

Aflatoxin effect on erythrocyte profile and histopathology of broilers given different additives

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Abstract. The aim of this study was to evaluate erythrocyte profile and microscopic changes effect of AF induces by low level (57.18 ppb) and chronic exposure (34 days) with administration of additive (*Lactobacillus plantarum* G7 and methionine). Aflatoxin-contaminated corn was prepared by inoculate *Aspergillus flavus* FNCC 6002 on corn. Total number of 576 broiler Lohman strain (MB202) unsexed DOC were allocated completely randomized into four treatments and 12 replicates, with 12 broiler chicks each. The treatments as follows: T1= aflatoxin-contaminated diet, T2= aflatoxin-contaminated diet + 1% of LAB (w/w), T3= aflatoxin-contaminated diet + 0.8% of methionine (w/w), and T4= aflatoxin-contaminated diet + 1% of LAB + 0.8% of methionine (w/w). The effect of treatments was evaluated using ANOVA and the difference among mean treatments were analyzed using DMRT. The result showed that administration of additives had no significant effect ($P>0.05$) on erythrocyte profile, liver, and bursa of Fabricius. The dose of additive in each treatment (T2, T3, T4) were insufficient to reduce adverse effect of chronic aflatoxicosis. It was concluded that the LAB dose for binding AF (57.18%) should be evaluated and the dose for methionine should be reduced for chronic treatment of aflatoxicosis.

1. Introduction

Mycotoxins are secondary toxic metabolites produces by fungi that occur as a contaminant in feed and feed ingredient universally [1]. More than 300 kinds of mycotoxins can cause toxicity sign in mammalian and poultry, aflatoxin (AF) and deoxynivalenol (DON) are two of the most common and harmful for the feed and animal industries [2,3]. Aflatoxins (AF) produced by *Aspergillus flavus* and *A. parasiticus* are a major concern in the poultry production, because it has association with illness and disease in poultry and domestic animals, immunosuppress effect, thermostable and hepatic lesions as the endpoint most associated with aflatoxicosis [4-6].

Erythron is defined by erythrocyte circulating in the body and erythropoietic tissue (bone marrow). On the normal conditions, erythron considers the balance of production, erythrocyte destruction by reticuloendothelial system (RES) in the liver, spleen and bone marrow. The method of erythron evaluation by using haematocrit values (Hct), haemoglobin (Hb), MCV, MCH, and MCHC examination [7].



The presence of small to moderate amount of mycotoxins in daily feed rations increases the susceptibility of animals to viral, bacterial and parasitic diseases, because mycotoxins can suppress immune system, even innate and adaptive immunity both of them [5]. AF can cause crucial macroscopic and microscopic changes of the liver, such as hepatomegaly, paleness, hydropic degeneration, fatty change, bile duct hyperplasia and periportal fibrosis [6].

Preventing management and decontamination technologies to minimize the effect of AF that contaminated feeds and feedstuffs is the important thing to develop. Procedure of decontamination focuses on degrading, destroying, inactivating, or removing AF by physical, chemical or biological methods has been developed [8,9]. The aim of this study was to evaluate erythrocyte profile and microscopic changes effect of AF induced induces by low level (57.18 ppb) and chronic exposure (4 days) with administration of additive (*Lactobacillus plantarum* G7 and methionine).

2. Material and methods

2.1. Material

Materials utilized in the present experiment contained raw material for diet formulation (corn, soybean meal, meat bone meal, palm oil, premix, NaCl, CaCO₃, and Ca₂PO₄), *Aspergillus flavus* FNCC 6002, dried culture of *Lactobacillus plantarum* G7, and methionine. The 576-days old chicks Lohman strain (MB202) were used in the present experiment.

2.2. Preparation of aflatoxin binder additive

Formulation of feed additive consisted of dried culture of *L. plantarum* G7 [10], methionine [11], and its combination. The dried culture of LAB was prepared using spray dryer [12]. *Lactobacillus plantarum* G7 was cultivated in de Man Rogosa Sharpe (MRS) Broth media at 37 °C for 18 hours. The culture was centrifuged at 4500 rpm for 10 minutes then the biomass/pellet was mixed with skim solution 20% w/v. The solution was homogenized before spray drying process. Spray dryer operating conditions as follows: inlet air temperature 120 °C outlet air temperature 68 °C, aspirator 90%, speed 25-30, and nozzle cleaner 2 (Buchi Mini Spray Dryer B-290). Dried culture obtained from spray dryer then added with skim as fillers and adjusted to 1x10⁷ CFU/g of cell density.

