Aflatoxin Detoxification by Esterified Glucomannan in Ducklings

การลดพิษอะฟลาทอกซินโดยเอสเทอริไฟด์กลูโคแมนแนนในลูกเป็ด

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บทคัดย่อ : อนงค์ บิณฑวิหค วิจิตร บรรลุนารา และ ธีรยุทธ แก้วอมตวงศ์. 2545. การลดพิษอะฟลาทอกซินโดยเอสเทอริไฟด์กลูโคแมนแนนในลูกเป็ด. วารสารวิจัยวิทยาศาสตร์การแพทย์ 16(2) : 135-148.

ศึกษาการลดพิษอะฟลาทอกซินโดยเอสเทอริไฟด์กลูโคแมนแนนในลูกเป็ดเนื้อพันธุ์เชอรี่วาเลย์ คละเพศ อายุ 1 วัน จำนวน 150 ตัว เลี้ยงด้วยอาหารเป็ดปกติเป็นเวลา 5 วัน จึงทำการทดลองโดยแบ่งลูกเป็ดแบบสุ่มออกเป็น 5 กลุ่ม กลุ่มละ 30 ตัว กลุ่มที่ 1 ได้รับอาหารเป็ดปกติและเป็นกลุ่มควบคุม กลุ่มที่ 2 ได้รับอาหารผสมอะฟลาทอกซินบี 1 (AFB1) 100 พีพีบี กลุ่มที่ 3 ได้รับอาหารผสมเอสเทอริไฟด์กลูโคแมนแนน (EGM) ขนาด 0.05% กลุ่มที่ 4 ได้รับอาหารผสม AFB1 100 พีพีบี และ EGM ขนาด 0.05% และกลุ่มที่ 5 ได้รับอาหารผสม AFB1 100 พีพีบี และ EGM ขนาด 0.1% เลี้ยงลูกเป็ดแต่ละกลุ่มด้วยอาหารผสมเป็นเวลา 30 วัน และเปลี่ยนให้กินอาหารตามปกติที่ไม่มี AFB1 และ EGM เป็นเวลา 20 วัน วันที่ 30, 35, 40, 45 และ 50 ของการทดลอง สุ่มเป็ดกลุ่มละ 6 ตัว เจาะเลือดแยกซีรั่มมาตรวจหาระดับเอ็นไซม์ ALP, GGT, AST และ ALT ผ่าชากนำตับมาตรวจรอยโรคทางจุลพยาธิวิทยา นำกล้ามเนื้อและตับมาตรวจหาอะฟลาทอกซินตกค้าง ผลการทดลองพบว่า ตั้งแต่วันที่ 30 ของการทดลอง เอ็นไซม์ ALP ในกลุ่มที่ได้รับ AFB1 มีค่าสูงกว่ากลุ่มควบคุม และกลุ่มที่ได้รับอาหารที่มี EGM และ AFB1/EGM ผสมปนอยู่อย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ส่วนเอ็นไซม์ GGT ให้ผลคล้ายคลึงกับ ALP ตั้งแต่วันที่ 35 ของการทดลอง กลุ่มที่ได้รับ AFB1/EGM พบเซลตับและเซลบุท่อน้ำดีมีรอยโรค bile duct proliferation, fatty degeneration และ intermediate morphology ลดลงเมื่อเปรียบเทียบกับกลุ่มที่ได้รับ AFB1 ระดับอะฟลาทอกซินบี1 (AFB1) และ อะฟลาทอกซินเอ็ม1 (AFM1) ตกค้างในตับสูงกว่าในกล้ามเนื้อ ระดับที่ตรวจพบสูงที่สุดในกลุ่มที่ได้รับ AFB1 และพบต่ำกว่าในกลุ่มที่ได้รับอาหารผสม AFB1/EGM ผลการศึกษาแสดงว่า EGM สามารถลดพิษ AFB1 100 พีพีบี ที่มีในอาหารได้ แต่ระยะเวลา 20 วันซึ่งไม่ได้รับ AFB1 และ EGM ไม่เพียงพอที่จะลดรอยโรคที่ตับ และลดสารพิษตกค้างจนหมดได้ ดังนั้นระดับอะฟลาทอกซินบี1ในอาหารเป็ดควรให้อยู่ในระดับต่ำเพื่อหลีกเลี่ยงความเสี่ยงอันตราย

คำสำคัญ : อะฟลาทอกซิน เอสเทอริไฟด์กลูโคแมนแนน ลูกเป็ด ค่าเคมีในเลือด วิการที่ตับ สารตกค้าง

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Abstract : Anong Bintvihok, Wijit Banlunara and Theerayuth Kaewamatawong. 2002. Aflatoxin detoxification by esterified glucomannan in ducklings. Thai J Health Res 16(2) : 135-148.

