

Haematological effect of *Aspergillus species* metabolites on broiler chicks

Samuel Toba Anjorin¹, Catherine Oluebube Cyriacus²

¹Department of Crop Science, Faculty of Agriculture, University of Abuja, Nigeria

²Department of Biological Sciences, Faculty of Science, University of Abuja, Nigeria

Corresponding author: Anjorin Toba Samuel, B. Agric., M. Tech. (Crop Prot.) Ph.D. (Plant Pathology) Department of Crop Science, Faculty of Agriculture

University of Abuja, Abuja-FCT, Nigeria

E mail oyindamola35@yahoo.com, Mobile No.: (+234)8062242866

Abstract

The effect of crude metabolites of *Aspergillus flavus* and *A. ochraceus* on the haemogram and leucogram values of broilers was evaluated. One hundred and twenty newly hatched, unsexed broilers were randomly distributed into four groups. They were fed with the contaminated diets containing the fungi metabolites singly and in combination, at levels of 0 and 10 mg/kg from 10 to 31 day-old. All the broilers fed with contaminated feed with combined *A. flavus* and *A. ochraceus* metabolites showed significant ($p < 0.05$) lower values of white blood cell count (WBC), red blood cell (RBC), haematocrit (Hct) and haemoglobin (Hgb) with values of $207.3 \times 10^9/L$, $1.6 \times 10^{12}/L$, 23.3% and 7.3 g/dl respectively compared to the control with values of $241.1 \times 10^9/L$, $2.3 \times 10^{12}/L$, 31.0% and 9.5 g/dl respectively. These were haematological evidence for anemia and leucopenia in the broilers. Healthy fowls feed should be free from toxigenic fungi contamination.

Keywords: *Aspergillus flavus*, *Aspergillus ochraceus*, broiler chicks, haematology, metabolites

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1. Introduction

Exposure of farm animals to toxigenic fungi and their toxins occurs mainly by ingestion of contaminated feeds which contain corn, peanut or sorghum [1-2]. The consumption of mycotoxin-contaminated diet by broilers have been reported to have induced haematological, biochemical and liver physiological changes and growth depression thus leading to economic losses to animal industries[3]. Haematological studies cover the cellular and serum composition of blood, the coagulation process, blood-cell formation, haemoglobin synthesis, and disorders of all these processes [4-5].

Mycotoxicosis of poultry results in increased mortality, decrease blood cell count, lower egg production, lower feed consumption, impairs resistance to infectious disease, reduces vaccination efficiency and induces pathological damage to the liver and other organs [6]. In the studies on dietary aflatoxin-induced genotoxicity using two *in vivo* bioassays, dietary aflatoxin was shown to be genotoxic and mutagenic to male Swiss albino mice [7]. The capacity of these mycotoxins to alter normal immune function when present in food at levels below observable overt toxicity is of particular interest. Of further concern is the possible transmission of fungal mycotoxin residues to meat and eggs from infected chicken which is potentially hazardous to human health. Heterocyclic metabolites of the genera *Aspergillus* are aflatoxin and ochratoxin [8]. *Aspergillus flavus* is the predominant specie responsible for aflatoxin contamination of crop-

based feed ingredients prior to harvest or during storage [9]. Celik *et al.* [10] reported that both lower and higher doses of AFB₁ affect the haematological parameters of the broiler chick resulting into a depressed cellular immunity due to suppression of the phagocytic activity of microphages and decrease in T-lymphocyte. Also the liver damage witnessed led to deficiency in the humoral immunity [11]. *A. ochraceus* strains are more commonly associated with warmer climates than *Penicillium* spp. [12]. *A. ochraceus* grows more slowly than *A. flavus* or *A. parasiticus*; It grows at temperatures ranging from 8 to 37°C with an optimum between 24 and 37°C (ICMSF[13]), with ochratoxin being produced at 12 to 37°C with an optimum at 31°C.[14] Due to paucity of information on this subject especially in the developing countries, this study investigated the effect of crude metabolites of *A. flavus* and *A. ochraceus* on the haemogram and leucogram values of broiler chicks.

2. Materials and method

2.1 Isolation of fungi:

A. flavus and *A. ochraceus* were isolated from naturally infected sesame seeds was cultured on Potato Dextrose Agar (PDA) and incubated at 28° C for 5 to 7 days. Seeds were examined under stereoscopic microscope. Fungi appearing on Petri plates were directly identified up to the species level with the help of a compound microscope (Leitz Ortholux) and relevant literature (22). The two and half kilogram (2.5 kg) of healthy yellow maize seeds to be used for subculturing was soaked overnight in 1 L of water. They were then autoclaved at 121°C for 15 mins. to kill the microorganisms present. Subsequently, the 2.5kg of the autoclaved seeds after cooling was sub-divided into three portions of 833g each and inoculated with *A. flavus* only, *A. ochraceus* only and *A. flavus* and *A. ochraceus* in combination respectively. It was then left to produce metabolite within 14 days at room temperature according to the procedure of Kamalavenkatesh *et al.*[6].