2.3. Experimental diet

An experimental diet composed of corn, aflatoxin-contaminated corn, soybean meal, meat bone meal, then formulated to meet nutrient requirements of broilers [13]. Experimental diet could be seen in Table 1. Aflatoxin-contaminated corn was prepared in several steps [14]. Corn was sterilized at 130 °C for 2 hours. *Aspergillus flavus* FNCC 6002 was cultivated in potato dextrose agar (PDA) media at 30 °C for five-seven days. Corn was inoculated by *A. flavus* and incubated for five days. After incubation, corn was dried under the sun heat then homogenized. Aflatoxin production from each treatment diet was determined using AgraQuant® Total Aflatoxin Assay (Romer Lab) by ELISA (Enzyme-Linked Immunosorbent Assay) reader (Thermo Scientific Multiscan Go). Aflatoxin dose in diet was 57.18 ppb=57.18 µg/kg.

Table 1. Composition and nutrient content of ingredients in the experimental diet

Ingredients	Composition (%)
Corn	59.0
Soybean meal	23.0
Meat bone meal	11.5
Palm oil	3.5
Premix	0.5
NaCl	0.5
CaCO ₃	1.5
Ca ₂ PO ₄	0.5
Total	100

2.4. *In vivo experiment*

A total number of 576 broilers Lohman strain (MB202) unsexed one-day-old broilers were allocated randomly into four treatments each. All chicks kept under a similar condition of management throughout the experimental period lasting for 35 days of age. The initial brooding temperature was 33 °C in the first week and reduced gradually two °C per week to 24 °C. Diets and water were provided *ad libitum* all over the experimental period. The experiment was designed in a completely randomized design with four treatments, 12 replicates with 12 broiler chicks each. The treatments as follows: T1= aflatoxin-contaminated diet, T2= aflatoxin-contaminated diet + 1% of LAB (w/w), T3= aflatoxin-contaminated diet + 0.8% of methionine (w/w), and T4= aflatoxin-contaminated diet + 1% of LAB + 0.8% of methionine (w/w).

Feed intake was recorded daily by subtracting the amount of offered feed with the residual for each treatment. Final body weight was assessed basis from initial day to the final day of the experiment. Mortality was recorded daily, and percentage of mortality was calculated. Feed conversion ratio (FCR) was calculated as total feed intake divided by final body weight of live chicken [15].

Vaccination was given at the age of 4 days old (ND-IB vaccine, Newcastle Disease, Infectious-Bronchitis-eye drop), eight days old (IBD vaccine, Infectious Bursal Disease), and 18 days old (ND Lasota vaccine, Newcastle Disease-oral). At the end of experimental period, five broilers per treatment were randomly selected and weighed. Blood samples were collected for erythrocyte profiles analysis, and then the necropsy was performed to observe pathological lesions, especially in livers and bursa of Fabricius.

2.5. *Hematological examination*

Five broilers (18 and 34 days old) from each treatment were selected at random and bled by wing vein puncture. The blood was collected into tube with 10% of EDTA as an anticoagulant [16]. The red blood cells (RBC) counts were determined by hemocytometer method. Haematocrit values were measured by the microhaematocrit method. Haemoglobin amounts were determined by the cyanmethaemoglobin method [8]. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated using formulation [7].

The formulation could be seen below:

$$\text{Mean corpuscular volume (MCV)} : \frac{PCV \times 10}{RBC} (\mu\text{3/FL}) \quad (1)$$

$$\text{Mean corpuscular hemoglobin (MCH)} : \frac{Hb \times 10}{RBC} (\mu\mu/\text{pg}) \quad (2)$$

$$\text{Mean corpuscular hemoglobin concentration (MCHC)} : \frac{Hb \times 100}{PCV} (\%) \quad (3)$$

2.6. *Pathological examination*

At the end of the experiment (34 days), five broilers from each treatment were selected at random and killed for pathological examination. All organs were examined for pathologic anatomic lesions. The samples of tissue, especially liver and bursa of Fabricius were collected and fixed in 10% normal buffered formalin.

Microscopically, hepatocellular degeneration in livers was graded as follows; Slight (degree 1): Mild hepatocellular swelling due to hydropic degeneration and fatty changes only in centrilobular areas. Moderate (degree 2): Clear hepatocellular swelling in both centrilobular and mid zonal areas. Severe (degree 3): Diffuse and severe hepatocellular swelling, cytoplasmic paleness and rupture, necrotic coagulative and cholangioma [6] with modification.

