''Reproductive response of laying chickens to ameliorative method of aflatoxin

**Olayinka Abosede Ojo, *[1]Immanuel Bitto and *[2] Emmanuel Olubisi Ewuola

* [1]Department of Animal Breeding and Physiology, University of Agriculture, Makurdi, Nigeria

* [2]Department of Animal Science, Animal Physiology Unit, University of Ibadan, Nigeria

**Department of Animal Production, Fisheries and Aquaculture, Kwara State University,

** Corresponding author: ykmelodya1@yahoo.com +2348064221539

Abstract

Aflatoxin is toxic, carcinogenic and ubiquitous in nature, affecting both crops and livestock. Mitigating aflatoxin effect using toxin binders has not been very effective. Information on the use of biological methods in aflatoxin mitigation has not been adequately documented. Consequently, influence of bio-control method of aflatoxin on some reproductive hormones, ovarian weight and histopathological parameters of laying chickens (LC) were investigated. Point-of-lay Bovan Nera (n=700) were harphazardly distributed to four dietary treatments; Aflasafe maize-based diet (AMBD), farm feed (FF), aflatoxin-contaminated diet with toxin binder (ACDTB) and aflatoxin-contaminated diet without toxin binder (ACDWTB). The contaminated diets contained 306.3ppb aflatoxin and the experimental design was completely randomized into four treatments (n= 175) of five replicates (n=35) per treatment for a period of 14 weeks. Blood (5mL) was collected at 14th week for LC to determine the estrogen, luteinising hormone (LH), follicle stimulating hormone (FSH), histopathology of the ovary using standard procedures. Data were analysed using descriptive statistics and ANOVA at a0.05. The ROW (%) ranged from 0.34±0.2 (ACDTB) to 0.93±0.3 (AMBD). Estrogen (mg/dL) value was highest in LC fed ACDWTB (2.75±1.08) and least in FF (2.07±0.52). The LH (iu/L) value was highest in LC fed AMBD

(1.29±1.68) and least in ACDTB (0.36±0.32). Histopathology of the ovary showed cysts, observed along the oviduct wall in LC fed ACDTB. AMBD enhanced active laying period in LC with no sign of aflatoxocosis. The use of aflasafe maize grain in poultry diet is recommended.

Keywords: Aflatoxin-contaminated diet, Aflatoxicosis, ovary, hormones, histopathology and laying chickens

1. Introduction

Aflatoxin (AF) has been confirmed as most toxic, tetratogenic, mutagenic and carcerous mycotoxins so far known (Williams et al., 2009; Khan et al., 2010). The detrimental effect of aflatoxin on various animals has been well documented (Keyl and Booth, 1971; Lawloy and Lynch, 2001; Zain, 2010) and this include yellow ocher discolouration with multifocal haemorrhage with proliferation of the bile ducts (Ehklas, 2012). Hepatomegaly and hypertrophy in some organs and higher relative weights of the organs (Huff et al., 1992; Ologhobo et al., 2015) have been reported. Other effects include accelerated follicular atresia (Siloto et al., 2011), associated by discontinuance of egg laying during the feeding trial (Hafez et al., 1982). Aflatoxin has also been documented to cause disturbances in the hormonal profile of domestic animals, usually resulting in reduced fertility potential (Clarke et al., 1987; Tiemann and Vanselow, 2003; Hasanzadeh et al., 2011). Different approaches have been investigated to mitigate the effect of aflatoxin contamination in livestock feeds. This include use of enteroabsorbants such as hydrated sodium, calcium alumino-silicates. Although, it selectively bind aflatoxin B1 without depleting micronutrients (Williams et al., 2004), but, few successes have recorded with this method. A combination of heat with ammonia has also been stated to irreversibly detoxified aflatoxin (Brown, 2010), but the limitation is that it can only make the blue glow go away, without protecting the feed. Also, microorganisms resembling Mycobacteriumis, Stenotrophomonas maltophilia and Trichoderm viride have also been attested to succeed in breaking down aflatoxins (Liu et al., 2001 and Kasmani et al., 2012). However, they have been noted to pose health risks to

consumers. Physical and chemical methods of degradation have been known to cause reduced product nutrient, organoleptic qualities and undesirable health effects (Teniola et al., 2005). Consequent to these limitations, novel bio-control method with biological mechanism has been developed, which involves displacing toxigenic strains of Aspergillus flavus from agricultural fields with strains of Aspergillus flavus that do not produce aflatoxin (atoxigenic strains). This causes encumbrance with the contamination process by physically excluding the toxigenic strain as at the time of microorganism invasion and contending for nutrients needed for aflatoxin biosynthesis by the toxigenic strains, thereby abridging the total toxigenicity of A. flavus population. The significance of this research is to evaluate the changes in reproductive performance during chronic aflatoxicosis and compare it with the use of aflasafe maize-based diet (aflatoxin-free diet) in laying chickens.