2.2 Extraction of mycotoxin:

The mycotoxin extraction was done by the clean-up method as described by Pholo *et al.*[14] This method of extraction involves the use of methanol/water. The fermented maize was ground and 60g was weighed and submerged into 300ml of methanol/water (70%:30%) . The wet mass was then shaken using an orbital shaker at 300rpm in 3 mins and then filtered. After filtration, the methanol/water was then eluded using a water bath, leaving the crude extract which was then collected. Adequate amount of the crude metabolite (10 mg/kg) was mixed in clean water daily to obtain experimental liquid sample.

2.3 Animal and experimental groups:

A total of one hundred and twenty newly hatched day old, unsexed broiler chicks were used. They were wing-banded and housed in electrically heated batteries with *ad-libitum* supply of feed and water. The broilers were kept in close observation for a period of 10 days to be acclimated at the University of Abuja biological garden.

The broiler chicks were randomly divided into four groups of ten each. The chicks were housed in a disinfected and temperature controlled colony cage. The first group was used as a control and was fed standard diet, while the remaining three groups were fed diet containing crude metabolites of *A. flavus*, *A. ochraceus*, and the combination of the two at 10mg/kg respectively. The experimental design was completely randomized design and in three replicates.

The broiler chicks were fed for 21 days from 10 days after hatch. The broilers were vaccinated with infectious bursa disease (IBD) orally 10ml/bird after 7 days of hatch. Feed consumption was monitored daily. The broiler starter feed contained crude protein, 20% (min); fat, 10% (max.); crude fibre, 9.0% (max.); calcium, 1.0% (min); available phosphorus, 0.45%

(min) and metabolisable energy, 2800 Kcal/kg (min). Other ingredients are salt, antioxidants, probiotics and enzymes. It was confirmed to be free from aflatoxin contamination by using RIDA® QUICK mycotoxin tests.

2.4 Haematological analysis:

The analysis was carried out at the University of Abuja teaching hospital (UATH), Gwagwalada Abuja, Nigeria. The sysmex KX-21N haematology analyzer (an automatic multi-parameter blood cell counter for *in vitro* diagnostic use in clinical laboratories) marketed by Sysmex corporation, US was used. The analyzer is a 3-part differential hematological analyzer that could differentiate the white blood cell into three populations, based on their sizes, neutrophils, lymphocytes, and mixed population which consists of eosinophiles, monocytes and basophils. The haematology analyzer, displays on the liquid crystal display (LCD) screen the particle distribution curves of white blood cell count (WBC), red blood cell count (RBC) and platelet count, along with data of 19 parameters as the analysis results. The quantitative hematology analyzer directly measures the white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HGB), hematocrit (HCT), platelet (PLT), absolute lymphocytes count (LYM), absolute mixed count (MXD) and absolute neutrophil count (NEUT).

2.5 Procedure of the blood analysis:

The blood samples were collected *via* the coxygeal vein in ethylenediamine tetra-acetic acid (EDTA) anti-coagulant. The EDTA anticoagulated blood was then diluted with cell pack in the WBC counting chamber. The cell pack is a whole blood diluent used in the determination of haemoglobin and electric counting and sizing of blood cells. A fixed volume of stromatolyser-WH solution (1 volume of stromatolyser-WH to 2 volumes of cell pack) was added to the

automatic sysmex KX-21N to obtain a final dilution of 1:500. The stromatolyser-WH was added to lyse the RBC and so leaving the remaining cell stroma at a level undetected by the sysmex KX-21N at the same time, the WBC membrane is preserved and WBC are stabilized at a level detectable by the sysmex KX-21N. The WBC was counted by direct current method. The haemoglobin was released during RBC lyses, and then converted to the red methemoglobin and read photometrically at 555nm. A portion of the diluted sample was then transferred automatically to the haemoglobin detector where the absorbance of the red pigment was measured to give the blood haemoglobin level.