2.7. Statistical analysis

The effect of treatments on broilers were evaluated using the analysis of variance of completely randomized design and the difference among mean treatments were analyzed using Duncan's Multiple Range Test [17].

3. Result and Discussion

The results of the present study are presented in Table 2 and 3. The data showed that the effect of treatments (T2, T3, and T4) on erythrocyte profile were not significantly different ($P > 0.05$), especially at the 34 days of blood sampling (Table 3). From the erythrocyte profile evaluation, T1 treatment (AF) occurred anemia microcyte-hyperchromic, which is marked by the decline of MCV value at the 18 days of sampling (Table 1) but not significantly different ($P > 0.05$) with another treatments (T2, T3, T4). At the 34 days of sampling, MCV and MCHC values were not shown anemia. MCV is an indicator for the size of erythrocyte and MCHC is indicator Hb concentration in the RBC.

Erytron is circulated RBC and the erythropoietic organ are called bone marrow. Erytron defense equilibrium between production, destruction of RBC by reticulo endothelial systems (RES) occurs in the liver, spleen and bone marrow. The condition influences the erytron is polycythemia, anemia, hydremia or hemodilution, and dehydration. Anemia is decrease of RBC, Hb or both of them in the blood circulation [7]. Base on the etiology of anemia at the present study, the anemia occurred at the 18 days of sampling all of treatments, but it did not occur at the 34 days of blood sampling.

The result of treatments (T1, T, T3, T4) on histopathological lesions of livers and bursa of Fabricius at 34 day old shown in the Table 4. From descriptive of liver histopathological changes the best result was found in T2 treatment (57.18 ppb of AF + 1% of LAB). Histopathological lesions in the livers observed in T2 are bile duct hyperplasia, edema, necrotic and dilated sinusoid (Figure 1-4). Bursa of Fabricius in the T2 treatment had focal follicular cyst (Figure 4), which is the same as in T4 for bursal organ.

Liver and immune system organs are considered to be target organs for AF and these organs are primarily affected in aflatoxicosis [6]. The particular aim of this study was induce chronic aflatoxicosis in broiler (34 days) with lower AF level (57.18 ppb), which may naturally occur under field conditions. The study of Ortatatli et al.[6] showed that low doses of aflatoxicosis were between 50-100 ppb.

Table 2. Effect of treatments (T1, T2, T3, T4) on some hematological parameters for the first of blood sampling (18 days of age)

Treatment	1 st blood profile sampling (18 days)					
	2.16 (N) RBC (10 ⁶ /mm ³)	7.7(N) Hb (g/dL)	26.3(N) PCV (%)	121(N) MCV (fL)	35(N) MCH (pg)	29(N) MCHC (%)
T1	2.51 ± 0.59 ^a	7.70 ± 0.84 ^a	25.60 ± 4.22 ^a	99.63 ± 27.37 ^a	31.01 ± 8.91 ^a	30.35 ± 2.98 ^a
T2	2.09 ± 0.45 ^{ab}	6.70 ± 0.45 ^a	25.00 ± 2.12 ^{ab}	125.95 ± 37.09 ^a	33.70 ± 9.81 ^a	26.93 ± 2.64 ^a
T3	2.44 ± 0.29 ^a	6.50 ± 0.50 ^a	24.00 ± 2.35 ^{ab}	100.23 ± 20.11 ^a	26.95 ± 3.96 ^a	27.42 ± 4.60 ^a
T4	1.77 ± 0.48 ^b	6.60 ± 1.43 ^a	20.60 ± 3.58 ^b	135.43 ± 52.09 ^a	40.43 ± 18.18 ^a	32.20 ± 4.92 ^a

^{a,b} Values within columns with no common superscripts are significantly different ($P < 0.05$), according to Duncan's multiple range tests. N: normal value (21 days of age [18]); RBC: red blood cell; Hb: Hemoglobin; PCV: packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

Table 3. Effect of treatments (T1, T2, T3, T4) on some hematological parameters for the second of blood sampling (34 days of age)