MATERIALS AND METHODS

This study was carried out at God's Grace Farm, located in Lagun Town, along Ibadan- Iwo road, Oyo State. The Aflatoxin-contaminated maize grain and aflasafe maize grains used for this experiment were obtained from the plant pathology unit, International Institute of Tropical Agriculture, (IITA), Ibadan, Nigeria. Other ingredients used for the feed formulated were obtained from God's Grace feed Mill, the host farm. Maize grain, used as the aflatoxin carrier was inoculated with toxigenic strain of *Aspergillus flavus* of Nigerian origin. The culturing and inoculation was done at the plant pathology unit, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, using 5% V8 juice and 2% agar, with PH 5.2 and a spore load of 2.475 x 106 per ml. Aflatoxins, quantified using scanning densitometer, CAMAG TLC scanner 3 with – CATS 1,4,2 software (Camag AG, Muttenz, Switzerland) (Suhagia et al., 2006)

EXPERIMENTAL BIRDS AND MANAGEMENT

A total of 700, 30-week old Bovan Nera black hens with a mean body weight of 2.0kg was used for this experiment The experimental birds were randomly allotted into 4 treatments consisting of 175 birds per treatment, replicated thrice with 35 birds/ replicate. The laying chickens were housed in battery cages having linear feed troughs and nipple drinkers for running water. The birds were fed basal feed for 14 days (acclimatization period), after which experimental diets and fresh water were provided *ad libitum*. Data collection was done weekly throughout the duration of the study which lasted for 14 weeks

2.2 EXPERIMENTAL DIETS

Four (4) experimental diets were formulated based on nutrient requirement of laying hens. The experimental diets are as shown in Table 1 comprising of the following; Treatment 1 (AMBD) – Aflasafe maize-based diet, Treatment 2 (FF+Toxin binder) –Farm feed with toxin binders (Control diet), Treatment 3 ((ACDTB)– Aflatoxin contaminated diet with toxin binder, Treatment 4 (ACDWTB)– Aflatoxin- contaminated diet without toxin binder. The diets were subjected to chemical analysis, to obtain proximate composition as stated in Table 1 under the Analyzed nutrient segment. At the end of the experiment (14th week), 80 birds were randomly selected from each treatment and three (3) mls of blood sample was collected through the jugular vein in the earliest hours of the day, into anticoagulant-free bottles. These bottles were kept in slanting position and allowed to clot. The samples were spun at 3000 rpm for 10 minutes, serum samples obtained were then separated into sterile tubes for analysis. Enzyme Linked Immunosorbent Assay (ELIZA) kits were used for the assay of estrogen, follicle stimulating hormone and luteinizing hormone. Equipment used was ELIZA microplate reader/ absorbance (IRE 96), a fully automated 8-channels measurement system having absorbance as its detection mode (Bourne et al., 2003). After feeding trial, 80 birds (20 birds per replicate) were randomly selected per treatment, weighed and sacrificed. Dissection was done through the lower abdominal incision. Samples of the ovary and reproductive organ were harvested for histopathological investigation.

The tissues were cut into small pieces of not more than 4mm thick and placed into pre-labelled cassettes. These were further immersed in 10% formal saline for 24 hours to fix. Tissue processing was done using hematoxylin and eosin technique (Avwioro, 2010).

2.3 Statistical Analysis

All data obtained from the study was subjected to descriptive statistics and one-way analysis of variance (ANOVA) in a completely randomized design, using statistical analysis software (SAS, 2008). Means were separated using Duncan multiple range test (Duncan, 1957).

Table 1: Composition of the experimental diet (%) (Layers Mash)

Ingredients (kg)	AMBD	FF+Toxin binder	ACDTB	ACDWTB
Aflasafe Maize	50.220	-	-	-
Farm Maize	-	50.220	-	-
Contaminated Maize	-	-	-	-
Soyabean Meal	23.100	23.100	23.100	23.100
Wheat offal	13.460	13.460	13.460	13.460
Salt	0.350	0.350	0.350	0.350
Bone Meal	3.010	3.010	3.010	3.010
Oyster Shell	9.040	9.040	9.040	9.040
Lysine	0.200	0.200	0.200	0.200
Methionine	0.200	0.200	0.200	0.200
Vitamin C	0.006	0.006	0.006	0.006
Layer Premix	0.300	0.300	0.300	0.300
Toxin Binder	-	0.100	0.100	-
Oxytetracycline	0.100	0.100	0.100	0.100

