Research Article

Seasonally Feed-Related Aflatoxins B$_1$ and M$_1$ Spread in Semiarid Industrial Dairy Herd and Its Deteriorating Impacts on Food and Immunity

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To comparatively determine the levels of aflatoxin (AF) B$_1$ in feedstuffs and of AFM$_1$ in milk from semiarid industrial cattle farms in northeastern Iran during four seasons and to elucidate the effects of mixed AFB$_1$ and AFM$_1$ on bovine granulocytes, 72 feedstuffs (concentrate, silage, and totally mixed ration (TMR)) and 200 bulk milk samples were simultaneously collected for ELISA-based AFs detection. Isolated blood and milk neutrophils ($n=8$/treatment) were also preincubated with mixed of $10\text{ng/ml AFB}_1$ and $10\text{ng/ml AFM}_1$ for $12\text{h}$; the impact was assessed on neutrophils functions. AFB$_1$ levels in feedstuffs averaged $28\mu\text{g/kg (4–127}\mu\text{g/kg)}$, with TMR maximal ($38\pm6.3\mu\text{g/kg}$), concentrate ($32\pm6.5\mu\text{g/kg}$), and silage ($16\pm1.5\mu\text{g/kg}$). The levels of AFB$_1$ and AFM$_1$ in feedstuffs and milk averaged $42\pm9.3$, $27\pm2.8$, $26\pm4.1$, and $18.5\pm2.8\mu\text{g/kg}$ and $85\pm7.3$, $62\pm6.1$, $46\pm6.2$, and $41\pm6.5\mu\text{g/kg}$ in winter (maximal), autumn, spring, and summer, respectively. Mix of AFB$_1$ and AFM$_1$ weakened various functions of granulocytes. It adds new reason why during winter semiarid raised food-producing animals show more immune-incompetence.

1. Introduction

Aflatoxins (AFs) are highly carcinogenic and immunotoxic secondary metabolites produced mainly by Aspergillus (A) flavus and A. parasiticus in feed/foods [1–4]. Environmentally, AFs exposure is inevitably rising [1, 5, 6], and their seasonal spread and impacts in semiarid industrial dairy farms’ microenvironment remain unaddressed. Inappropriate environmental condition leading to the formation of aflatoxigenic molds varies and could be maximal during harsh seasons [7–10].

By contaminating agricultural commodities, AFs alarmingly cause immunodeficiency and cancer risks in mammals [2, 3, 5, 11]. Unfortunately, pivotaly food animals increasingly consume aflatoxigenic mold-contaminated feed, worldwide [5, 7, 10]. Advanced analytical methods on AFB$_1$/M$_1$ detection have revealed that even in Europe AFs routinely circulate in the body of food-producing animals and humans through feed/foods [12, 13]. Predominantly hydroxylated AFB$_1$ metabolite (AFM$_1$) is as carcinogen as AFB$_1$, and bioaccumulates everywhere (meat, milk, etc.) in vivo [5, 14, 15].

It is worrying reality that AFs levels in feedstuffs can be far higher than what we apparently measure. Inextricable link of various cancers to environmentally relevant levels of AFs, existence of masked AFs, and limited analytical capabilities for detection of AFs in agricultural commodities, sera, and dairy products have rendered the aspects of AFs’ detection and impact the focus of most concern [1–3, 5].

With EU’s tolerable levels for AFB$_1$ in feed (2–8\mu\text{g/kg}) and AFM$_1$ in milk (0.05\mu\text{g/kg}) (the EU, 2010) and Iran’s permissible AFB$_1$ in feed (5–20\mu\text{g/kg}) and foods (5–15\mu\text{g/kg}) and AFM$_1$ in milk [(0.5\mu\text{g/kg}, kg is equal to liter in case
for milk) (Anonymous, 2002) (due to contaminated milk, the risk for lactating mammals is much higher compared to nonlactating ones) and globalization, routine monitoring of AFs is more urgent [16]. Due to highly carcinogenic and immunosuppressive nature of AFs [even permissible level of AFB$_1$ is hazardous for immune cells/molecules in humans and animals [2–4]] and the lack of information on the immunotoxic influence of mixed AFB$_1$ and AFM$_1$ we aimed to (1) determine the seasonal levels of AFB$_1$ in various feedstuffs and of AFM$_1$ in bulk milk obtained from industrial cattle farms in agroecologically and geopolitically important semiarid northeastern Iran and (2) examine the in vitro effects of environmentally relevant levels of mix of AFB$_1$ and AFM$_1$ on different functions (free radicals or ROS production, phagocytic and killing capacity, and necrosis) of blood-and-udder’s key innate immune cells, neutrophils (PMN), using luminometry, flow cytometry, and bactericidal assays for two major vertebrates’ superbugs (i.e., Staphylococcus (S.) aureus and Escherichia (E.) coli).