Treatment	2 nd blood profile sampling (34 days)					
	2.16 (N)	7.7(N)	26.3(N)	121(N)	35(N)	29(N)
	RBC (10 ⁶ /mm ³)	Hb (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (%)
T1	2.17 ± 0.24 ^a	9.16 ± 1.05 ^a	25.60 ± 4.22 ^a	130.01 ± 2.84 ^a	42.29 ± 1.39 ^a	32.53 ± 0.48 ^a
T2	2.11 ± 0.31 ^a	9.04 ± 1.61 ^a	25.00 ± 2.12 ^{ab}	131.29 ± 2.39 ^a	42.69 ± 1.56 ^a	32.51 ± 0.72 ^a
T3	2.28 ± 0.09 ^a	9.74 ± 0.61 ^a	24.00 ± 2.35 ^{ab}	132.38 ± 2.81 ^a	42.66 ± 1.28 ^a	32.22 ± 0.32 ^a
T4	2.23 ± 0.06 ^a	9.34 ± 0.29 ^a	20.60 ± 3.58 ^b	131.10 ± 2.50 ^a	41.94 ± 1.52 ^a	31.99 ± 0.81 ^a

^{a,b} Values within columns with no common superscripts are significantly different ($P < 0.05$), according to Duncan's multiple range tests. N: normal value (35 days of age [18]); RBC: red blood cell; Hb: Hemoglobin; PCV: packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

The previous study reported that administration of 1% of LAB + 1.2% of methionine in aflatoxin-contaminated diet (6.1 ppb) resulted the lowest damage organ (64.4 %) of liver, kidney, spleen, thymus, bursa of Fabricius, pancreas, duodenum and ileal, but did not significantly affect the body weight gain and feed conversion of broiler [20]. The organs damage caused by AF-contaminated diet are hematopoietic organs, Immune system, and reticuloendothelial systems (RES) [21-23].

Target organs that considered of AF exposure are liver, kidney and the immune system organs and these are primarily affected in aflatoxicosis cases [6]. The particular aim of this study was to induce chronic aflatoxicosis in broilers (34 days) with low levels of AF (57.18 ppb) to the erythrocyte profile, liver, and bursa of Fabricius. The range of AF include low level of aflatoxicosis are 50 and 100 ppb and those may naturally occur under field conditions [6]. Based on SNI standard, the maximum level of AF in the diet of broilers is 50.0 µg/Kg (50.0 ppb) for all phases and broilers concentrate [24-26].

Table 4. Effect of treatments (T1, T2, T3, T4) on liver and bursa of Fabricius histopathology for 34 days of age

Change/Lesion	Treatment			
	T1	T2	T3	T4
Liver				
Bile duct hyperplasia	0/5	3/5	0/5	3/5
Periportal fibrosis	1/5	0/5	0/5	0/5
Hepatic necrosis, edema, dilated sinusoid	1/5	0/5	1/5	0/5
Bursa of Fabricius				
Focal follicular cyst and lymphocytic hyperplasia	0/5	2/5	0/5	1/5
No pathologic lesion	5/5	3/5	5/5	4/5

The values represent the number of chicks showing changes/number of chicks examined in each treatment group

In this study, the toxic effect of AF on erythrocyte profile, liver and bursa of Fabricius have been clearly observed by feeding 57.18 ppb of AF fed for 34 days. Whether 50 ppb of AF level causes aflatoxicosis in broiler as no significant differences was observed [6]. In this study all treatments has no significant differences ($P > 0.05$) for binding or detoxification AF contamination diets event observed from erythrocyte profile and histopathological (liver and bursa of Fabricius). Level of LAB (1%) or methionine (0.8%) or combine between LAB and methionine not adequate for binding or detoxification the level of AF contaminated diets (57.18 ppb). LAB that has functioned as binder of AF and methionine

that have function as detoxification agent need to be increased twice until three time level for looking for the best doses for binding and detoxification function.

Feed supplementation with methionine could prevent liver necrosis in the right dose because methionine supplementation could increase the glutathione (GSH) production. AFB1 transformation in liver yielding a very radical AFB1 epoxides. AFB1 transformation could be prevented by glutathione conjugation (γ -glutamic-cysteinyl-glycine, GSH), catalyzed by glutathione-S-transferase (GST) become a mercapturic-AFB1 molecule (8,9-dihydro-(S-Cysteinyl-(N-acetyl))-9-hydroxy aflatoxin BI), that molecule is polar and could be taken out from the body via urine [11]. Over dosage of methionine supplementation could become toxic, so that the right dosage needs to be well managed for obtaining a good result [11].