This study was undertaken to detoxify aflatoxins using esterified glucomannan in the ducklings feed. One hundred and fifty 1-day-old, Cherry Valley ducklings were fed conventional feed for 5 days. The ducklings were then randomly divided into 5 groups, 30 birds in each. Five dietary treatments were given consisting of 1) conventional feed as a negative control, 2) 100 ppb aflatoxin B_1 (AFB1), 3) 0.05% esterified glucomannan (EGM), 4) 100 ppb AFB1 plus 0.05% EGM, 5) 100 ppb AFB1 plus 0.1% EGM. The test diets were offered for 30 days followed by the negative control diet for 20 days. On the 30^{th}, 35^{th}, 40^{th}, 45^{th} and 50^{th} day of experiment, six birds from each group were sacrificed. Blood samples were tested for alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), aspartate amino transferase (AST) and alanine amino transferase (ALT). Liver samples were collected for histopathological findings. Muscle and liver tissues were analyzed for aflatoxin residues. On day 30, ALP was higher ($P<0.05$) in birds given dietary AFB1 than in those given the control diet, AFB1/EGM or EGM supplemented diets. The pattern of GGT activity was similar to the ALP activity from day 35. The liver of EGM supplemented birds given AFB1 showed markedly decreased bile duct proliferation, fatty degeneration and intermediate morphology of the hepatocyte and bile duct epithelial cells, in comparison to the AFB1 group. The residual levels of AFB1 and AFM1 were markedly higher in the liver than in the muscle. The levels in the liver and the muscle were highest in the AFB1 supplemented group and lower in the AFB1/EGM group. The study indicate that addition of EGM to diets containing 100 ppb AFB1 appears effective in reducing toxicity, however the 20 day withdrawal time was not sufficient for a complete resolution of the liver lesions and tissue residues. Even lower levels of AFB1 in duckling feeds should be required if all risk is to be avoided.

Key words : aflatoxin, esterified glucomannan, duckling, blood chemistry, liver lesion, residue

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Introduction

Aflatoxins are known to be hepatotoxic and hepatocarcinogenic agents in several animal species, especially young poultry (Shank, 1981; Angsubhakorn, 1983a, 1983b ; Bintvihok *et al.*, 1987a, 1987b, 1991a, 1991b). The most common acute response in most species is acute hepatitis with microscopic changes including diffuse degeneration in parenchymal cells, enlarged nuclei and extensive bile duct proliferation (Norred, 1986; Bintvihok *et al.*, 1991a, 1991b). Hepatocarcinogenicity of aflatoxins has been observed in several experimental animals and humans because of ingestion of aflatoxin contaminated feeds or foods in areas of Asia and Africa by epidemiological studies (Bruce, 1990). A large number of the observations have shown that hepatic metabolism plays a prominent role in determining the biological action of aflatoxin B1. Conversion of aflatoxin B1 to aflatoxin M1, aflatoxin P1, aflatoxin Q1, aflatoxin B2a and aflatoxicol are regarded to be detoxification steps, because the toxicity or carcinogenicity of these metabolites are lower than those of aflatoxinB1 (Campbell and Hayes, 1976; Shank, 1981; Larsson and Tjalve, 1992). Additionally, residues of aflatoxins and their metabolites might be present in meat, milk, eggs and other products of animals receiving aflatoxin contaminated feeds and could subsequently create health problems in man. The close relationship between aflatoxin intake and human liver cancer has been demonstrated in various areas of Asia and Africa (Bintvihok *et al.*, 1987a, 1987b, 1991a, 1991b; IARC, 1993; Trucksess *et al.*, 1983). The regulatory level for aflatoxin B1 in foods adopted in many countries is 5 ppb (WHO, 1979; Gilbert, 1991; Van Egmond, 1991).

