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Pattern of partitioning of aflatoxins from feed to urine and its effect on serum chemistry in Nili-Ravi buffalo heifers

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Abstract. The objectives of the present study were (1) to monitor the pattern of excretion of aflatoxinM1 in urine after its conversion from aflatoxinB1 and (2) to observe the effects of different levels of aflatoxinB1 in feed on serum concentrations of key metabolites glucose, total protein, cholesterol and urea as indicators of metabolic status. Nili-Ravi buffalo heifers ($n = 12$) of similar age and weight were randomly distributed to four groups. Animals in Groups A, B and C were offered a contaminated cottonseed cake-based concentrate ration at 0.5%, 1.0% and 1.5% of bodyweight, respectively. Control animals in Group D were fed with aflatoxinB1-free green fodder. Based on the level of contamination of the concentrate ration with aflatoxinB1 (554 µg/kg), Groups A, B and C consumed 953, 2022, 3202 µg of aflatoxinB1 daily. Feed samples were analysed at Romer Laboratories Pty Ltd, Singapore by high performance liquid chromatography. AflatoxinM1 quantification in urine samples was conducted using a competitive enzyme-linked immunosorbent assay with kits supplied by Helica Biosystems, Inc., USA. Serum samples were analysed for concentrations of glucose, total protein, cholesterol and urea using clinical chemistry kits provided by Human diagnostics (HUMAN, Biochemica und Diagnostica mbH, Germany). Carry-over rate of aflatoxinM1 in urine for Groups A, B and C was 15.51%, 15.44% and 14.04% of aflatoxinB1 while there was no detectable aflatoxinM1 in the urine of the control group (D). There was no significant difference in the concentrations of serum glucose, total protein and cholesterol between treatment groups. However, the concentration of serum urea was significantly higher ($P < 0.05$) in the group offered the highest level of aflatoxinB1-contaminated concentrate. This result suggests that mycotoxicosis may compromise protein metabolism and accretion in affected animals. This leaves open the possibility that high concentrations of aflatoxins in milk may ultimately affect the health status of human milk consumers.

Additional keywords: AFLB1, AFM1, mycotoxins, transfer rate.

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Introduction

Aflatoxins (mycotoxins produced by *Aspergillus flavus* and *A. parasiticus*) are readily absorbed and distributed to almost all vital organs and body fluids after ingestion (Stubblefield *et al*. 1981). The microsomal cytochrome P450 system in liver facilitates both activation and deactivation of aflatoxinB1 (AFB1). Oxidation of AFB1 results in the formation of the biologically active metabolite, AFB1–8, 9-epoxide (Kuilman *et al*. 2000). This metabolite can then react with RNA and DNA leading to hepatocellular carcinomas or with liver protein (Judah *et al*. 1993) to cause liver toxicity. AflatoxinB1–8, 9-epoxide is then converted into several less toxic metabolites such as aflatoxinM1 (AFM1), aflatoxinQ1 (AFQ1) and aflatoxinP1 (AFP1) after hydroxylation (Kuilman *et al*. 2000). The body has a mechanism to regulate toxicity through conjugation of AFB1 with glutathione, facilitated by glutathione S-transferases (Hayes *et al*. 1991).

The kidneys, lungs, liver and mammary glands were found to sequester the highest concentrations of total aflatoxins (Stubblefield *et al*. 1983) while brain, gall bladder, bile, small intestine, heart, skeletal muscles, spleen, supra mammary lymph nodes and tongue were also found to retain considerable amounts of aflatoxins. Truckness *et al*. (1983) found that the transfer of aflatoxicol, AFB1 and AFM1 to milk, plasma and red blood cells of the cattle is very rapid, reaching high levels within 1 h of dosing. Consistent with this rapid increase, the circulatory system is highly efficient at eliminating aflatoxin metabolites through milk and urine. Stubblefield *et al*. (1983) found concentrations of AFM1 in kidneys to be almost 40 times higher than the intact AFB1, showing extensive metabolism of the original feed contaminant in cattle. Thus urine is one of the major routes for excretion of AFM1 after its conversion from AFB1 in the liver (Nabney *et al*. 1967).

