (Fig. 5).

Moreover, groups A and D presented hepatocytes hydropic degeneration, proliferated bile ductules and parenchymal haemorrhages. Kupffer cells proliferation and mild fibrotic reaction were evident in portal triat of livers in group D only (Table 2).

Aflatoxins residues determination in livers:

Four out of 17 (23.5%) liver samples collected from Group A chicks fed aflatoxins contaminated ration of 90 ppb were positive for aflatoxins residues which varied between 0.2-0.9 ppb, and 3 out of 17 (17.6%) liver samples collected from group B fed aflatoxins contaminated ration of 90 ppb and fresh garlic water (10 mg/ml daily) were positive with a range of 0.3-0.4 ppb. One sample out of 17 (5.9%) liver samples collected from group C fed aflatoxins contaminated ration (90 ppb)mixed with HSCAS 1gm/kg, was positive (0.1 ppb).Nine out of 17 (52.9%) liver samples collected from group D fed aflatoxins contaminated ration of 103 ppb were positive with a concentration ranging between 0.1- 1.5 ppb. The liver samples collected from group E fed aflatoxins contaminated ration of 24.6 ppb were negative for aflatoxins residues (Table 3).

It can be seen from Tables 4,5&6 that both the independent Sample T-test and the one wayANOVA revealed no significant differences between the mean residual aflatoxin concentrationin the livers of groups A,B,C, but the mean aflatoxin concentration in the livers of group D was significantly higher when compared to the mean in the other groups.

Table 2: Microscopic lesions in Different Groups

	Group A (ration 1)	Group B (ration 1+ garlic)	Group C (ration 1+ antimycotoxin)	Group D (ration 2)	Group E (control)
Hydropic degeneration	+	–	–	++	–
Leucocytic infiltration	+++	+	+	+++	–
Bile duct proliferation	+	–	–	+	–
Lymphoid follicles	++	+	+	++	–
Parenchymal haemorrhage	+	–	–	++	–
Subcapsular haemorrhage	+	+	+	+++	–
Dilatation of portal vein	+	+	+	+	–
Leucocytic infiltration in the portal area	+	++	–	+	–
Periportal fibrosis	–	–	–	+	–
Kupffer cells proliferation	–	+	+	–	–
necrosis	++	+	+	+++	–
Collapsed hepatocyte	+	+	+	+	–
Dilatated sinasoids	++	+	+	++	–

Ration 1 ≡ 90 ppb.

Ration 2 ≡ 103 ppb.

+ ≡ positive

_ ≡ negative

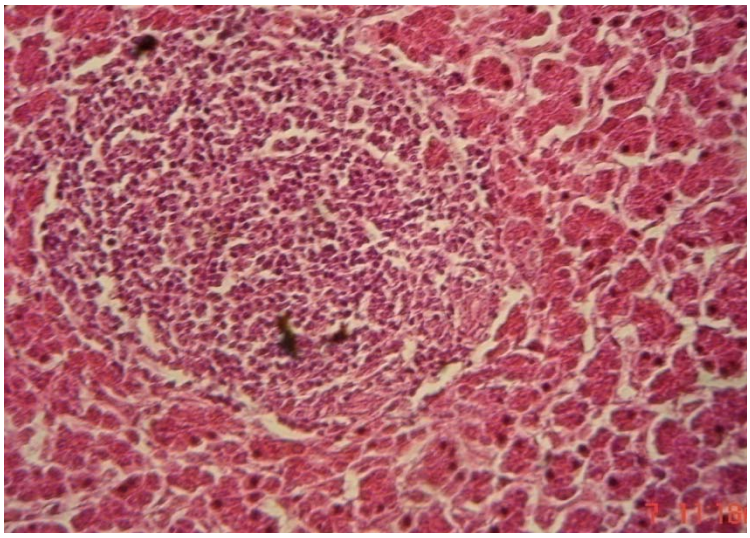

Fig. 4. Liver section from chicks of group A treated with aflatoxins at 90 ppb. The changes are characterized by lymphoid Follicle (H&E, magnified 400x)