In order to reduce aflatoxin residues and their metabolites in foods of animal origin, aflatoxin in feed must be detoxified. Appropriate mean include chemical treatment, physical separation and toxin binders (Bintvihok *et al.*, 1987a, 1987b, 1991c; Cole, 1999). The toxin binders are the most practical because of their low cost, minimum adverse effects on other nutrients and ease of application. Bound aflatoxins are not absorbed from the intestine and are excreted in the feces before they can harm the animals. Esterified glucomannan is a toxin binder consisting of functional carbohydrates extracted from yeast cell walls. It has a large surface area containing pores of difference sizes to trap a wide range of mycotoxins (Cole, 1999). In studies with food animals, most reports have dealth with responses in chickens, swine and dairy cattle, however little is known about response to toxin binders in ducks. The objectives of the present study were to examine detoxification of aflatoxins by

esterified glucomannan in the duckling in relation to hepatic metabolism as well as residues of aflatoxin B1 and its metabolites in edible tissues.

Materials and methods

Preparation of aflatoxin B1

Aspergillus flavus (local strain of Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand) was cultured on potato dextrose agar (PDA) and incubated for 7 days. The cultured mixtures were dissolved in distilled water, mixed in 30% moisture-commercial corn and kept in burlap or gunny bags for 1-2 weeks for aflatoxin B1 production (Bintvihok *et al.*, 1993). The aflatoxin B1-contaminated corn was then evaporated and adjusted to 14% moisture. Samples were ground and aflatoxin B1 content determined using an ELISA method (Tecna R&D Diagnostics-Biotechnology test kits). Diluted aflatoxin B1-contaminated corn and/or esterified glucomannan were mixed in feed and fed to ducklings. The feeds were prepared weekly and refrigerated until use.

Animals and management

One hundred and fifty 1-day-old Cherry Valley ducklings of both sexes were fed commercial conventional feeds for 5 days. Ducklings were then randomly divided into 5 dietary treatment groups of 30 ducklings each. The first group were fed commercial conventional feed and served as the control. The second and third groups were given the commercial diets containing 100 ppb aflatoxin B1 (AFB1) contaminated corn and 0.05% esterified glucomannan (EGM, Mycosorb, Alltech Inc. Nicholasville, KY, USA), respectively. The fourth and fifth groups received AFB1-contaminated diets (100 ppb) containing either 0.05 or 0.1% EGM, respectively. Test diets were offered continuously for 30 days followed by conventional feed for 20 days. Six ducks of each group were sacrificed by 70% alcohol intracerebral injection on the 30^{th}, 35^{th}, 40^{th}, 45^{th} and 50^{th} day of experiment (table 1). Blood samples were analyzed for hepatic enzyme activities. The tissues samples were collected for histopathology and residue analysis.

Analysis of hepatic enzyme activities

Alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) were determined as in IFCC methods, 1983. Aspartate amino transferase (AST) and alanine amino transferase (ALT) were measured as described by Willard and John, (1970).

Table 1 Dietary treatments of ducklings

	Treatment	Number [a]	Body Weight [b]
Group 1	Control (conventional feed)	30	115.7 ± 0.2
Group 2	100 ppb AFB1	30	113.7 ± 0.4
Group 3	0.05% EGM	30	113.7 ± 0.4
Group 4	100 ppb AFB1 plus 0.05% EGM	30	112.7 ± 0.2
Group 5	100 ppb AFB1 plus 0.1% EGM	30	112.8 ± 1.1

[a]Six ducks of each group were sacrificed on the 30th, 35th, 40th, 45th and 50th day of experiment.

[b]Body weight of ducklings are mean ± SD (gm/duckling).

Histopathology

Gross pathological findings were recorded throughout the necropsy date. Liver samples were removed from each bird and fixed in 10% buffered formalin. The conventional histopathological method for paraffin embedding, sectioning and hematoxylin and eosin staining (Sheehan and Hrapchak, 1980) were employed.