Pakistan is second in the world in buffalo milk (22.96 million tonnes) and meat (0.775 million tonnes) production after India (FAO 2011). Ingredients used as concentrate are often contaminated with fungi, which secrete mycotoxins that are then incorporated into the feed base (Sultana and Hanif 2009).

The pattern of secretion of AFM1 in buffalo urine may provide a means for measurement of mycotoxin likely to be stored in carcasses providing meat entering the human food chain. The objectives of the present study were (1) to determine the relationship between feed AFB1 status and urinary AFM1 contents and (2) to observe the effect of mycotoxins on key serum constituents in Nili-Ravi buffalo heifers.

Materials and methods

The government Buffalo Research Institute, Pattoki (latitude: 31°05′N and longitude: 73°52′E) district Qasur of province Punjab in Pakistan provided Nili-Ravi buffalo heifers (n = 12) of similar age (18.7–20.4 months) and liveweight (339–387 kg), which were randomly assigned to four groups offered with different levels of AFB1 naturally contaminated concentrate feed. Groups A, B and C were fed with contaminated concentrate at 0.5 (1.72 kg), 1.0 (3.65 kg) and 1.5 (5.78 kg) % of bodyweight, respectively; while Group D was kept as the control (animals were only fed with fresh green fodder, free of AFB1). Animals in each group were offered individually above-mentioned contaminated concentrate and *ad libitum* AFB1-free green fodder (Berseem, *Trifolium alexandrium*) for 10 days before the start of the experimental period of 5 days. The concentrate ration was highly contaminated with AFB1 (554 μg/kg). Total daily intakes of AFB1 for animals in Groups A, B, C and D were 953, 2022, 3202 and 0 μg over the 5 days, respectively. The AFB1-free green fodder was also available *ad libitum* during the 5-day test period and daily intake was recorded by weighing back feed residuals. Water was made available *ad libitum* and intake was measured on a daily basis. Total daily dry matter (DM) intake was calculated by adding the DM % of concentrate and green fodder. Total daily excretion of AFM1 was calculated by multiplying concentration of AFM1 (μg/L) by total urine production of that day.

Sample collection

Feed samples

A representative sample of green fodder was dried and preserved on Day 1 together with a representative concentrate sample. These were analysed for AFB1 at Romer Laboratories, Singapore.

Serum samples

Blood samples (10 mL) were collected by venipuncture using disposable syringes (19-gauge needles) and stored at 23°C for 2 h for serum to form. They were then centrifuged at 1200*g* at 25°C for 20 min to collect the serum, which was stored at −20°C pending analysis. Concentrations of glucose, total protein, cholesterol and urea were determined by using clinical chemistry kits provided by Human diagnostics (HUMAN, Biochemica und Diagnostica mbH, Wiesbaden, Germany). Serum samples were analysed using a chemistry analyser (Microlab 300) provided by the ELITech Group, Paris, France.

Urine samples

Foley balloon catheters (24-gauge, Ningbo Greatcare Meditech Co. Ltd, Zhejiang, China) were passed through the urethra into the urinary bladder of all animals on Day 1 after the adjustment period of 10 days and kept there for 5 days. These catheters were directed to airtight plastic bottles (20 L). Urine samples (5 mL) were collected every 24 h after mixing and stored at 4°C pending analysis. All animals were monitored continuously after catheter introduction.

AflatoxinB1 analyses

Samples of green fodder and contaminated concentrate ration were sent to Romer Laboratories, for AFB1 analysis and were analysed by high performance liquid chromatography (HPLC). Samples were also analysed for AFB2, deoxynivalenol, fumonisinB1, ochratoxinA and zearalenone.