Total (kg)	100.00	100.00	100.00	100.0
Calculated Nutrients				
Crude Protein (%)	17.30	17.30	17.30	17.30
Crude Fibre (%)	4.56	4.56	4.56	4.56
Metabolizable Energy(Kal/kg)	2598.25	2598.25	2598.25	2598.25
Avail. Phoshorus	0.68	0.68	0.68	0.68
Lysine	1.05	1.05	1.05	1.05
Methionine	0.46	0.46	0.46	0.46
Avail. Calcium	4.34	4.34	4.34	4.34
Analysed Nutrients				
Met energy(kcal/kg)	2874.38	2926.22	3048.16	3052.60
Crude protein	20.50	18.78	18.22	18.01
Ash	18.50	16.50	13.50	13.00
Crude fibre	7.05	7.16	7.21	7.25
Dry matter	90.49	90.09	90.40	90.29

Vitamin A 10,000 IU, Vitamin D_3 2,000 IU, vitamin E, 100 IU, Vitamin K, 20mg, thiamine B_1, 15mg; Riboflavin B_2 40mg, , Vitamin B_6 (pyridoxine) 1 5mg, Niacin 150 mg; Vitamin B_{12} 0.015mg, Pantothenic acid 50mg, Folic acid 5mg, Biotin 0.2mg. Choline chloride, 12mg; , Antioxidants 1.25g; Mn, 0.8g; Zinc, 0.5g; Iron, 0.2g; copper, 0.05g; iodine, 0.12g; Selenium, 2mg; cobalt, 2mg. AMBD= Aflasafe maize-based diet, FF =Farm feed + Toxin binder, ACDTB =Aflatoxin-contaminated diet with toxin binder and ACDWTB = Aflatoxin-contaminated diet without toxin binder.

4. Results and discussion

Table 2: Weight of Reproductive Organ of laying chickens fed experimental diets

PARAMETERS	AMBD	FF+TOXIN BINDER	ACDTB	ACDWTB
Ovary (%)	0.93±0.3[a]	0.99±0.5[a]	0.34±0.2[b]	0.44±0.4[b]
Repr. Organ (%)	2.47±0.4	2.44±0.7	1.48±1.2	2.25±1.9

AMBD= Aflasafe maize-based diet, FF =Farm feed + Toxin binder, ACDTB =Aflatoxin-contaminated diet with toxin binder and ACDWTB = Aflatoxin-contaminated diet without toxin binder.

The relative ovary weight of birds fed AMBD (0.93%)) and FF+toxin binder (0.99%) were not different, however, both were significantly higher than the values observed in birds which received ACDTB (0.34%) and ACDWTB (0.44%) respectively. The result is in tandem with the observation of Ajani et al. (2014), who noticed ovary atresia i.e degeneration of immature ovarian follicles during follicular phase in laying hens fed a diet containing 8000ppb AFB for 7 days. According to Hafez et al. (1982), aflatoxicosis causes pathological changes in the chicken ovaries, which has detrimental effect on egg production. Therefore, it can be inferred that aflatoxin could have adverse effect on egg production, due to aflatoxin-related pathological changes in the ovary as reported in this study. The chickens fed ACDTB, showed ovarian cycts along the oviduct wall. This could also have been due to the aflatoxin-contamination effect. Cysts are fluid-filled cavities that may occur along the oviduct wall, resulting into tumor, when larger than 2mm in diameter. It has been noted that production of progesterone is usually by the largest growing follicles and its receptors are found along different points in the oviduct wall. The observed cycts could have blocked or reduced the number of progesterone receptors, causing a depression effect in the mechanism of progesterone production and its activity, with a negative influence on oviduct

134 contraction and egg transport. Perhaps, this have caused a drastic reduction in egg oviposition,
135 resulting into re-absorption of yolk into the system of the hens. This result is in accordance with
136 the reports of El-Azab et al. (2009) and Hasanzadeh et al. (2011), who stated that, aflatoxin has
137 been shown to disrupt the reproductive system in both male and female animals, causing
138 alterations in the form of growing and mature ovarian follicles.