2. Materials and Methods

Cluster random 72 feedstuffs (silage, concentrate, and totally mixed ration, TMR) and bulk milk (n = 200) samples were simultaneously seasonally obtained from industrial cattle farms (see Table 1 and Figure 1; n = 25 farms) in sterile cold condition accordingly (ISO 6497 2002 [10]). Much lower detected levels of AFB$_1$ and AFM$_1$ were eventually used for in vitro effects of mix of AFB$_1$ and AFM$_1$ on isolated bovine blood and milk PMN.

Indirect ELISA were performed according to the test kits for feedstuffs (AFB$_1$ ELISA, EuroProxima, Beijerinckweg, The Netherlands) and bulk milk (Ridascreen$^\text{®}$ AFM$_1$, RBiopharm, Germany), by measuring absorbance at 450 nm with ELISA reader (ELx 800, BioTek Instruments, USA). The lower detection limit for AFB$_1$ and AFM$_1$ was 0.05–0.1 µg/kg.

For in vitro effects of AFs on blood and milk PMN a group of 12 physiologically healthy dairy cows were used for PMN isolation and analyses, accordingly [2, 17].

AFB$_1$ and AFM$_1$ were obtained from Sigma and prepared according to Mehrzad et al. [2], with some modification (i.e., AFM$_1$ was directly dissolved in DPBS); they were then separately but simultaneously added, at a final concentration of 0 (control) and 20 ng/ml (10 ng/ml AFM$_1$ plus 10 ng/ml AFB$_1$) to the PMN cultured in complete RPMI 1640 medium (12 h, 37°C, 95% humidity, 5% CO$_2$). PMN were then washed and chemiluminescence (CL), flow cytometry-based neutrophils phagocytosis, and necrosis were measured according to the reported procedure [2, 3].

To evaluate the effect of AFs on superoxide anion ($O_2^-$) production and myeloperoxidase (MPO) activity of blood PMN, $O_2^-$ production was measured in post-AFs treated PMN by converting to nM of cytochrome $c$ reduced using the extinction coefficient $E_{550\text{ nm}} = 2.1 \times 10^4$ M$^{-1}$ cm$^{-1}$ as described [18]. Post-AFs treated PMN MPO activity was measured based on the oxidation of ortho-dianisidine (0.8 mmol/l) of supernatant of sonicated PMN extract containing 0.1 mmol/l of added H$_2$O$_2$ using microtiter plate spectrophotometer at 450 nm (Multiskan Plus Type 314, Lab-systems, Helsinki, Finland). The analyses of phagocytosis and killing of E. coli and S. aureus were done accordingly [2, 17].
Figure 1: Upper panel, map of the study area showing many intensively large scaled industrial cattle farming in part of semiarid northeastern Iran, where feed and milk samples were seasonally obtained. Lower panel (a), chemical structure of aflatoxins (AFs) B₁ (C₁₇H₁₂O₆) and M₁ (C₁₇H₁₂O₇) routinely found in animal feed and milk. AFM₁ is derivative of hydroxy AFB₁ and predominant secondary metabolite of AFB₁. One oxygen atom more in AFM₁ leads to a polar lipophobic toxin. Middle, aflatoxin B₁ levels in different types of cattle feed (n = 72 feedstuffs), and lower, aflatoxin M₁ levels in bulk milk (n = 200) during four seasons (in y-axes, kg is equal to liter). Different letters with their symbols (i.e., #, ¥, and € for concentrate, silage, and TMR/totally mixed ration, resp.) represent significant difference between their respective treatments (P < 0.05 versus control).
3. Results

Of 72 feed samples, more than 52 (average 72.2%) were contaminated with far above permissible AFB₁ in feed (Table 1), from which the levels of AFB₁ contamination in concentrate, corn, silage, and TMR were 75%, 62.5%, and 79.2%, respectively. The intensity of AFs spread was higher in autumn-winter (Table 1 and Figure 1B). AFB₁ level in feedstuffs ranged from 4 to 127 μg/kg with average of 28 μg/kg. Among feedstuffs TMR showed maximal level of AFB₁ (38 ± 6.3 μg/kg) followed by concentrate (32 ± 6.5 μg/kg) and corn silage (16 ± 1.5 μg/kg). Maximal level observed in winter followed by autumn, spring, and summer averaged 42.9 ± 3.2, 27 ± 2.8, 26 ± 4.1, and 18.5 ± 2.8 μg/kg, respectively (Figure 1B). Similarly, bulk milk AFM₁ level ranged between 85 ± 7.3, 62 ± 6.1, 46 ± 6.2, and 41 ± 6.5 μg/kg, in winter, autumn, spring, and summer, respectively, (Figure 1C) in all cases, kg is equal to liter.