In the direct current detection method, blood sample was aspirated, measured and diluted at the specific ratio, then fed into each transducer. The transducer chamber has a minute hole called the aperture. On both side of the aperture, there was the electrode between which flows direct current. The blood cells suspended in the diluted sample passed through the aperture, causing direct current resistant to change between the electrodes. As direct current resistance changes, the blood cell sizes was then detected as electric pulses. The cell count is calculated by counting the pulses. The blood samples used were free from clots and fibrin strands. The spiral blood mixer was used to ensure adequate sample mixing because excessive mixing may induce platelets clumping and alter white cell membranes resulting in false results.

2.6 Statistical Analysis:

All data were expressed as mean \pm SD. Data were analyzed by one-way ANOVA using SPSS 16.0 software. Least significant difference tests were applied to compare treatment means. All statements of differences were based on significance at $P < 0.05$.

LSD is calculated thus:
$$\text{LSD } 0.05 = t_{.05} \sqrt{\frac{2s^2}{r}}$$

Where

r = replication,

t = the tabular t- value at the df of the error and

s² = error mean square

3. Results

Haematological analysis on broiler chicks fed with contaminated feed with crude metabolite of *A. flavus* and *A. ochraceus* singly showed no significant difference ($p > 0.05$) in the proportion of white blood cell count (WBC), red blood cell count (RBC), hematocrit (HCT), haemoglobin (Hgb) compared to the chicks used as control (Table 1). The chicks fed with the combination of the two fungi metabolite showed significant ($p < 0.05$) decreased values of WBC, RBC, Hct and Hgb with values of $207.3 \times 10^9/\text{L}$, $1.6 \times 10^{12}/\text{L}$, 23.3% and 7.3 g/dl respectively compared to the control with values of $241.1 \times 10^9/\text{L}$, $2.3 \times 10^{12}/\text{L}$, 31.0% and 9.5 g/dl respectively.

The proportion of lymphocyte, neutrophil and mixed population in broiler chicks fed with feed contaminated with crude metabolite of *A. flavus* and *A. ochraceus* singly and in combination showed significantly ($P < 0.05$) lower values compared to the control (Table 2). Table 3 shows the summary of the haematological results of the broiler chick after the experiment and is stated in mean \pm SD.

The adverse effects of mycotoxins on animal health are expressed in a diverse range of symptoms including homeostasis blood system damage, immunosuppression, hepatotoxicity, nephrotoxicity, neurotoxicity, and even death [14]. Haematological parameters of animal are determined as an index of their health status. RBC, Hgb, Hct (PCV), WBC and lymphocyte depletion observed invariably indicated the immunosuppressive potential of these fungi crude metabolite due to their cytotoxic effect on the lymphoid cells. In our study, the mould contaminated diet decreased the levels of WBC, RBC, HCT (PCV) and Hgb level and caused an increase in neutrophil and mixed population (basophil, monocyte and eosinophil). Our results indicated that homeostasis blood system damage was induced by mycotoxins. However, these findings concurred with RBC, WBC and lymphocyte depletion of broiler chick treated with higher levels of 200 ppm, 400ppm [2]. The reduction of WBC and lymphocyte is of serious concern since there is increased susceptibility of the affected animal to secondary infection. Braz¹⁵ reported that in chickens fed a diet of 2.5mg/kg AF, the delayed-type hypersensitivity skin reaction to tuberculin was reduced.

Tuzcu *et al.*[2] gave haematological evidence for immunosuppressive effect of *A. flavus* crude metabolite on cell mediated immunity were it decreased significantly ($P < 0.001$) the proportion of WBC and lymphocyte count. Mycotoxin can act as immunosuppressive agents affecting cell-mediated and humoral immune compartments [16]. Celik *et al.*[9] have shown that AF depresses cell functions besides decrease in the lymphocytes in the lymphoid tissues. Significant ($p < 0.05$) decreases were observed in the chicks fed AFB₁ (1mg/kg diet) in their blood lymphocyte counts compared to the control group [15]. Sur and Celik [16] observed that low level administration of AFB₁ at the beginning of early embryonic development decreases blood lymphocyte percentages at the hatching period.

Table (1): Proportion of mean WBC, RBC, HCT and Hgb in broiler chicks fed with feed contaminated with fungi metabolites

Treatment	WBC 10 ⁹ /L	RBC 10 ¹² /L	Hct (%)	Hgb (g/dl)
<i>A. flavus</i> metabolite	227.3	1.9*	26.7	8.4
<i>A. ochraceus</i> metabolite	229.2	1.9	27.0	8.5
<i>A. flavus</i> + <i>A. ochraceus</i> metabolites	207.3*	1.6*	23.3*	7.3*
Control	241.1*	2.3*	31.0*	9.5*
LSD (0.05)	22.23	0.40	5.91	1.79

Data is mean of 10 replicates * = Data in a column with mean difference significant at the 5% level (P<0.05) by LSD test.