The right doses of methionine for detoxification 1000 $\mu\text{g/Kg}$ (1ppm) between 0.75%-1.25%, the 1.5% of methionine exactly cause hepatic necrosis [11]. GSH production has homeostatic mechanism in liver, methionine supplemented in feed could not always change the GSH, because production of GSH could not inhibited by comethionine, and its derivatives like buthionine and neutral or non polar amino acid (glycine, alanine, phenylalanine, and proline) acidic amino acid (glutamic acid), basic amino acid (lysine), or synthesis (2-amino-2-norbornan carboxylic acid and omethylaminoisobutyric acid) [27] and AFB1 bundled by GSH not always in linear curve, on equilibrium point GSH-AFB1 would get weaken [28], that is why 0.8% of methionine could be toxic for liver with the 57.18 ppb of AF, even though that dosage suitable for 1000 ppb (1 ppm) of AF. 1% of LAB could reduce 44.5% of lesions at 21 days and 31.1% of lesions at 35 days for 2.71 ppb of AF [20], could not adequate enough for binding 57.18 ppb of AF. This also applied to a combination between 1% of LAB and 0.8% of methionine could liver toxicity and got necrosis and interfere erytron as a homeostasis of hematological function.

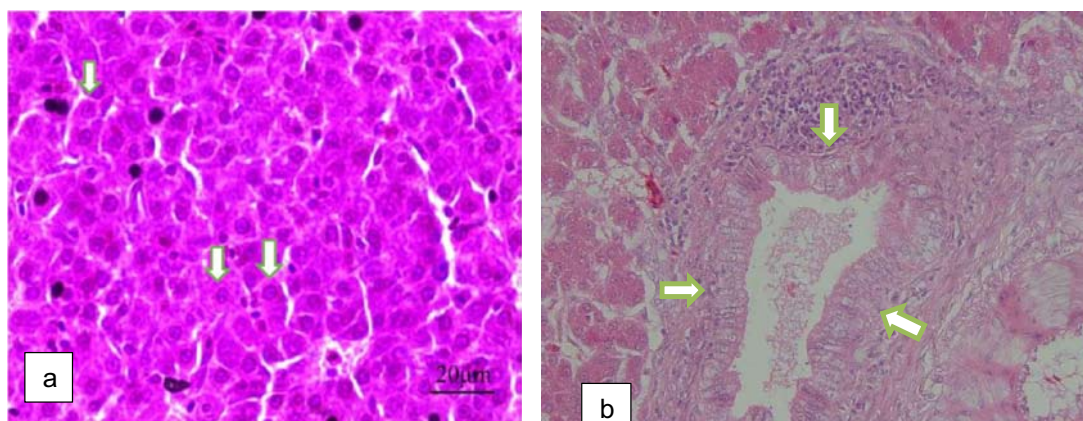


Figure 1. (a) Normal hepatocyte in chicken. The hepatic lobule has complete structure and the liver cell has regular shape (H & E, 400x) [19], (b) Mild to moderate bile duct hyperplasia occur on T2 (57.18 ppb of AF + 1% of LAB) and T4 (57.18 ppb of AF + 1% of LAB + 0.8% of methionine) (H & E, 1000x).

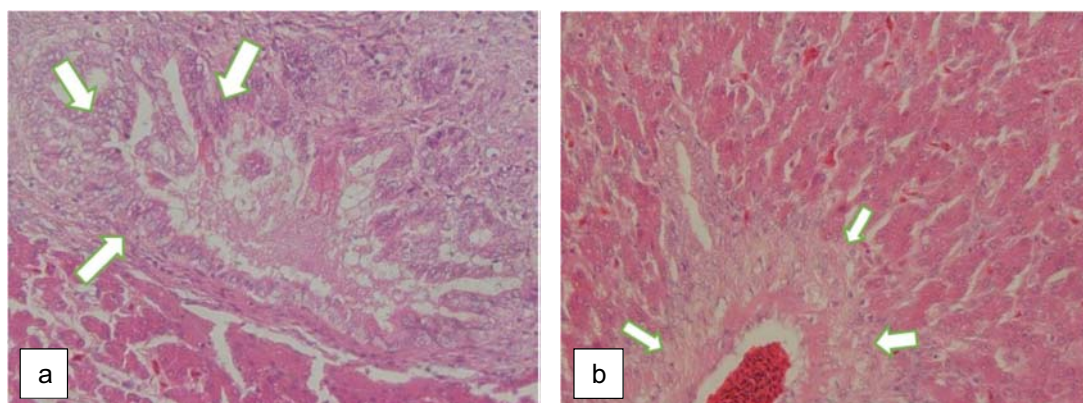


Figure 2. (a) Severe bile duct hyperplasia only occurs on T4 (57.18 ppb of AF + 1% of LAB + 0.8% of methionine) (H & E, 500x), (b) Periportal fibrosis only occurs on T1 (57.18 ppb of AF) (H & E, 1000x).