The PMN phagocytosis-(in)dependent CL/(non)particle-stimulated luminol-enhanced CL (Figures 2(a1) and 2(a2)) and the flow cytometry-based phagocytosis assay of blood (Figure 2(c1)) and milk (Figure 2(c2)) PMN consistently revealed a significant decrease in phagocytic activity and killing capacity by AFs-exposed PMN. Among PMN stimulated with PMA, latex beads, or Pansorbin®, the AUC for the mix of AFs-exposed blood and milk PMN were, respectively, 16, 38, and 30% and 18, 37, and 31% lower than control PMN. Further, T_max in the AFs treated blood PMN stimulated with PMA, latex, and Pansorbin was slightly decreased (data not shown). Interestingly, the luminol-dependent CL arising from added hypochlorite (HClO) was significantly higher in mix of AFB₁ and AFM₁-treated group (Figure 2(b), upper panel insert). Furthermore, while low levels of mix of AFB₁ and AFM₁ showed little effects on blood PMN necrosis (Figure 2(c)), it decreased both the production of O₂⁻ (P < 0.01) by PMN (Figure 2(d)), and the MPO activity (P < 0.05) of PMN (Figure 2(e)). Unlike post-AFs treated necrotic PMN, neutrophils exposed to mix of AFB₁ and AFM₁ were less efficient to phagocytose and kill S. aureus and E. coli (see Figures 2(a) and 2(b)).

4. Discussion

The level which is alarmingly higher than the permissible levels of AFs in feedstuffs and milk (5 μg/kg for feed AFB₁ and 5 ng/kg for milk AFM₁) is largely attributed to climate conditions and inappropriate management of grasses and grains during harvest, transportation, drying and mixing stages [7, 19]. For example, improper processing/storage condition of higher dry matter corn silage likely boosts AFB₁ production [6, 7, 10], due mainly to weakening anaerobic bacterial mediated lactic acid production, increasing mi(a)croenvironment pH, thereby boosting aflatoxicogenic fungal growth. Owing to being categorized as group-1 carcinogens, many countries and regulatory agencies have harshly imposed tolerable limits on AFs. For example, the European Commission has set tolerable limit for AFB₁ and total AFs (B₁, B₂, G₁, and G₂) 2–8 μg/kg and 4–15 μg/kg, respectively, in crops such as nuts and grains (EU, 2010); furthermore, Iran has set the tolerable limit for AFB₁ for feedstuffs and foods 5–20 μg/kg and 5–15 μg/kg, respectively (Anonymous, 2002); also, maximal tolerable for milk AFM₁ in EU and Iran is 0.05 and 0.5 μg/kg, respectively. As such, routine harsher monitoring of AFs levels in different feedstuffs is urgent in semiarid northeastern Iran. For the detection of AFB₁ and AFM₁, herein we used ELISA with sensitivity and specificity of above 98% with acceptable recovery rate, LOD/LOQ, and precision; nevertheless it is worth simultaneously confirming their quantification with some more advanced analytical tool like HPLC or LC/MS/MS as done by others [12, 13].

Often, in droughty and poor crop years semiarid northeastern Iran, cows are normally more exposed to mold/AFs contaminated feed, and so are warm-wet weather, delayed harvest, snow cover conditions, infrequent daily preparation/provision of TMR, and so on [6]. Also, in Iran almost all agricultural commodities are sent into commercial channels with zero pasture-based cattle farming; further, key component of animal feed, corn, is mainly imported and thus not fresh; in contrast, in developed countries ~30% feeds are retained for on-farm use, efficiently lessening AFB₁ exposure. Mean (minimum, maximum) relative temperature (°C) and absolute humidity (%) in our study area during April-May, July-August, November, and February were 21.9 (15.1, 28.7), 28.1 (20.4, 35.7), 9.6 (3.9, 15.4), and 5.1 (−3, 11.6) and 45 (25, 66), 25 (11.5, 37.5), 63.5 (42, 84), and 64 (40, 87), respectively, potentially easing fungal growth and AFs spread in feed and milk (Figure 1), thereby worsening feed/food quality in autumn-winter.

During harsh seasons more contaminated moldy feeds are often used in feedlots; analytical incapability to detect very low (insensitive) levels of AFs and lack of AFs toxicity data in cows [5] exacerbate the complexity of AFs exposure in food animals and humans.