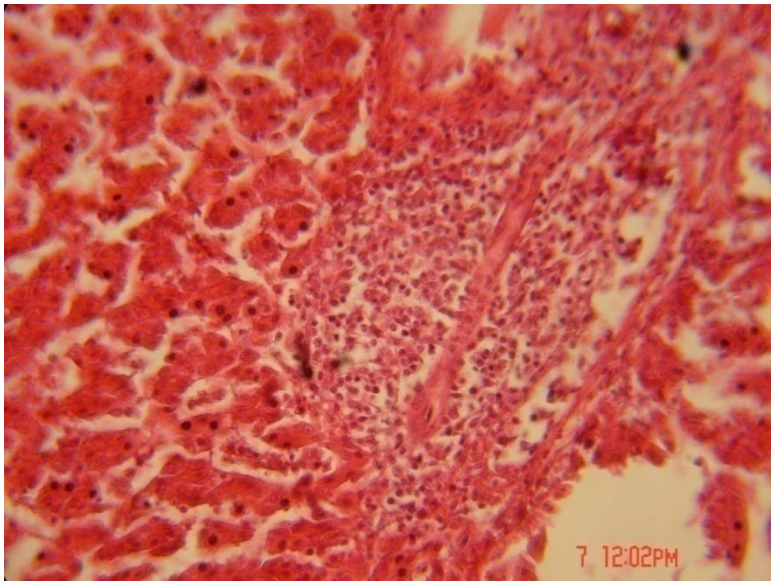

Fig. 5. Liver section from chicks of group B treated with aflatoxins at 90ppb+garlic. Note leucocytic infiltration in the portal area, collapsedhepatocyte, dilatated sinasoids (H&E, 400x)

Table 3: Aflatoxins Residues (ppb) in Livers in Different Groups measured by ELISA technique

Group	Number of chicks	positive samples	Range (ppb)	Percentage (%)
A	**17**	**4**	**0.20-0.90**	**23.529**
B	**17**	**3**	**0.30-0.40**	**17.647**
C	**17**	**1**	**0.10**	**5.882**
D	**17**	**9**	**0.10-1.50**	**58.824**
E	**17**	**0**	**0**	**0**

Table4: Independent sample T-test

	group	Number of samples	Mean	SD	Sig	
T	**A**	**17**	**0.1**	**0.23184**	**0.094**	**Not sig**
E	**B**	**17**	**0.058824**	**0.132565**	**0.086**	**Not sig**
S	**C**	**17**	**0.005882**	**0.024254**	**0.332**	**Not sig**
T	**D**	**17**	**0.235294**	**0.390418**	**0.024**	**Sig**
Control	**E**	**17**	**0.0**	**0.0**	**-**	**-**

SD: Standard deviation

Table 5: Independent samples T test

	Group	Number of samples	Mean	Std. Deviation	Sig	
T E S T	A&D	34	0.167647	0.323542	0.005	Sig
T	B&C	34	0.032353	0.09761	0.062	Not Sig
Control	E	17	0.0	0.0	-	-

Table 6: One-Way ANOVA Test; Groups are significantly different from each other

	Number	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	17	0.1	0.23184	0.05623	-0.0192	0.219201	0	0.9
B	17	0.058824	0.132565	0.032152	-0.00934	0.126982	0	0.4
C	17	0.005882	0.024254	0.005882	-0.00659	0.018352	0	0.1
D	17	0.235294	0.390418	0.09469	0.03456	0.436029	0	1.5
E	17	0	0	0	0	0	0	0
Total	85	0.08	0.224032	0.0243	0.031677	0.128323	0	1.5

P value =0.011 (significant)