Sample preparation and clean up

A total of 25 g of sample was ground. Ground sample was mixed well and then extracted with 100 mL acetonitrile/water (84 : 16). After blending for 3 min, it was filtered through folded filter paper. Tween 20 (33 mL of 1%) in PBS was then added in 2 mL of filtrate (acetonitrile/water sample extract). All diluted sample extract was applied to Aflastar Fit (immuno affinity column) columns. The sample was allowed to pass through the column at the rate of 1–3 mL/min. The column was then washed with 10 mL of PBS. All excessive liquid was removed and toxins were eluted from the column by applying two times 0.5 mL methanol followed by two times 0.5 mL of deionised water. After mixing, 100 μL was injected into HPLC.

HPLC

HPLC analyses were performed using an HPLC series 1100 from Agilent Technologies (Waldbronn, Germany). Chromatographic separation of AFB1 was conducted by use of an Agilent Zorbax SB-Aq column (4.6 mm × 150 mm, 5 um). The mobile phase applied was water/acetonitrile/methanol mixture (5/1/1), including 100 μL nitric acid and 0.3 g potassium bromide per L. The flow rate was 2 mL/min, column oven temperature 30°C, injection volume 100 μL. A Kobra cell was used for post-column derivatisation, fluorescence detector settings were 360 nm (excitation), 440 nm (emission).

AflatoxinM1 analyses in urine

AFM1 quantification in urine samples was conducted with a competitive enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. 991AFLMO1Y-96 Helica Biosystems Inc., Santa Ana, CA, USA). Mean recovery of AFM1 in spiked samples (0.5 and 2.0 ng/L) according to the manufacturer's specifications were 96.40% and recovery range was 78–111%.

Carry-over rate of aflatoxinM1 in urine

The carry-over rate of AFM1 in urine was calculated by following the formula:

$$\text{Carry-over rate} = \text{Total daily AFM1 excreted} / \text{Total daily AFB1 intake} \times 100.$$

Statistical analyses

Data were measured 5 times on each animals so they were analysed using linear mixed models with Group*Day as fixed effects and Animal/Day as random effects. As data for serum

concentrations of glucose, total proteins, cholesterol and urea were measured at only one time point, they were analysed by one-way ANOVA using a completely randomised design. GENSTAT 16th edition (Hemel Hempstead, UK) was used for all analyses.

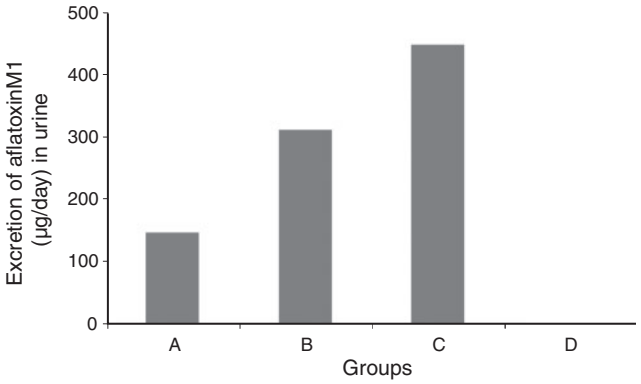

Fig. 1. Total daily mean excretion of aflatoxinM1 in urine of buffaloes exposed to different levels of aflatoxinB1 ($P < 0.001$: s.e.d. = 30.07). Note: Animals in Groups A, B, C and D were exposed to 953, 2022, 3202 and 0 µg/day, respectively.

Results

There was a significant ($P < 0.001$) difference in total daily excretion of AFM1 in urine among the four groups; higher levels of AFM1 in urine were associated with higher levels of consumption of AFB1 (Fig. 1).

There were no significant differences in urinary excretion between days, with levels remaining constant over the 5 days of treatment (Fig. 2).