Table (2): Proportion of mean lymphocytes, neutrophils and mixed population in broiler chicks fed with feed contaminated with fungi metabolites

Treatment	LYM (%)	NEU (%)	MXD (%)
<i>A. flavus</i> metabolite	86.3*	12.6*	1.1*
<i>A. ochraceus</i> metabolite	86.3*	12.3*	2.4*
<i>A. flavus</i> + <i>A. ochraceus</i> metabolite	85.0*	13.0*	2.0*
Control	96.3*	3.7*	0.01*
LSD (0.05)	5.63	2.97	1.27

Data is mean of 10 replicates; * = Data in a column with mean difference significant at the 5% level (P<0.05) by LSD test.

Table (3): Haematological parameter of broiler chicks fed with feed contaminated with fungi metabolite

Haematological parameter	Control	<i>A. flavus</i> metabolite	<i>A.ochraceus</i> metabolite	<i>A. flavus</i> + <i>A.ochraceus</i> metabolites
WBC ($10^9/L$)	241.1±0.01	227.3±0.08	229.2±0.14	207.3±0.14
RBC ($10^{12}/L$)	2.3±0.04	1.9±0.1	1.9±0.27	1.6±0.13
Hct (%)	31.0±0.03	26.7±0.06	27.0±0.3	23.3±0.09
Hgb (g/dl)	9.5±0.00	8.4±0.07	8.5±0.23	7.3±0.21
LYM (%)	96.3±0.01	86.3±0.00	86.3±0.01	85.0±0.01
NEU (%)	3.7±0.00	12.3±0.00	12.3±0.07	13.0±0.06
MXD (%)	0.01±1.0	2.4±0.2	2.4±0.23	2.0±0.09

Data is mean of 10 replicates

4. Discussion

In the present study, fungi crude metabolite decreased significantly ($P < 0.05$) the proportions of RBC, hematocrit and haemoglobin. This result is similar to that reported by Javed *et al.*[18] were a decrease in RBC and haemoglobin levels of broilers fed 125 and 274mg/kg AFB₁ from 1 to 14 day of age were reported. The decrease in red blood cell count indicates anaemia which is considered to be due to inflammations, infections and toxemia.

It was indicated in this study that fungi crude metabolite caused striking changes in the leucocyte formula of the experimental groups although there was significant increase in the neutrophil and mixed population (basophil, eosinophil and monocyte). This finding is in line with the report that the neutrophil percentages were gradually increased whereas the lymphocyte was decreased [2]. The reason for the increasing of neutrophil percentage (neutrophilia) was

considered due to general inflammation by intoxication and acute hemorrhage. Haemolytic anemia can cause a high neutrophil count [19]. Haemolytic anaemia is a condition in which the RBC are destroyed earlier than they should be. The high eosinophil percentage (eosinophilia) is considered to be due to allergies, infections and toxin sensitivity. High basophil percentage is considered as a result of chronic hemolytic anaemia and fungi infection. However, high neutrophil count can lead to leukocytosis which is associated with bacterial infection and leukemia. Low lymphocyte percentage (lymphocytopenia) is associated with mononucleosis and anemia. The low level of AFB₁ has been shown to play a direct immune-suppressive effect on the cell-mediated immune (CMI) reactions by inhibiting phagocytic and microbiocidal activity of macrophages, and decreasing peripheral blood T-lymphocyte counts in the broiler chick [20] .

There is no doubt that the best method to control contamination by mycotoxins in foods is to prevent fungal growth. Wendell²⁰ found out that the contamination of grains can occur as a result of inadequate storage conditions, high level of moisture on harvested grains and also on the field during the pre-harvest period, and the use of plant genotypes that are more resistant to fungal contamination at storage is very important.

5. Conclusion

It was found from this study that *Aspergillus*-contaminated diets altered hematological parameters. The result obtained from this study give some haematological evidence to explain the immunosuppressive effects of *A. flavus* and *A. ochraceus* crude metabolite individually and in combination and induces significant declines in the proportion of red blood cell, haematocrit, haemoglobin, white blood cell and lymphocyte level. These findings pointed to a potential threat for predisposition of toxin fed birds to feed biochemical hazards. The solutions to this obstacle

to poultry production should start with a strict selection of healthy raw materials, a correct feed storage and monitoring and when necessary, an efficient and safe inclusion of additives such as propionates that can control the contaminating mycotoxins in the feed. Further studies involving the effect of selected mycotoxins on the immune system and physiology of different types of poultry is imperative.

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