Pakistan J. Zool., vol. 52(1), pp 405-408, 2020. **DOI**: https://dx.doi.org/10.17582/journal.pjz/2020.52.1.sc10

Short Communication

Effects of Dietary Aflatoxin B1 on Physiological Biomarkers with Special Reference to Udder Health of Lactating Goats

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Article Information
Received 27 September 2017
Revised 22 May 2018
Accepted 11 June 2019
Available online 04 November 2019

Authors' Contributions
HAU and AZD conceived and designed the study. HAU and IH analysed mycotoxins and wrote the article. MI and MS statistically anaalysed the data. AZD and AJ supervised the study.

Key words
Aflatoxin B1, Lactating goats, HPLC, Aflatoxin M1.

ABSTRACT

The aim of present study was to determine carcinogenic metabolite aflatoxin M1 (AFM1) in milk and to investigate the effects of low doses of dietary aflatoxin B1 (AFB1) on physiological biomarkers with special reference to udder health and serum parameters of lactating goats. Thirty two lactating Beetal goats of 3-4 y age, weighing 40.91±0.285, were randomly selected, and equally divided into four groups. Group A was kept as control while animals of groups B, C, and D were individually fed daily with 30µg, 40µg and 50µg of AFB1, respectively, through naturally contaminated cotton seed cake for a 10 days period. Milk samples were tested for aflatoxin M1 (AFM1) through high performance liquid chromatography (HPLC), somatic cell count (SCC) and total viable count (TVC). Blood samples were analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels. Aflatoxin M1 (AFM1) was detected in all milk samples of the Group B, C, and D in concentration higher than 0.05 ppb. The AFB1 was excreted in milk as metabolite AFM1 @ 1.35-1.59%. Udder health and milk quality deteriorated as SCC and TVC increased. Levels of serum enzymes AST and ALT increased with ingestion of dietary AFB1. It is concluded that ingestion of very low level of AFB1 by lactating goats results in excretion of carcinogenic metabolite AFM1 in milk beyond the permissible level. Dietary AFB1 has role in sub-clinical mastitis and causes injurious effects on general health status of lactating goats.

Aflatoxins are produced by the genus *Aspergillus* (several species) of fungi as secondary metabolites, which contaminate plants and their products (Iqbal *et al*., 2010). In a study Aspergillus flavus and Aspergillus parasiticus were major isolates found in food samples. Aflatoxin B1 (AFB1) is acutely toxic (Ei-Gohary, 1995) and causes liver cancer (Etzel, 2002). The types of aflatoxin B1, G1, B2, and G2 are frequently encountered in feeds. Dairy cows receiving AFB1 contaminated diet excrete AFM1 in the milk as a metabolite of AFB1 that may also be transferred to other dairy products (Creppy, 2002). The metabolite aflatoxin M1 (AFM1) excreted in milk is carcinogenic (Firmin *et al*., 2011). In a study, the mould-contaminated diet significantly reduced feed intake and body weight gain in poultry (Liu *et al*., 2011). AFM1 attains its maximum concentration in 3 days while after 4-5 days of withdrawal of AFB1 it cannot be detected in milk (Yiannikouris and Jouany, 2002). The maximum allowable concentrations of European Communities for AFB1 in feeds and concentrates for dairy animals are 20 µg/kg and 5 µg/kg, respectively (Galvano *et al*., 1996) and the concentration of AFM1 in animal milk is limited to 0.050 µg/kg in the European Union (European Commission, 2003a). AFM1 cannot be destroyed by storage or processing, such as autoclaving, pasteurization (European Commission, 2006). The AFM1 excretion in milk after AFB1 ingestion depends upon various factors like species of animal, milk yield, frequency of milking (Tajkarimi *et al*., 2008). The milk products from contaminated milk may also have AFM1 (Haris and Staples, 1992). Blood acts as an indicator of the status of the animals exposed to toxicants and other conditions. Physiological status of an animal has impact on blood constituents. Several factors like genetic make-up, breed, age, sex, and management conditions are responsible to influence blood parameters of domestic

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0030-9923/2020/0001-0405 $ 9.00/0

animals (NseAbasi *et al*., 2014).

Pakistan is the home tract of high yielding beetal goats with average milk yield of 290 L/lactation of 130 days thus playing an important role in country's economy. These goats are known as poor man cow because of high milk production. These goats are usually fed with concentrate feeds in the prevailing feeding systems in the country. Feed can be a potential source of aflatoxins if not properly handled. Moreover Pakistan's climate can support fungal growth. In light of these facts the present study was conducted to evaluate the effects of dietary AFB1 on udder health, quality of milk and blood profile in lactating Beetal goats.

