

THE RELATIONSHIP BETWEEN AFLATOXICOSIS, OXIDATIVE STRESS AND INCIDENCE OF MASTITIS IN DAIRY CATTLE

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ABSTRACT

Received at: 27/12/2012

Accepted: 20/1/2013

The aim of this study was to determine the effect of aflatoxin B1 on redox status and incidence of mastitis in dairy cows. The current study was done on 32 lactating Holstein cows; the first group (10 cows) were received ration, with aflatoxins not exceed permissible limits, while the second group (22 cows) received ration containing high levels of aflatoxins. Two blood samples were collected from each cow the first sample was taken heparinized for plasma separation and estimation of oxidant/antioxidant parameters (total antioxidant capacity, glutathion peroxidase, catalase & superoxide dismutase, malondialdehyde, nitric oxide and determination of the cell mediated immune response. The second blood sample was taken for serum separation for evaluation of copper & zinc levels. In the same time, after California Mastitis Test (CMT) was done, double milk samples were taken aseptically from 32 lactating Holstein cows. One sample was examined for bacteriological studies while the other was examined for milk fat %, protein % and somatic cell count (SCC). The results of second group showed significant reduction at ($p < 0.05$) in antioxidant parameter; total antioxidant capacity, glutathion peroxidase, catalase & superoxide dismutase, while increased significantly at ($p < 0.05$) in oxidant stress parameters; malondialdehyde, nitric-oxide. In contrast copper and zinc showed non significant decreased. Concerning Cell mediated immune response; phagocytosis (%), killing (%) of polymorph-nuclear cells and lymphocyte stimulation index the data revealed a significant reduction at ($p < 0.05$). As a result of the impaired cellular immunity, all cows in second group suffered from subclinical mastitis. The changes in CMT scores and SCC levels in cow's milk in subclinical infections were due to some important mastitis pathogens., *S.aureus* was the most prevalent organism (31.25%) followed by *E.coli* (25.0%) then *str. dysgalactia* (15.63%) and Coagulase Negative Staphylococci (CNS) was (15.63%). Significant decrease at ($p < 0.01$) was observed in milk fat % and protein %. We observed that aflatoxin B1 increase oxidative stress, immunosuppression, which may decrease resistance to infection disease, predispose cows to incidence of mastitis resulting in increase SCC, decrease milk fat % and protein %.

Key words: *Aflatoxicosis, oxidative stress, Mastitis.*

INTRODUCTION

Contamination of animal feed with mycotoxins is a worldwide problem in animal production. The complex diet of ruminants, consisting of forage, concentrates and silage can be a source of diverse mixtures of mycotoxins that contaminate individual feed components concomitantly, there has been an increase in feed intake to meet the greater nutrient demand which often exposes cows to mycotoxins contaminated feed (Bennet and Klich, 2003 and

Queiroz *et al.*, 2012). The aflatoxins are a group of closely related to mycotoxins that are widely distributed in nature. Aflatoxins constitute toxic metabolites of the fungi as *Aspergillus Flavus* and *Aspergillus Parasiticus* (Payne, 1998 and Kourousekos, 2011). The most important of the group is aflatoxin B1 (AFB1) which has a range of biological activities and disorders depending on the duration of consumption and quantity of the toxin (Abdel-Wahab *et al.*, 2002 and Eraslan *et al.*, 2004). Such disorders could seriously affect animal

production, incidence of metabolic disorders, mastitis and elevated somatic cell count; consequently decrease milk production after aflatoxin is consumed by lactating animals (Singh *et al.*, 1996). It is metabolized to aflatoxin M1 which is excreted into the milk, factors affect carry over into milk include mammary alveolar cell membrane health, low molecular weight of aflatoxins (Masoero *et al.*, 2007).

Consumption of lesser amount of aflatoxin may result in impaired immunity and decreased resistance to infectious diseases and may attack nucleophilic nitrogen, oxygen and sulphur heteroatom in cellular constituents which could be due to the induction of oxidative stress which is classical defined as an imbalance between pro-oxidant and antioxidants resulting in overall increase in cellular levels of reactive oxygen species (Umberto *et al.*, 2011). As consequence dairy cow could predispose to incidence of mastitis which is directly related to the broad immunosuppressive effect of aflatoxin on cellular and humeral mediated immune response and decrease host resistance which could cause intramammary infection or make the gland more susceptible to infection (Oswald *et al.*, 2005)

The aim of the present study was designed to determine the relationship between aflatoxins as a feed born stress factor, its effect on redox status and their relation to the incidence of mastitis in dairy cattle.