139 **4.1 Dietary effect on hormonal assay of laying chickens**

140 The results of the luteinizing hormone (LH), estrogen and follicle stimulating hormone
141 (FSH) assays of laying chickens fed experimental diets during treatment period are illustrated in
142 Table 3. Except the FSH values that was not significant, the luteinizing and estrogen hormone
143 values showed significant differences. The LH value (1.29±1.68 IU/L) obtained in birds fed the
144 AMBD, was not significantly ($P<0.05$) different compared with those given FF+toxin binder
145 (0.64±1.27 IU/L), the LH values (0.36±0.32 IU/L and 0.42±1.03 IU/L) of laying birds fed
146 aflatoxin-contaminated diets with and without toxin binder were significantly lower. The mean
147 value of estrogen recorded in birds fed AMBD (2.10±0.52pg/mL) showed no influence compared
148 to the FF+toxin binder (2.07±0.83pg/mL). The mean values recorded for birds fed ACDTB
149 (2.38±0.90pg/mL) and ACDWTB (2.75±1.08pg/mL) were not statistically influenced by the
150 dietary treatment. The FSH values of laying chicken were not significantly ($P<0.05$) different
151 among the treatments.

152 **Table 3: Hormonal Assay of laying hens fed experimental diets**

PARAMETERS	T1	T2	T3	T4
Luteinizing hormone (LH)	1.29±1.68^{a}	0.64±1.27^{ab}	0.36±0.32^{b}	0.42±1.03^{b}
Estrogen	2.10±0.52^{b}	2.07±0.83^{b}	2.38±0.90^{ab}	2.75±1.08^{a}

Follicle Stimulating Hormone (FSH)	2.66±1.33	2.24±0.84	2.13±0.68	2.22±0.79

ab: means on the same rows but with different superscripts are significant (P<0.05).AMBD =Aflasafe maize-based diet, FF+Toxin binder = Farm feed + toxin binder, ACDTB= Aflatoxin-contaminated diet + toxin binder, ACDWTB= Aflatoxin-contaminated diet without toxin binder.

Reproductive hormones of birds fed experimental diet was significantly influenced by the dietary treatment. Luteinizing hormone is a primary hormone of reproduction, produced from the anterior lobe of the pituitary gland. The epithelial cells lining the empty follicular cavity has been reported to multiply under the influence of luteinizing hormone, to form corpus luteum. In poultry, ovulation and oviposition processes are controlled by luteinizing hormone and progesterone. A higher concentration of serum LH of experimental laying birds, as observed in birds fed AMBD, is an advantage as it would improve the egg laying capacity of the birds which would translate into higher profitability. The depressed values of LH in this study corroborate the findings of Hasanzadeh et al. (2011) and Ismail (2012), who reported a significantly lowered luteinizing hormone level in the aflatoxin-treated groups of male rats compared to the control, which received zero dosage of aflatoxin. This result might be attributed to the adverse effect of AF on the hypophysis, resulting into hypophysotoxicity, according to Clarke et al. (1987). Estrogen is a steroid hormone produced in the gonads, which performs a prominent role in the development of female secondary sex characteristics and in ovarian cycle. Trophic hormones FSH and LH are noted for stimulating the synthesis and release of estradiol from the theca interna of matured follicle. It has been documented that toxins cause a cascade of events called acute phase response, which induced local inflammation and activation of the hypothalamic-pituitary-adrenal axis (HPA), as stated by Sheng *et al.* (2021). Perhaps, the local inflammation of the HPA caused by AF effect had resulted into a significantly lowered concentration of LH and FSH in laying birds, which could have caused a concormitant decrease in the estrogen value. Documentation showed that FSH and LH release from the pituitary gland occurs in a rhythmic manner, as regulated by the hypothalamic biologic clock, known as pulse frequency (Squires, 2003). The frequency of GnRH (Gonadotropic

Releasing Hormone) by the hypothalamus, differentially affect FSH and LH release, which should also influence estrogen synthesis and release. Contrary to this, the estrogen level of birds in ACDWTB is significantly higher compared to the control (FF+Toxin binder). In literature, it has been stated that cholesterol is the precursor of estradiol and it is present as low density lipoprotein (LDL) in plasma. In the serum biochemical response of hens, the cholesterol level of the birds in ACDWTB is highest. Perhaps, the high level of LDL (low density lipoprotein) in the plasma of the laying hens acted as a precursor for increased synthesis of estrogen in the laying birds in ACDWTB, with the recorded highest value. FSH functions in maintaining the hierarchy of size in the developing follicles and the rate of follicular atresia. It also works synergistically with luteinizing hormone to cause an elaborate secretion of estrogen during ovulation. An increased FSH concentration in birds fed AMBD suggested a normal hierarchical follicular development and ovulation, as well as normal egg production (Squires, 2003).