Analysis of aflatoxin B1 and its metabolites residues

Muscle and liver tissue samples were removed from each bird and frozen at -20°C for subsequent analysis of aflatoxin B1 and metabolites. Residues of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and their metabolites in the tissues were determined using a method of Association of Official Analytical Chemists (AOAC, 1995). Samples of 50 gm each were extracted and cleaned-up with sep-pak florisil cartridge and quantitatively analysed by HPLC. AFB1, AFB2, AFG1 and AFG2 were determined by HPLC with a normal phase column using water-saturated with chloroform:cyclohexane:acetonitrile:ethanol (50:15:2:1, v/v/v/v) as mobile phase at a flow rate of 1 ml/min, 40°C column temperatures and fluorescence detector. Aflatoxin M1 (AFM1) and aflatoxicol (AFR0) were determined by HPLC with a reverse phase column using 45% methanol as mobile phase at a flow rate of 1 ml/min, 40°C column temperatures and fluorescence detector. Standard aflatoxins for HPLC were purchased from Sigma Chem., Co., USA.

Data analysis

Statistical significance ($P<0.05$) was computed by using analysis of variance (ANOVA,two-way) and was conducted using the SAS program (SAS Institute Inc., 1989).

Results

Hepatic enzyme activities

From day 30 ALP activity was significantly higher in birds given AFB1 than in those given control or EGM-supplemented diets ($p<0.05$) (table 2). Beginning day 35, treatment-related responses in GGT activity were similar to those in ALP activity (table 3). AST and ALT did not show consistency of the results because of variation within treatment (Table 4-5)

Table 2 Alkaline phosphatase (ALP) activity (mean ± SE, U/L) in ducklings

Day sacrificed	Control	100 ppb AFB1	0.05% EGM	100 ppb AFB1	
				+ 0.05% EGM	+ 0.1% EGM
30	861.7 ± 5.1*	1040.0 ± 9.2*	893.8 ± 13.7*	896.7 ± 9.8 *	847.5 ± 7.9*
35	908.0 ± 8.4	1176.7 ± 17.7	975.0 ± 6.7	921.0 ± 17.1	940.0 ± 7.6
40	921.0 ± 14.2	1219.0 ± 22.3	983.0 ± 2.5	925.0 ± 13.6	821.3 ± 46.3
45	935.0 ± 2.5	1183.3 ± 19.4	942.0 ± 10.7	952.5 ± 5.3	926.0 ± 8.8
50	925.6 ± 4.8	1190.0 ± 77.8	957.5 ± 4.2	760.8 ± 14.4	871.3 ± 1.5

*Significant differences ($P<0.05$) of ALP activity in comparison among the dietary treatment groups was found from day 30.

Table 3 Gamma-glutamyl transferase (GGT) activity (mean ± SE, U/L) in ducklings

Day sacrificed	Control	100 ppb AFB1	0.05% EGM	100 ppb AFB1	
				+ 0.05% EGM	+ 0.1% EGM
30	998.3 ± 8.3	1008.3 ± 5.9	994.5 ± 6.4	995.0 ± 8.4	1008.3 ± 3.3
35	959.4 ± 19.5*	1054.0 ± 22.9*	960.5 ± 6.0*	945.2 ± 4.3*	954.2 ± 7.2*
40	954.4 ± 6.3	1039.2 ± 3.7	953.8 ± 3.6	985.3 ± 6.0	959.5 ± 10.2
45	979.3 ± 8.5	1031.5 ± 4.6	983.8 ± 6.4	950.5 ± 4.6	963.0 ± 2.0
50	978.1 ± 2.8	989.0 ± 6.4	985.3 ± 3.4	1001.0 ± 2.0	958.9 ± 3.2

*Significant differences ($P<0.05$) of GGT activity in comparison among the dietary treatment groups was found from day 35.

Table 4 Aspartate amino transferase (AST) activity (mean ± SE, U/ml) in ducklings

Day sacrificed	Control	100 ppb AFB1	0.05% EGM	100 ppb AFB1	
				+ 0.05% EGM	+ 0.1% EGM
30	37.0 ± 10.1	38.5 ± 2.8	39.6 ± 4.0	37.3 ± 4.8	34.8 ± 6.3
35	53.6 ± 2.0	41.8 ± 1.4	47.5 ± 2.4	66.0 ± 5.3	32.0 ± 1.9
40	36.6 ± 2.3	35.6 ± 1.9	33.0 ± 2.1	45.0 ± 6.3	40.0 ± 1.5
45	49.5 ± 1.0	54.8 ± 1.4	54.8 ± 2.7	58.5 ± 1.8	45.0 ± 2.6
50	51.0 ± 1.3	53.0 ± 0.0	44.8 ± 2.4	33.8 ± 1.1	55.9 ± 2.2