MATERIALS and METHODS

Animal Grouping:

The current study was done in a private farm on 32 lactating cows. All animals in the course of this study were fed total mixed rations (TMR), nutrient concentrations met nutritional requirement for lactation according to NRC (2001), examined periodically for mycotoxins. All cows according to previous considerations were classified into two groups: first group (10 cows) were received TMR mixture, where the percentage of aflatoxins not exceed permissible limits which is 5ppb (FAO, 2004), the second group (22 cows) received ration containing high levels of aflatoxins (16-60 ppb)

Blood Samples:

Two blood samples were collected from each cow via jugular vein puncture the first sample was taken in heparinized vacuum tubes for plasma separation and estimation of oxidant/antioxidant parameters (total antioxidant capacity (TAC), glutathion peroxidase (GSH-Px), catalase (CAT) & superoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide (NO) and determination of the cell mediated immune response. The second blood sample was put on plain centrifuge tubes for serum separation and evaluation of serum copper & zinc levels

Oxidant/Antioxidant determination:

Samples were examined using commercial diagnostic kits (Biodiagnostic) for the following parameters; TAC according to Koracevic & Koracevic (2001), GSH-Px according to Paglia and Valentine (1967), CAT according to Aebi (1984), SOD according to Nishikimi *et al.* (1972) MDA according to Ohkawa *et al.* (1979) and NO according to Rajaraman *et al.* (1980). Copper & zinc levels were estimated by using atomic absorption spectrophotometer (Model, 3300, Parkin Elmer USA)

Cell mediated immune response:

The cellular immune response of first and second groups was assessed by the lymphocyte transformation using MTT lymphocyte blastogenesis micro assay was conducted previously according to Denizot and Lang (1991) with some modifications Maslak and Reynolds. (1995). Isolation from blood by the method described by Rouse *et al.* (1980). Estimation the percentage of bacteria phagocytised according to Woldehiwet and Rowant (1990) the percentage of killed bacteria was estimated according to the formula described by Woldehiwet and Rowant (1990).

Mycotoxins analysis in feed and milk:

Mycotoxins B1 were analyzed in feed by high performance liquid chromatography (HPLC-CBC-7210H Austerila) with fluorescent detection (FLD). We offer a variety of clean up columns according to Scott (1997). Mycotoxins M1 were estimated on milk by HPLC technique according to Baccaca *et al.* (2008).

Milk Sample:

Samples:

A number of 32 milk samples were taken from dairy cows. Each sample were collected in clean, sterile and dry McCartney glasses in duplicate and preserved in ice tank till examination. One sample was examined for bacteriological studies while the other was examined for milk composition and somatic cell count (S.C.C.).

Field Test (California Mastitis Test, CMT):

According to APHA(1992) for detection of subclinical mastitis in cattle, California mastitis test was performed on individual milk samples collected from each quarter udder of every cattle to detected subclinical mastitis. The C.M.T gives an indirect estimation of S.C.C. and it is based upon a gelling reaction between the nucleic acid of the cells and a detergent reagent depending on the amount of gel formation, samples were assigned to 3 categories negative or positive reaction in 2 grades (++ and+++).

Bacteriological examination:

All milk samples were collected from infected cows for bacteriological examination were cultured on different specific media (sheep blood media, Manitol salt agar, Edward's media MacConky agar media).

Isolation and identification of causative organisms:

Isolation and identification of causative organisms were done according to Toplly and Wilson (1998).

Measurements of milk constituents:

It was done by using infra milk analyzer 150, from Bentley. The following milk constituents were estimated, in both normal milk and milk from lactating cows suffered from subclinical mastitis: fat (%) and protein (%).

Statistical analysis:

The redox, milk composition and cell mediated immune parameters were subjected to T test analysis according to Senedecor & Cochran (1982).

RESULTS

The results in Table (1) showed significant reduction at (p<0.05) in antioxidant parameter (TAC, GSH-Px, CAT, SOD.), significant elevation at (P<0.05) in oxidant parameters (MDA, NO), while copper & zinc showed non significant decreased in group received aflatoxin B1 above permissible limit compared to group received aflatoxin B1 within permissible limit. Cell mediated immune response is elucidated in table (2) the data revealed that phagocytosis (%), killing

(%) of polymorphnuclear cells and lymphocyte stimulation index in the presence of phytohaemagglutinine (PHA) display a significant reduction at (P<0.05) due to exposure to aflatoxin B1 above permissible limit.

The present study in table (3) shown that *S.aureus* was the most prevalent organism (31.25%) followed by *E.coli* (25%) and *str. dysgalactia* (15.63%) while coagulase negative staphylococci (CNS) was (15.63%).