A highly significant difference ($P < 0.001$) was observed in urinary AFM1 concentrations (µg/L) among Groups A, B, C and D. The mean concentrations of AFM1 (µg/L) were 14.37, 18.96, 24.17 and 0 for the animals fed with 953, 2022, 3202 and 0 µg/day of AFB1, respectively. There was no difference in the daily pattern of excretion of AFM1 (µg/L) over the 5-day experimental treatment period. A strongly significant ($P < 0.01$) difference in water intake was observed among all four experimental groups (Table 1). It was positively related with daily intake of the concentrate ration. There was a highly significant ($P < 0.001$) difference in water intake from day

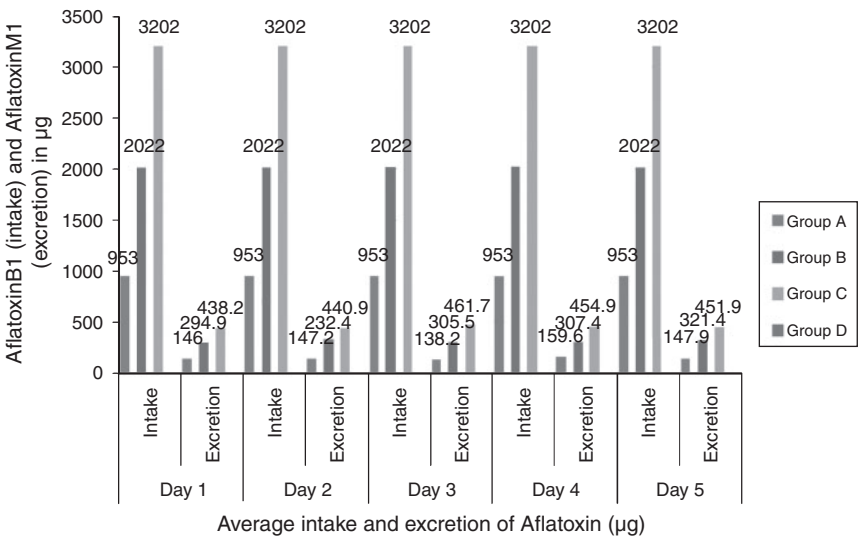

Fig. 2. Daily variation in excretion pattern of aflatoxinM1 in buffaloes exposed to various levels of aflatoxinB1 ($P > 0.05$: s.e.d. = 29.00). Note: animals in Groups A, B, C and D were exposed to 953, 2022, 3202 and 0 µg/day, respectively.

Table 1. Mean values for daily urine production, water intake, dry matter intake, carry-over rate, excretion of aflatoxinM1 and mean concentrations of serum constituents in buffaloes exposed to different levels of aflatoxinB1

Group	Urine production (L/day)	Water intake (L/day)	Dry matter intake (kg/day)	AflatoxinB1 intake in feed (µg/day)	AflatoxinM1 excretion in urine (µg/day)	Carry-over rate of aflatoxinM1 in urine (%)[A]	Glucose (mg/dL)	Total protein (mg/dL)	Cholesterol (mg/dL)	Urea (mg/Ll)
A	10.35	23.01	8.139	953	147.8	15.51	79.0	6.40	67.7	46.0
B	16.59	29.04	10.002	2022	312.3	15.44	65.7	6.17	88.0	62.7
C	18.67	33.35	12.252	3202	449.5	14.04	70.3	6.27	76.0	69.0
D	9.39	20.81	6.623	n.a.	0	n.a.	73.3	6.30	73.3	46.0
P-value	<0.001	≤ 0.01	<0.001	n.a.	n.a.	n.a.	0.744	0.990	0.617	0.030

[A]Carry-over rate = aflatoxinM1 excretion in urine/aflatoxinB1 intake in feed ×100.

to day. Mean water intake on Day 1 for all animals was 21.72 L, which was significantly ($P < 0.001$) lower than mean intakes for the subsequent days i.e. 26.37, 27.68, 28.78 and 28.21 L for second, third, fourth and fifth day, respectively (s.e.d. = 1.368). Consequently urine production in animals from each group was significantly ($P < 0.001$) different (Table 1). There was no significant difference in urine production from day to day. A highly significant ($P < 0.001$) difference in total DM intake was detected between groups and days. Total DM intake was significantly ($P < 0.001$) lower for the control animals (6.623 kg/day) relative to Groups A, B and C (8.139, 10.002 and 12.252 kg/day, respectively). Total DM intake for all groups on Day 2 was 8.945 kg, which was significantly lower ($P < 0.001$) than the values for the other days. No interactions were apparent between groups and days for total AFM1 excretion per day and per litre of urine, daily water intake, daily urine production or daily total DM intake.