Table 5 Alanine amino transferase (ALT) activity (mean ± SE, U/ml) in ducklings

Day sacrificed	Control	100 ppb AFB1	0.05% EGM	100 ppb AFB1	
				+ 0.05% EGM	+ 0.1% EGM
30	90.0 ± 14.7	78.8 ± 4.5	66.0 ± 6.4	68.7 ± 2.7	76.0 ± 4.0
35	68.2 ± 2.7	66.2 ± 1.4	69.0 ± 2.3	63.8 ± 1.7	74.8 ± 3.4
40	82.0 ± 2.7	82.4 ± 1.7	82.6 ± 4.9	56.0 ± 8.3	78.3 ± 6.9
45	57.0 ± 2.7	63.7 ± 0.9	73.0 ± 3.0	76.3 ± 1.1	72.0 ± 1.2
50	65.1 ± 0.9	80.0 ± 1.4	63.3 ± 1.8	64.3 ± 0.8	71.3 ± 1.2

Histopathology

Livers of birds given AFB1-contaminated diets supplemented with EGM showed markedly less bile duct proliferation, fatty degeneration and intermediate morphology of cells between hepatocyte and bile duct epithelial cell in comparison to the AFB1 group during the experimental period (figure 1-6). The bile duct proliferation was primarily increased on the 30^{th} day of experiment (figure 2), while the fatty degeneration of hepatocytes were marked severe on the 35^{th} day of experiment in ducklings fed 100 ppb AFB1 (figure 3). In contrast, the bile duct proliferation of ducklings fed 100 ppb AFB1-0.05% EGMs on the 35^{th} day of experiment was mild lesions (figure 4). Until the 45^{th} day of experiment, the bile duct proliferation and intermediate morphology of ducklings fed 100 ppb AFB1 were moderate lesions (figure 5) while ducklings fed 100 ppb AFB1-0.1% EGMs showed only mild lesions of fatty degeneration of hepatocytes (figure 6). However, the other lesions of bile stasis, hepatic cell swelling and glycogen degeneration had no specific responsible for the experiment.