Moreover the study identified the changes in CMT scores and SCC levels in cow's milk in subclinical infections due to some important mastitis pathogens. As shown in table (4) there is a good correlation between CMT and SCC according to the infectious status of mammary quarters of examined cows. In the absence of infection, CMT score was negative and SCC range was 15-159×10³ cells/ml with arithmetic means 85.5×10³. In case of infection with minor pathogens, CMT score was ++ and SCC range was 210-245×10³ with mean 224×10³. While in case of infection with major pathogens CMT score was (+++) and SCC range was 250-800×10³ with mean 623×10³.

Table (5) showed the effect of udder infection on milk fat% and protein% the mean fat (%) in normal milk cows was (5.9±0.38) while in subclinical mastitis cow's milk the mean was (2.28±0.199) with significant decrease (p<0.05). While the mean protein (%) in normal milk cows was (4.39±0.17) while in subclinical mastitis cow's milk the mean was (2.65±0.139) with significant decrease (p<0.05).

Table 1: Effect of aflatoxin (B₁) exposure on oxidant/ antioxidant parameters in of dairy cows.

	First Group	Second Group
TAC mmol/ml	0.40±0.02	0.23±0.01**
GSH-P _x μ m/ml	78.47±3.58	60.71±3.91*
CAT μ/g	7.52±0.30	5.21±0.10
SOD μ/g	801.9±23.91	723.31±19.20*
MDA (nmol/ml)	4.3±0.13	6.93±0.27*
NO (μm/ml))	12.80±0.78	19.49±1.1**
Copper μg/dl	1.33±0.11	0.93±0.04
Zinc μg/dl	3.20±0.07	2.89±0.03

Mean significant from healthy at (p<0.05).

Table 2: Effect of aflatoxin (B₁) exposure on lymphocyte stimulation index (lymph.) phagocytosis % (phag. %) and killing % (killing %) in dairy cows in blood sample.

First Group			Second Group		
Lymph,	Phag. %	Killing %	Lymph.	Phag, %	Kiling %
1.44 ±0.12	85.34±1.82	80.2±2.06	0.89±0.08*	71.45±3.38*	68.4±1.9*

Mean significant from healthy at (p<0.05).

Table 3: Prevalence of M.O in cases of subclinical mastitis in cow's milk

Bacterial Isolates	No. Isolates	Prevalence
<i>S.aureus</i>	10	31.25%
<i>E.coli</i>	8	25.0 %
<i>St.dysgalactia</i>	5	15.63%
CNS	5	15.63%
Total no of milk samples	32	87.51%

Mean significant from healthy at (p<0.01).

Table 4: Effect of isolated M.O on CMT and mean S.C.C in examined samples of dairy cows.

Organism group	Samples No.	CMT Score	SCC (×1000)		
			Mean	SE	Range
Absence of infection	10	+	85	16	(11-159)
Minor infection	7	++	224	5	(210-245)
Major infection	15	+++	623	23	(370-800)

Mean significant from healthy at (p<0.01).

Table 5: Effect of aflatoxin (M₁) exposure on milk: fat (%) and protein (%) of dairy cows

Parameter %	Case of Udder	
	Normal	subclinical
Fat	5.9±0.38 (4.78-8.24)	2.28±0.19** (0.32-3.51)
Protein	4.39±0.17 (3.7-5.2)	2.65±0.13** (1.16-3.6)

Mean significant from healthy at (p<0.01).

DISCUSSION

Aflatoxins are highly oxygenated secondary metabolites produced by certain toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* growing on a variety of feed stuff (Manjulata, 2011). Data suggests a role for oxidative stress which is the pathogenesis of aflatoxicosis (Umarani *et al.*, 2008), induced by reactive oxygen species (ROS) which believed to be a primary factor in various cattle diseases including mastitis (Pietro, 2011). In the present study our results of oxidant and antioxidant status revealed that there was a significant decrease of antioxidant net work (TAC, GSH-Px, CAT & SOD) and increase oxidant levels of lipid peroxidation & NO in mastitic animals received contaminated ration with aflatoxin B1 above permissible limit, this results

agree with Umberto *et al.* (2011); Valko *et al.* (2007); Mclean Dutton (1995). This may be attributed to that aflatoxin B₁ in order to exert its effect, it must be converted to its reactive epoxide by the action of the mixed function mono-oxygenase enzyme system (cytochrome p450-dependent) in the tissues this epoxide is highly reactive and can reacts with several macromolecules including DNA, RNA and protein (Mclean and Dutton, 1995), in addition, Umberto *et al.* (2011) revealed that the induction of oxidative stress of AFB1 on bovine peripheral blood mononuclear cells revealed increase of intracellular the reactive oxygen metabolites (ROM) without any time dependent effect. On the other hand. some mycotoxins may induce the production of free radicals and/or the reduction of antioxidant defense since during toxicity (Meissonnier *et al.*, 2008),