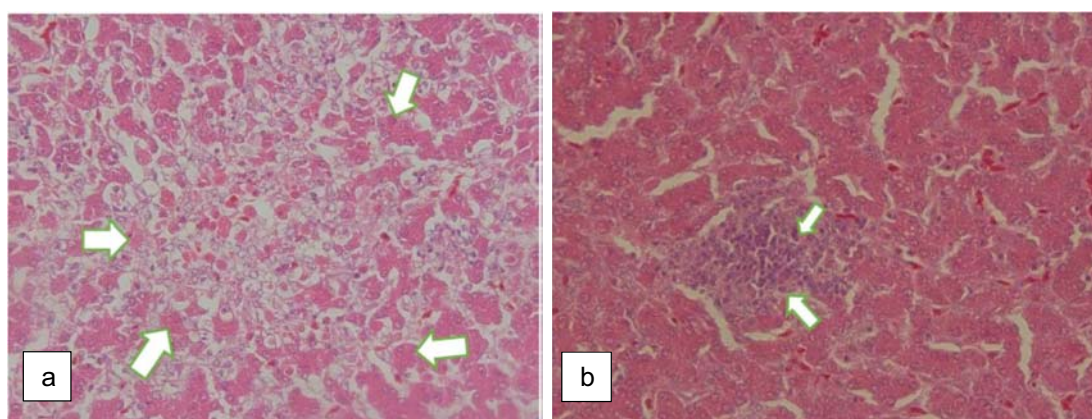


Figure 3. (a) Edema, dilated sinusoid and necrotic hepatocyte occurs on T1 (57.18 ppb of AF) and T3 (57.18 ppb of AF + 0.8% of methionine) (H & E, 1000x), (b) Focal lymphocytic infiltration in the liver occurs on all treatments (T1, T2, T3, and T4) (H & E, 1000x).

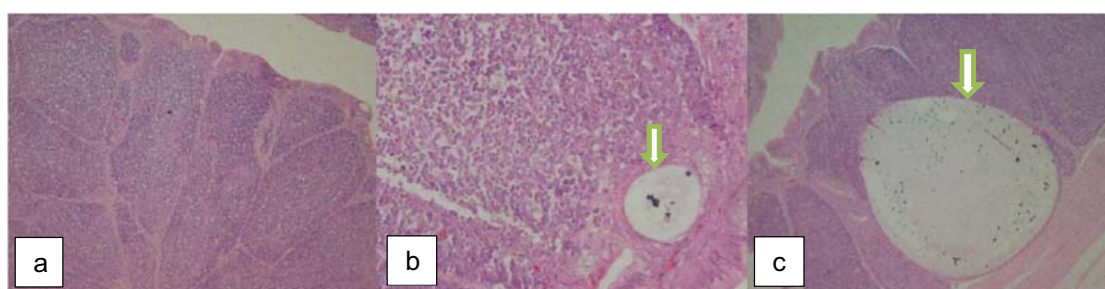


Figure 4. (a) No pathological lesion occurs on all treatments (T1, T2, T3 and T4) (H & E, 100x), (b-c) Bursal cyst and lymphocytic hyperplasia occurs on T2 (57.18 ppb of AF + 1% of LAB) two cases of five samples, and T4 (57.18 ppb of AF + 1% of LAB + 0.8% of methionine) one case of five samples (H & E, 500x (b) and H & E, 1000x (c)).

4. Conclusion

A number of all treatment doses (T2, T3, T4) is not adequate or not exact to reduce adverse effect of chronic aflatoxicosis. Needs to be evaluated of LAB dosage for binding AF (57.18%) and to reduce the dosage of treatments especially for methionine dosage for chronic treatments aflatoxicosis.

Acknowledgement

The authors gratefully acknowledge to all who have assisted and supported this research project (Faculty of Agricultural Technology UGM, Faculty of Animal Science UGM, Faculty of Veterinary UGM and technician in BPTBA LIPI). Particularly the Ministry of Research Technology and Higher Education (Kemenristekdikti) for funding this Insinas 2015 programed. Also special thanks to Ahmad Sofyan, Ph.D. for statistical support.

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