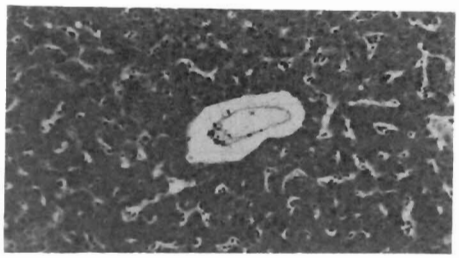
Fig.1 

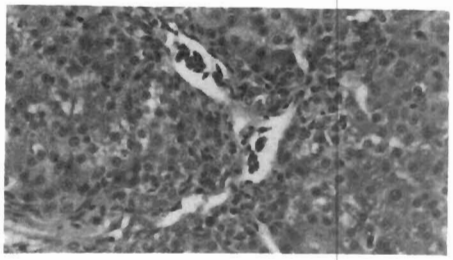
Fig.2 

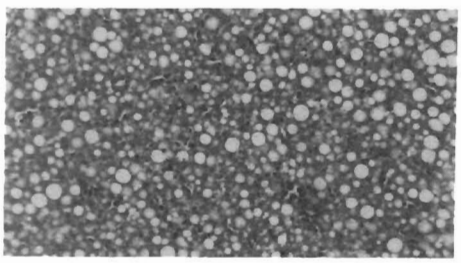
Fig.3 

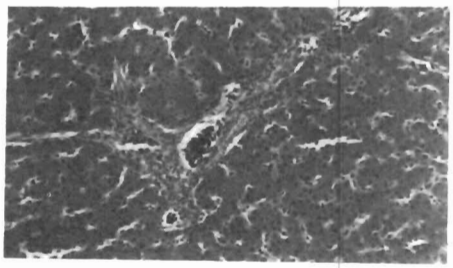
Fig.4 

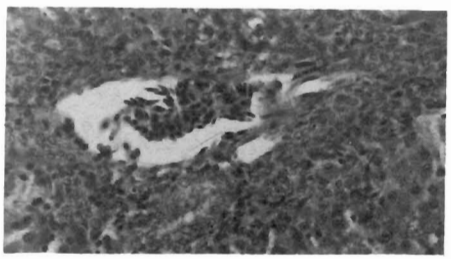
Fig.5 

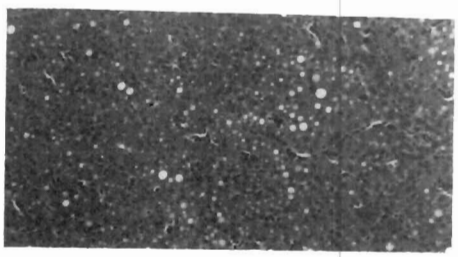
Fig.6 

Figure 1 Normal hepatic cell cord of control ducklings on the 30th day of experiment. **Figure 2** Moderate bile duct proliferation of ducklings fed 100 ppb AFB1 on the 30th day of experiment. **Figure 3** Severe fatty degeneration of hepatic cell of ducklings fed 100 ppb AFB1 on the 35th day of experiment. **Figure 4** Mild bile duct proliferation of ducklings fed 100 ppb AFB1-0.05% EGMs on the 35th day of experiment. **Figure 5** Moderate bile duct proliferation and intermediate morphology of ducklings fed 100 ppb AFB1 on the 45th day of experiment. **Figure 6** Mild fatty degeneration of hepatic cell of ducklings fed 100 ppb AFB1-0.1% EGMs on the 45th day of experiment.

Aflatoxin residues

Figures 7 and 8 showed aflatoxin M1 residues in muscle and liver of the ducklings. From day 30 of the experiment, residual levels of AFM1 were markedly higher in liver than in muscle. Figure 9 showed aflatoxin B1 residues in liver of the ducklings. On day 30 and 35 of the experiment, residual levels of AFB1 were found only in birds given dietary AFB1 whereas AFB1 residues were not found in the muscle. Residual levels in liver and muscle were highest in the AFB1 group, and residual levels decreased over the experimental period. However, AFM1 residues in liver were yet detected at the end of experiment. The AFB2, AFG1, AFG2 and AFR0 residues were not found in the tissues.