leading to the induction of the oxidative stress which has to be regarded as a cause or as a consequence of the action of toxicant on cellular system and variable effects of mycotoxins on different cell systems which has been suggested that this may be due to different sensitivity related to the metabolic characteristic of different cell types (Muller *et al.*, 2004). Whatever, ROM content in animal cells may be increased by several factors including xenobiotics such as mycotoxin with the onset of oxidative stress conditions as a result of increase level of lipid peroxidation (Klaunig and Kamendulis, 2001) (Valko *et al.*, 2007).

AFB₁ has been shown to reduce T-lymphoblastogenesis, impair delayed cutaneous hypersensitivity and graft-versus-host reaction (Williams, 2004). Our results revealed that Cell mediated immune response phagocytosis%, killing % of polymorphnuclear cells and lymphocyte stimulation index reduced this results agree with (Meissonnier *et al.*, 2008; Valko *et al.*, 2007 and Jiang *et al.*, 2005). These results indicated that AFB₁ dietary exposure decreased cell-mediated immunity while inducing an inflammatory response these impairments in the immune response increased susceptibility to infectious diseases (Meissonnier *et al.*, 2008). At exposure levels of a strong reduction of the blastogenic potential in bovine lymphocytes hypothesizing a general inhibition of T-lymphocyte functions such as killer, helper, effector or other immune processes which may compromise the immunological surveillance mechanisms (Valko *et al.*, 2007). As the immune system is primary responsible for defense against invading organisms, the sensitivity of the immune system to mycotoxin induced immunosuppression arise from the vulnerability of the continually proliferating and differentiating cells that participate in immune mediated activities and regulate the complex communication network between cellular, humoral components and mycotoxins induced immunosuppression may manifest as depressed T or B lymphocyte activity and impaired macrophage/neutrophil-effector functions (Oswald *et al.*, 2005), suppressed immune function by mycotoxns may eventually decrease resistance to infectious diseases. Several reports show that mycotoxins such as aflatoxin are able to affect the inflammatory response (Contreas and Sardillo, 2011) Aflatoxin is able to affect the inflammatory response, it act at different levels they can directly affect the viability of phagocytes (macrophages and neutrophils, alternatively they can impair the of the activity of the secretory functions of these cells. AFB₁ihibits phagocytosis, intracellular killing and spontaneous production of oxygen radicals in rat also AFB₁ have suppressive effect on inflammatory cytokines the molecular-cellular basis and general

mechanism responsible for the broad immunosuppressive effect of AFB₁ appears to impaired protein synthesis (Oswald *et al.*, 2005)

Regarding to microbial count aflatoxin effect on udder health, resulting in mastitis, somatic cell count and total microbial count change agree with (Kourousekos, 2011 and Brown *et al.*, 1981), aflatoxin B₁ at a high enough dose are often bacterial counts of *St.agalctiae* and *S.aureus* in milk from infected quarter increased (Valko *et al.*, 2007) who found associated with suppressed immune response of cows, increased the oxidative damage in a cell or tissue, increased SCC as a result of mastitis, moreover These results are in a harmony with the results recorded by (Dohoo and Meek, 1982 and Sharma, 2003) who found good correlation between CMT score, SCC and infection status of udder halves.

AFM₁ is excreted in milk after aflatoxinB₁metabolism in the body. As a consequence of aflatoxin influence on the general condition of the animals together with the excretion into milk. The effect on milk quality, including changes in some milk components, concentrations of the milk fat and protein which reduced significant at (p<0.01) this results agree with (Kourousekos, 2011; Akbar and Majid, 2010; Queroz *et al.*, 2012). Other researcher did not recod any aflatoxin effects on fat values (Kutz, 2009), while the reduction of milk fatty acids in these results were attributed to the inhibition or decrease production of some enzymes (Kourousekos, 2011). Moreover aflatoxin found to cause chemical change in milk attributed to damage of lysosomes by aflatoxinB₁and possibly increased capillary permeability. Thus any chemical change in milk caused by aflatoxiin udder may cause obvious physical change in milk (Brown *et al.*, 1981) Opposite result (Kutz, 2009) found that the milk protein content no aflatoxin effects on it. Other make reason for a slight increase of total protein in blood level after aflatoxin consumption as well as for RNA polymerase suspended action, resulting in the inhibition of a lot of metabolic reactions, such as protein synthesis. Another explanation that decrease milk productions are related to decrease dry matter intake from feed contamination to mycotoxin and aflatoxin, however, feed consumption was affect by exposure time of diet with aflatoxin, age of animal and aflatoxin level in diet Dietary aflatoxin could have effect on protozoa population, rumen flora and cause decrease in cellulose utilization, volatile fatty acid, ammonium production and also it leads to increase inactivity of alkaline phosphatase enzyme in the rumen. These factors influence feed consumption, milk yield and composition (Akbar and Majid, 2010). Any chemical change in milk caused by aflatoxin udder resuling in physical change in milk. An increase in SCC during mastitis infection increase the