Among various feedstuffs analyzed in central and northeastern Iran, concentrate was maximally contaminated to both aflatoxicogenic molds and AFB₁ [9, 10]. Though a tiny amount of feed AFB₁ appears in milk of AFB₁-consumed dairy cattle, AFB₁ simply transmit into milk as AFM₁ with high cancer risks for consumers, especially neonates and children, who consume far more milk and dairy products. AFM₁ is hardly degradable by pasteurization procedure [5, 15]; nonetheless, fermentation could lead to biodegradation of AFs in fermented dairy products and thus less toxicity.

The background of the selected doses examined here was (1) in line with the first part of this study on AFB₁ and AFM₁ quantification, (2) our current understanding on the metabolism and toxicodynamics of AFB₁, and (3) from researchers [2–5, 20] in bovine, porcine, and human in vitro models. Researchers have even used higher doses of AFs [21]. Although our knowledge on interaction(s) of AFs effects in animals, especially food/dairy-producing ruminants, is...
Figure 2: Upper panel, luminol-dependant chemiluminescence (CL) in blood (a1) and milk (a2) neutrophils incubated with(out) 20 ng/ml of mix of aflatoxins (AFs) B₁ and M₁ for 12 h and stimulated with PMA, latex beads, and opsonized Pansorbin; values are expressed as the area under the curve (AUC) of relative light units (RLU)/s of 1000 viable neutrophils during 30 minutes. The inserted images representatively confirm the high purity and similar light microscopic appearance of neutrophils in both groups. Effect of above-mentioned level of mix of AFs on luminol-dependent CL from added hypochlorite (HClO) (b), necrosis (c), O₂⁻ production (d), and myeloperoxidase (MPO) activity (e) in neutrophils. Lower panel, mix of aflatoxins (AFs) B₁ and M₁ weakens phagocytic activity and killing capacity of blood neutrophils. Effect of 20 ng/ml of mix of AFB₁ and AFM₁ (non)treated for 12 h on the phagocytosis and killing activity of neutrophils against E. coli (a), S. aureus (b), and phagocytosis of 1 μm fluorescent microparticles by blood (c1) and milk (c2) PMN. Values are means ± SEM of 8 (*P < 0.05; **P < 0.01 versus control).
limited, nevertheless it is worth mentioning the point of intake of AFB\textsubscript{1} with the diet and its transfer to milk in high yielding lactating cows. Given that a cow with average daily milk production of 50 litter is consuming ~40 kg TMR per day and ~50% of AFB\textsubscript{1} in contaminated TMR goes to the blood with a tenth (mainly converted to AFM\textsubscript{1}) to milk [20, 22], the assumed AFB\textsubscript{1} and AFM\textsubscript{1} in blood/milk can accumulatively reach above our \textit{ex vivo} tested levels.

The inhibiting effect of AFs on the killing capacity of PMN for \textit{E. coli} and \textit{S. aureus} was manifested mainly by reduction of MPO activity and intracellular ROS production; this further confirms the negative effects of mix of AFB\textsubscript{1} and AFM\textsubscript{1} on the innate immunity; that is why AFB\textsubscript{1}-exposed dairy cows are more susceptible to environmental infections, specially mastitis and metritis (unpublished data). Nevertheless, we know little on how mix of AFB\textsubscript{1} and AFM\textsubscript{1} behaves in the phagosome and lysosome, where extremely large amounts of oxidants and granule constituents, through MPO-H$_2$O$_2$-HOCl system, are released [2, 17, 23]. What happened on different stages of PMN necrosis and apoptosis remained further investigation.

In short, a relatively high level of feed and milk AFs with more pronounced AFB\textsubscript{1} spread in cereal crops during winter and considering the economic, health, and cancer importance of AFs, percussion measures, hugely requires boosting food/health quality and lowering the risk of AFs exposure in farm animals and human in the study region.

**Additional Points**

**Practical Applications.** Poor harvesting, drying, transportation, and storage of feed/agricultural commodities lead to mold growth and particularly AFs formation in feedstuffs and milk and thus many animal and public health risks. Feedstuffs and bulk milk were sampled from various industrial dairy farms during 4 seasons in semiarid northeastern Iran for AFB\textsubscript{1} and AFM\textsubscript{1} detection and their \textit{ex vivo} effect on peripheral granulocytes. Alarmingly, maximal immunotoxic levels of AFB\textsubscript{1} and AFM\textsubscript{1} in feedstuffs and milk occurred in winter followed by autumn, spring, and summer. More controls of feed materials would be needed to prevent an undesirable contamination of feed, foods, and milk/dairy products.

**Conflicts of Interest**

No potential conflicts of interest were reported by the authors.

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