Discussion

Aflatoxins have gained considerable importance due to its toxicity and its frequent occurrence in feed ingredients used in poultry rations. Recent studies have shown that, aflatoxicosis is emerging as a serious problem facing poultry industry in Khartoum State (Mursal, 2009; Babiker, 2009).
All feed samples examined from poultry farms in different localities in Khartoum State, were found positive for aflatoxins, with concentration varying between 10- 97 ppb, and that 17 % of the livers examined, were found to contain aflatoxins residues, the concentration of which varied between 2-12 ppb (Mursal, 2009). On the other hand, Babiker, 2009 examined poultry and dairy feed samples and found that all samples were positive for one, two or three fungal genera namely Aspergillus, Penicillium and Fusarium which commonoly contaminate the feedstuffs with potent mycotoxins e.g: aflatoxin, ochratoxin and trichothecene.
The particular aim of this study was to induce experimental chronic aflatoxicosis in laying chicks for 100 days with low aflatoxins levels (90 and 103 ppb), a situation that may naturally occur under field conditions. Chicks fed ration containing aflatoxin showed obvious clinical symptoms similar to those reported by Espada *et al,* (1992); Fernandese *et al,* (1994); and Mursal, (2009).The symptoms were more evident in groups A&D
.On the other hand, the addition of antimycotoxin and garlic to the aflatoxins-containing diet, provided low amelioration in toxicity and improvement in weight gains, this finding is similar to those obtained by other researchers (**Ledoux et *al*, 1999**; Guyonnet *et al,* 2002; Berges *et al*, 2004; Tsai *et al,* 2005). Similarly, addition of HSCAS to aflatoxin contaminated diets did not seem to have any benificial effect on performance and weight gain. This appears to agree with Watts et al,(2003) and Santin et al,(2002). In contrast, Ledoux *et al,* (1999) found that HSCAS was effective in preventing the toxic effects of aflatoxins at levels up to 4 mg/kg feed. This may be due to the high quality & high level of HSCAS used in his study.

This study also showed that garlic given at 10 mg/ml/day, had little effect on body weight loss induced by aflatoxicosis. However, Berges *et al,* (2004) reported that, garlic can partially increase enzymes involved in aflatoxin B1 detoxification, and Guyonnet *et al*, (2002) who showed that cytosol from (DAS) and (DADS) [garlic components] treated rats produced inhibition of aflatoxin B1-8,9 epoxide (AFBO) induced mutagenecity and increased the cytosolic formation of AFB1-glutathione conjugates. They also reported that DADS is a potent inducer of AFB1 aldehyde reductase one (rAFAR1). This could be attributed to the low dose of garlic and the animal species used in the different studies.

The daily weight gains recorded for the treated groups (A,B,C&D) were far less than those obtained for control groups(E). By the end of the experiment ,the average weight of control chicks (800gm)was about double that of treated birds (400-450gm) constituting a significant weight loss..

Aflatoxins affect all poultry species. Although high levels may cause mortality, low levels can be detrimental if continually fed. The liver is the mostly affected organ (Eaton and Groopman, 1994). The present study showed that aflatoxin were detected in 60%of livers in group D chicks (concentration,0.15--1.5ppb),23% in group A(0.2--0.9 ppb),18% in group B(0.3--0.4ppb) and 6% representing one case in group C(0.1ppb) .The latter group received HSCAS in their rations. In this respect, Mursal(2009)found that 10-60% of livers examined in different broilers farms in khartoum state contained aflatoxins in concentrations ranging from 2 to 12 ppb.which are higher than the concentrations obtained in this study.

The gross hepatic lesions observed here (palenss,increased fragihty, haemorrhage etc) are similar to those reported previously by Bryden and Cumming, (1980), Khan, (1994) and Mahagan *et al*, (2002)

The microscopic lesions detected were almost similar in all groups but in addition group A and D(receiving 90 and 103 ppb aflatoxin in the ration respectively) showed evidence of parenchymal haemorrhages and proliferation of bile duct epithelium. On the other hand , birds in groups B

and C(receiving garlic and HSCAS ,respectively) showed kupffer cell proliferation.

In general, the liver lesions seen here are nearly similar to those observed by various authors in birds fed mycotoxin contaminated rations.Hamorrhage Sandhu *et al,* (1995). proliferation of bile duct epithelium(Ortatatli,2005; Tessari *et al,* (2006).) ,parenchymal lymphoid aggregation(Takhar and Sadana,2004),. Vascular degeneration and periportal fibrosis (Ortatatli *et al,* 2005).) have been reported. This indicates that the toxic effect of aflatoxins are mainly located in the liver and that. Chronic toxicity may be associated with poor performance and wieght loss (Dalvi,1986)

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