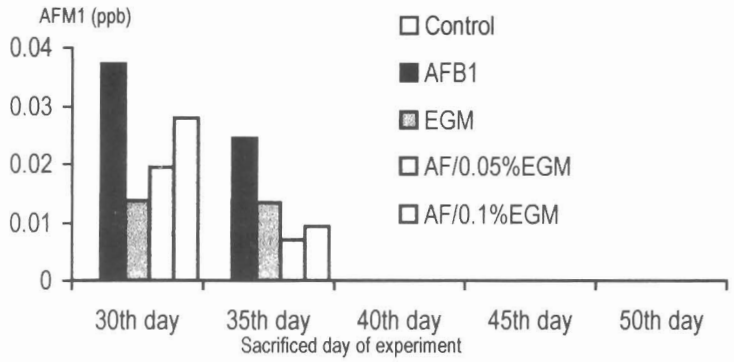


Figure 7 Aflatoxin M1 residues in muscle tissue of ducklings

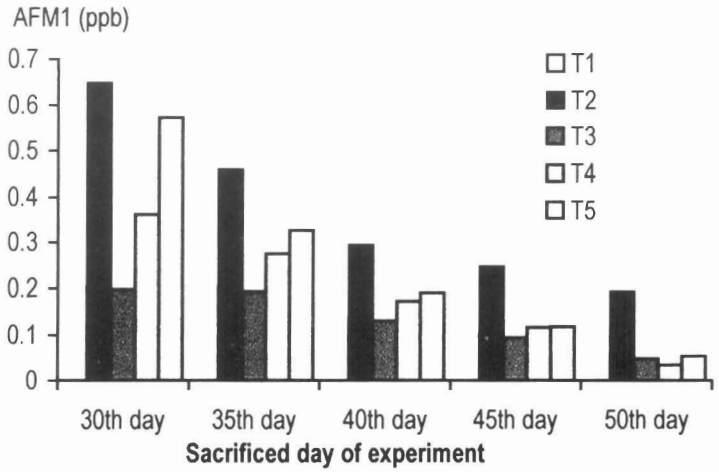


Figure 8 Aflatoxin M1 residues in liver tissue of ducklings

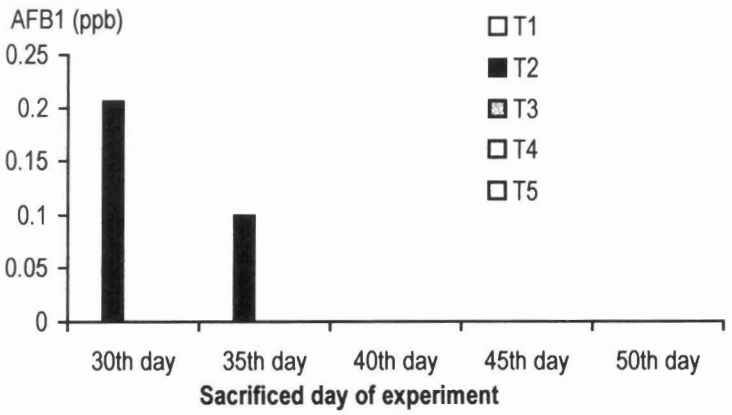


Figure 9 Aflatoxin B1 residues in liver tissue of ducklings

Discussion

From the above data, the hepatic enzyme activities data (ALP, GGT) agree with the data reports of Raju and Devegowda (2000) and Bintvihok *et al.* (1991a, 1991c) that the hepatic enzyme activities were increased by aflatoxins and were decreased by detoxifying agents or toxin binders. Moreover, the histopathological findings support the previous data reports of Bryden (1985), reporting on the response of one day old Pekin-Aylesbury ducklings fed 0.1, 0.2, 0.4 and 0.8 mg aflatoxin B1/kg diet at 7, 14 and 21 days of age, found the most severe bile duct proliferation in 14 day old animals administered 0.2-0.4 mg toxin. Bile duct proliferation was extensive in all treated groups. Clearly, duckling bioassay should be considered as only semiquantitative or qualitative (Butler 1974). Studies of the effects of aflatoxin B1 on liver histopathology in animals report swollen hepatocytes, fatty degeneration, bile duct proliferation, hepatic necrosis, hepatocellular necrosis, biliary cell proliferation of the periportal areas, liver cirrhosis, vacuolation in hepatocytes, interlobular fibrosis, periportal lipidosis, periportal lymphocytic infiltration in ducklings, chicks and quails (Uchida *et al.*, 1988 and Bintvihok *et al.* 1991b; 1997). After withdrawal of the food containing aflatoxins, all apparent gross lesions of aflatoxicosis disappeared, with no evidence of any lesions 8 days after removal of the contaminated diets (Chen *et al.* 1985). However, the liver lesions of this study have yet found at the end of experiment.

The aflatoxin residual data support the reports of Rizzi *et al.* (1998), and Bintvihok *et al.* (1987a, 1987b, 1990, 1991a, 1991c, 1998) that the levels of AFB1 and AFM1 in livers are higher than those data in muscles. The residual levels were decreased by detoxifying agents or toxin binders. Moreover, the conventional feeds contain AFB1 4.8 ppb and the birds given dietary EGM were found traceable AFM1 residues in muscle and liver. Since the early 1960, various studies have been undertaken to establish quantitatively the extent of conversion of AFB1 to AFM1 and also undertaken of the toxic effects of AFM1 in laboratory animals. However, in comparison to AFB1, relatively little is known about the toxicity of AFM1, primarily because of the difficulty in obtaining sufficient quantities of the pure compound necessary for extensive toxicity testing. The limited animal studies carried out to determine toxicity and carcinogenicity of AFM1 tend to come to the same qualitative conclusion that AFM1 has hepatotoxic and carcinogenic properties. Quantitatively, the subacute toxicity of AFM1 in ducklings and rats seems to be similar to or slightly less than that of AFB1. The carcinogenicity is probably one or two orders of magnitude less than that of the highly carcinogenic AFB1 (Eaton and Groopman, 1994). Therefore, metabolic

pathway of aflatoxins in the body should research furthermore. Results of this study indicate that addition of EGM to diets containing 100 ppb AFB1 for detoxification seem to be effective, but the 20 day withdrawal time was not sufficient for complete reduction of liver lesions and residues. Therefore, further lower levels of AFB1 in the duckling feeds should be required for avoiding the risk.

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