Table 4: Histopathological examination of the ovary given aflatoxin-contaminated diet

Parameter	AMBD	FF+Toxin binder	ACDTB	ACDWTB
Ovary	No visible lesion	No visible lesion	Cyst observed along the oviduct wall	Cyst observed along the oviduct wall

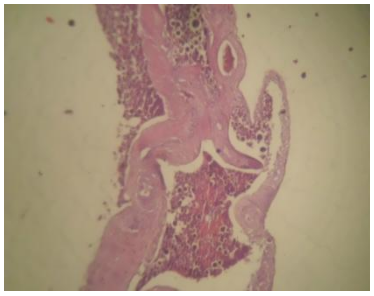

Plate 1: Showing the Ovary of laying chickens fed AMBD. No visible lesion observed.

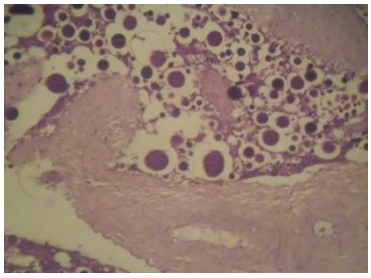

Plate 2: Showing the Ovary of laying chickens fed FF. No visible lesion was observed.

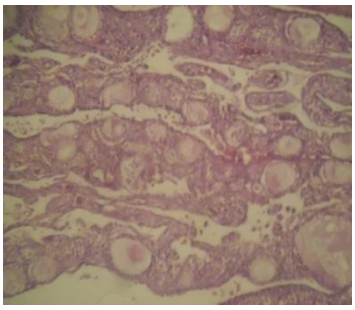

Plate 3: Showing the Ovary of laying chickens fed AF-CDTB. No visible lesions seen. There are cysts along the oviduct wall.

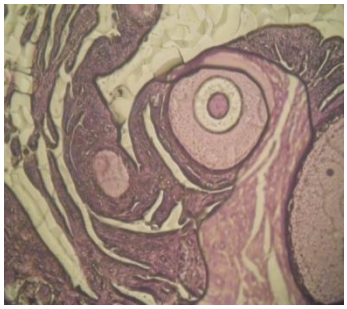

Plate 4: Showing the ovary of laying chicken fed AF-CDTWB. There is no observable visible lesion.

4.2 **Ovary Histopathology**

The microscopic investigation of various organs in previous studies showed that aflatoxin adversely affected the organs attributed with the hematopoietic, immune and the reticuloendothelial system (Qureshi et al., 1998; Ortatatli and Oguz, 2001; Ortatatli et al., 2005 and Eklas, 2012). The ovary of laying chickens fed AMBD, which showed no histopathological alterations may be due to the aflatoxin-free diet obtained through the use of aflasafe maize. Similar result was observed in birds fed the FF+Toxin binder diet and birds fed ACDTWB. Although the ACDTWB diet had a recorded high aflatoxin concentration, which resulted into cessation of egg production in the group, corroborating the findings of Hafez et al. (2008) and Ismail (2012) who observed that aflatoxin-treated mature domestic fowls showed follicular atresia, accompanied by cessation of egg production during the whole feeding period. Hasanzadeh et al. (2014) also observed that the micromorphological effects of the AFB1 on the ovary showed that the attic changes were seen in different ovarian follicular layers, including oocyte, granulose and theca, which increased both at the microscopic and macroscopic level (Siloto et al., 2011). Yet no visible lesion was observed and the concentration of the aflatoxin used showed no histopathological alteration on the ovary. Aflatoxin has also been shown to disrupt the

reproductive system in both male and female animals, causing alterations in the form of the growing and mature ovarian follicles. (El-Azab et al., 2009; Hasanzadeh et al., 2014).

Conclusion and recommendation

Adverse effects of aflatoxin in laying chickens can be prevented by the use of feed ingredients that contains no aflatoxin contamination through the mutual exclusion mechanism of *Aspergillus species* in the soil. Although aflatoxin binder is effective, but its cost implication could increase the overall production cost, especially on a large scale livestock farming. Aflatoxicosis in livestock can be mitigated with the use of aflasafe maize-based diets, it is therefore recommended for use in both small and large scale production.

Ethics Statement

This research was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Kwara State University .

Consent for publication

"Not applicable"

Availability of data and materials

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors of this research paper do not have any financial or non financial competing interest.

Authors' contributions

IB made immense contributions to the design of the study and drafted the hormonal inclusion in this research; **EO** made the collaborative funding acquisition for this research possible and substantively revised it till perfection,**OA** collected, statistically analyzed and interpreted the data used in writing this manuscript and was a major contributor in writing this manuscript.

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