amount may cause obvious of destruction enzymes present in the milk, which increase the rate of deterioration of milk fat and protein, (Jillian, 2006).

The conducted investigations confirmed that, aflatoxin B1 has variable effects on different cell systems resulted in induction of oxidative stress and suppressed immune function which may eventually decrease resistance to infectious disease and exacerbation in existing intramammary infection and predispose cows for incidence of mastitis, decrease milk production, increase SCC, in turn increase the rate of deterioration of milk fat and protein.

ACKNOWLEDGMENTS

Extend great thanks and gratitude for Doctor Manal Gallal Chief Researcher in Biology department in Reproduction Research Institute Pyramid on scientific effort that provided for the completion of this study.

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العلاقة بين الافلاتوكسين والاجهاد التاكسیدی و حدوث التهاب الضرع فى الماشية الحلاب

منى عبد المنعم محمود ، هناء علام ، احمد شعبان ، ايناس محمد جمال الدين

تهدف هذه الدراسة الى تقييم تأثير الافلاتوكسين ب 1 على الاجهاد التاكسیدی و حدوث التهاب الضرع فى الابقار الحلابية. وقد اجريت هذه الدراسة على 32 بقرة حلاب و تم تقسيمهم الى مجموعتين المجموعه الاولى (10 بقرات) تتغذى على عليقة لا تتجاوز الحد المسموح به من الافلاتوكسين ب 1 والمجموعه الثانيه (22 بقرة) تتغذى على عليقة بها مستويات عاليه من الافلاتوكسين ب 1 ثم اخذ عينتين دم من كل حيوان الاولى لفصل البلازما لتقدير مستويات مضادات الاكسدة الكلية ونشاط انزيمات البيروكسيداز جلوتاثيون، الكاتالاز والسوبر اوكسيد ديسموتاز والمالوندايديد، و اوكسيد النيتريك وتقييم استجابة المناعة الخلوية . وعينة الدم الاخرى يفصل منها السيرم لتقدير النحاس والزنك كما تم فحص كل الابقار باختبار كاليفورنيا للكشف عن التهاب الضرع الغير ظاهرى و تم جمع عينتين لبن فى انابيب معقمة من كل بقرة من جميع الحيوانات عينة للفحص البكتيرى والاخرى لتقدير نسبة الدهن والبروتين باللبن والخلايا الجسدية. و اظهرت الدراسة نقصا معنويا فى مضادات الاكسدة الكلية و نشاط انزيم البيروكسيداز جلوتاثيون ونشاط انزيم الكاتالاز و نشاط انزيم اوكسيد السوبر ديسموتاز وفى المقابل اظهرت النتائج ارتفاعا معنويا فى مستوى المالوندايديد و اوكسيد النيتريك. ومن ناحية اخرى تأثرت الاستجابة المناعية الخلوية سلبا فقد حدث انخفاض معنويا فى الخلايا الليمفاوية والنسبة المئوية للخلايا الاكولة والخلايا الفاتلة. ونتيجة لذلك فقد ارتفعت الأصابة بالتهاب الضرع الغير ظاهرى فى المجموعه الثانيه وحدث انخفاض معنوي فى كل من نسبة الدهن ونسبة البروتين فى اللبن تلك المجموعه. هذا وقد تم الكشف عن وجود الميكروبات الأتية: المكور العنقودى الذهبى (25.31%)، أى كولاى (25%) ، السبى ديس جالاكتيا والعنقودى سالب الكواجيلولاز (63.15%) لكل منهما. نستخلص من هذه الدراسة التأثير السلبى للافلاتوكسين على الابقار الحلاب مما ادى ارتفاع مستوى الاجهاد التاكسیدی وحدث انخفاض فى المناعة مما ادى ارتفاع نسبة تعرض الابقار الحلاب للأصابة بالتهاب الضرع الغير ظاهرى و حدوث ارتفاع عدد الخلايا الجسدية وانخفاض فى كل من نسبة الدهن ونسبة البروتين فى الالبان.