Materials and methods

Thirty two previously synchronized, lactating Beetal goats 3-4 y old having body weight 40.91 ± 0.285, were randomly selected. The goats with 6-8 weeks lactation period were randomly divided into 4 equal groups. After one week of adaptation period, group A was kept as control while group B, C, and D were fed with 200g, 267g and 333g of contaminated cotton seed cake to provide them 30µg, 40µg and 50µg of AFB1 per animal per day, respectively for 10 days. The concentration of AFB1 in experimental cotton seed cake was determined through high performance liquid chromatography (Masoero *et al*., 2007) with modifications. Briefly 25 g of grounded cotton seed cake were mixed with 84 ml of acetonitrile and 16 ml distilled water added with 5 g of sodium chloride. Acetic acid 70µl was added to 9 ml of the filtrate in a tube and was vortexed (Barnstead international company, M37610-33, USA). The mixture was eluted through immunoaffinity column (mycosep®, 226 aflazone + multifunctional columns, Romer labs, USA). HPLC column (Lichrospher® 100, RP-18, end capped 5µm, Germany) was used in the experiment. The concentration of AFB1 in experimental cotton seed cake was 150 µg / kg.

The milk samples were collected 24 h before the first AFB1 feeding and on days 5 and 10 of the experiment. The samples were shifted at 4°C to Quality Operations Laboratory, UVAS, Lahore for quantitative analysis of AFM1, SCC and TVC. AFM1 was determined in milk samples using HPLC technique (Masoero *et al*., 2007) with modifications. Briefly 50 ml defatted milk was passed through immunoaffinity column (AflaStar M1, Romer Labs, USA) at the flow rate of 2–3 ml min^{-1}. One ml acetonitrile was passed through the column, thus AFM1 was eluted. The amount of AFM1 per ml of milk was calculated by the formula; AFM1 = (area of sample/area of standard) × concentration of standard/50ml. Somatic cell count of all milk samples was determined through direct microscopic slide method (Rawool *et al*., 2007). TVC of all milk samples was determined by spread plate method of culturing according to the standard protocol (Welley *et al*., 2011). For analysis of serum enzymes AST, ALT, and ALP, three milliliter of blood sample from each goat was collected 24 h before and on days 5 and 10 of experiment through jugular venipuncture avoiding hemolysis. Commercial kits of Human diagnostics, Germany, were used for the determination of these enzymes according to the recommended methods.

The data were analyzed by one-way analysis of variance (ANOVA) using SPSS 18. Differences among mean values of experimental groups were tested using least significant difference (LSD) 95% confidence intervals. Paired t-test was used for the comparison of before and after treatment data of the same group.

Results and discussion

Different feedstuffs may become contaminated during improper storage and also during growing in the field (Richard *et al*., 2009). Cotton seed cake consists of certain components like protein and lipids that favor the growth and multiplication of fungi. These components act as nutrient source for fungi (Jones and King, 1990). As cotton seed cake usually contains high concentration of aflatoxins, so it was used as a source of dietary AFB1 to experimental animals in the study. Milk samples from all experimental goats were tested before AFB1 feeding and were found negative for AFM1 (Table I). AFM1 was detected in the milk of AFB1 treated groups, which indicates the metabolism of AFB1 into AFM1 in goats' body. AFM1 concentration in milk ranged from 0.210-1.073 ppb. AFM1 concentration showed linear increase with the dose of AFB1 (Table II). It was noted that even lowest dose (30µg/animal) of AFB1 used in this study resulted in excretion of AFM1 in milk beyond permissible level. Increase in AFM1 excretion in Greek indigenous goats' milk with increasing the amount of AFB1 administered has been reported by earlier researchers (Kourousekos *et al*., 2012).

SCC of milk increased in AFB1 treated groups B, C and D (Table II). The animals remained uninfected throughout the experimental period, therefore the increase in SCC cannot be attributed to clinical mastitis, which causes increase in SCC (Applebaum *et al*., 1982). The findings are not in agreement with earlier study, reported no changes in goats SCC when administered with pure AFB1 orally (Jones and King, 1990). However the results of current study agree with previous research in which high score of California mastitis test was observed in cows after AFB1 intake (Bansal *et al*., 2005). In one study differences in results were recorded regarding milk quality when cows were administered with same amounts of pure and impure

AFB1 (Brown *et al*., 1981). This increase of SCC can be attributed to sub-clinical mastitis in experimental animals as AFB1 causes immunosuppression (Applebaum *et al*., 1982).

TVC of all treated groups significantly increased after AFB1 consumption (Tables I, II). After feeding AFB1, the maximum TVC recorded in one goat was 6.0×10^4 cfu / ml of milk (Table II). These findings are contrary to the results previously described as after administration of AFB1 in pure form to lactating goats caused no changes in the TVC of milk (Applebaum *et al*., 1982). In present study TVC was positively correlated to SCC of the milk, while similar findings have been reported by earlier researchers (Cheng *et al*., 2002). The increase in TVC of treated groups may be attributed to the immunosuppressive effects of AFB1, as significant decrease in humoral and cell mediated immune response occurred in New Zealand white rabbits subjected to different levels of mycotoxins (Georgios *et al*., 2012).