Difference in the mean blood concentrations of glucose, total protein and cholesterol among treatment groups were not significantly influenced by treatment (Table 1). Mean concentration of urea in blood serum was significantly higher ($P < 0.005$) in Group C exposed to the highest AFB1 concentrate than all other groups.

Discussion

Deaths of several hundred calves in Australia (McKenzie *et al.* 1981), numerous animal deaths on a chinchilla farm in Argentina (Pereyra *et al.* 2008) and the death of 493 buffaloes in Landhi colony Karachi, Pakistan (Sultana and Hanif 2009) provide examples of the potential impact of acute aflatoxicoses in production animals. Nabney *et al.* (1967) reported a carry-over of AFM1 in urine of up to 5.94% in sheep. This result is clearly different from the results (14–15.5%) produced in the present study. The reason for this difference may relate to species differences as Nili-Ravi buffalo heifers were used in this experiment. Another reason may be the resistance of sheep to mycotoxins. AFM1was carried over at the rate of 1.23–2.18% of AFB1 in the urine of humans fed with contaminated corn and peanut oil in another study conducted by Zhu *et al.* (1987) when average daily intake of AFB1 was 58 µg/day. The reason for this reduced carry-over rate may be the difference in physiology between humans and ruminants.

A total of 4.52% of the aflatoxin ingested was excreted through milk (0.18%), urine (1.55%) and faeces (2.79%) in a study with beef cattle (Allcroft *et al.* 1968). The apparent lower transfer in this study could relate to the single dose of aflatoxin administered to animals. The sensitivity of the analytical technique (ELISA) used in the present study relative to the methodology in use 40 years ago may also contribute to the differences. The mechanisms of transfer may also vary between the heifers used in this study and lactating cattle. Different factors may influence the variation in carry-over rate (0.3–6.2%) of AFM1 in milk (Creppy 2002).

Transfer of mycotoxins to the human food chains is particularly common in developing countries. In Cameroon for example, 35.5% and 45.5% of the urine samples of children suffering from kwashiorkor and Marasmic kwashiorkor were positive, having mean values of 0.109–2.840 µg/L and 0.109–0.864 µg/L, respectively (Tchana *et al.* 2010). Another reason for the difference in AFM1 concentration in urine may be the synergistic effect with the presence of other mycotoxins. The concentrate ration used in the present study contained 50, 166, 230, 31.2 and 18 µg/kg of aflatoxinB2, deoxynivalenol, fumonisinB1, ochratoxinA and zearalenone, respectively. Various studies (Pozzi *et al.* 2001; Rajmon *et al.* 2001; Gelderblom *et al.* 2002) have reported synergistic effects of AFB1 with other mycotoxins at cellular and hepatic levels in different animals: similar effects may have occurred in the present study also.

Mean concentrations of glucose and cholesterol observed in this study were all higher than the concentrations reported by Hagawane *et al.* (2009). Concentrations of glucose and cholesterol were found to be 50.06 and 26.76 mg/dL, respectively, in healthy lactating buffaloes. This most likely is the result of these animals being in full lactation. Differences in the expression of urea with increasing mycotoxin levels could result from a disruption of protein synthesis either within the rumen in the synthesis of microbial protein or in the post-ruminal gastrointestinal tract. The mycotoxins may also be acting in muscle tissue and liver hampering protein synthesis and causing deamination and greater excretion of urea into the urine. This mechanism deserves further investigation as it may be important in inhibiting growth or lactational efficiency.

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