Results showed significant increase in AST level of groups B, C and D, while remained statistically unchanged in control group (Tables I, II). ALP remained statistically unchanged in groups A, B and D, while increased in group C. The enzyme ALT level significantly increased in treated groups B and D only. The findings are in agreement with those described by earlier researchers, who administered AFB1 in 3 different levels to ewes and noted significant effect on AST and ALT levels (McDougal *et al*., 2007). Regarding ALP he reported decrease in its level with AFB1 administration, while in this study, present doses of AFB1 exerted no significant effect on ALP level in goats. After aflatoxin administration, increased AST activity in goats was recorded in a previous study (Prabu *et al*., 2013). In the present study, the level of AST was dose dependent so it can be assumed that AFB1 has significant effect on hepatocytes. The increase in AST and ALT can be attributed to the cytotoxic effect of AFB1 on liver cells. It has been described that AFB1 caused oxidative damage through lipid peroxidation induction in rats (Battacone *et al*., 2003).

Conclusions

In conclusions, ingestion of very low level of AFB1 by lactating goats results in excretion of carcinogenic metabolite AFM1 in milk beyond permissible level. Dietary AFB1 has role in sub-clinical mastitis and causes injurious effects on general health status of lactating goats.

Table I.- SSC (cells/ml), TVC (cfu/ml), AST, ALT, and ALP (units/liter), and AFM1 (µg/kg) levels of goats before AFB1 administration (Mean±S.E).

Parameter	Group A	Group B	Group C	Group D
SSC	$1.5\times10^{6}\pm2.31\times10^{5a}$	$2.43\times10^{6}\pm1.47\times10^{5b}$	$2.0\times10^{6}\pm2.98\times10^{5acb}$	$8.75\times10^{5}\pm1.25\times10^{5cd}$
TVC	$2.5\times10^{4}\pm1.63\times10^{3a}$	$2.18\times10^{4}\pm1.87\times10^{3a}$	$2.06\times10^{4}\pm2.39\times10^{3a}$	$2.25\times10^{4}\pm2.83\times10^{3a}$
AST	77.75 ± 2.46^{a}	93.13 ± 10.75^{b}	73.50 ± 1.33^{a}	92.50 ± 2.62^{b}
ALT	13.50 ± 0.73^{a}	15.25 ± 0.881^{a}	18.63 ± 0.865^{b}	20.50 ± 0.567^{b}
ALP	60.50 ± 6.907^{a}	187.25 ± 24.22^{b}	99.38 ± 4.37^{a}	167.38 ± 13.79^{b}
AFM1 residues	n.d	n.d	n.d	n.d

*Means in each row having different superscript are significantly different at $p\leq0.05$. AFM1, aflatoxin M1; AFB1, aflatoxin B1; SCC, somatic cell count; TVC, total viable count; S.E, standard error; cfu, colony forming unit; n.d, not detected; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

Table II.- SSC (cells/ml), TVC (cfu/ml), AST, ALT, and ALP (units/liter), and AFM1 (µg/kg) levels of goats after AFB1 administration (Mean±S.E).

Parameter	Control	AFB1 administered/day for 10 days		
		30 µg	40 µg	50 µg
SSC	$1.44\times10^{6}\pm1.75\times10^{5a}$	$4.13\times10^{6}\pm4.09\times10^{5b}$	$3.63\times10^{6}\pm 4.60\times10^{5bc}$	$1.38\times10^{6}\pm1.56\times10^{5a}$
TVC	$2.4\times10^{4}\pm1.9\times10^{3a}$	$4.3\times10^{4}\pm1.6\times10^{3bcd}$	$4.8\times10^{4}\pm3.3\times10^{3bc}$	$3.6\times10^{4}\pm3.3\times10^{3bd}$
AST	79 ± 2.21^{a}	110 ± 5.38^{bcd}	104 ± 2.01^{bc}	126 ± 9.45^{bd}
ALT	14 ± 0.32^{a}	20 ± 1.11^{bc}	21 ± 1.10^{bc}	28 ± 1.25^{d}
ALP	62 ± 7^{a}	223 ± 22^{b}	147 ± 4^{cd}	175 ± 16^{cd}
AFM1 residues	0^{a}	0.4049 ± 0.1013^{b}	0.5801 ± 0.0846^{bcd}	0.7960 ± 0.0871^{cd}

*Means in each row having different superscript are significantly different at $p\leq0.05$. AFM1, aflatoxin M1; AFB1, aflatoxin B1; SCC, somatic cell count; TVC, total viable count; S.E, standard error; cfu, colony forming unit; n.d, not detected; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

Acknowledgments

The authors gratefully acknowledge Higher Education Commission of Pakistan, for providing funding to this research project under grant No. 213-55937-2AV2002.

Statement of conflict of interest

The authors have no affiliations with any organization which may influence the results of the study.

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