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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 mix of 10 ng/ml AFB_1 and 10 ng/ml AFM_1 for 12 h; the impact was assessed on neutrophils functions. AFB_1 levels in feedstuffs averaged 28 μg/kg (4–127 μg/kg), with TMR maximal (38 ± 6.3 μg/kg), concentrate (32 ± 6.5 μg/kg), and silage (16 ± 1.5 μg/kg). The levels of AFB_1 and AFM_1 in feedstuffs and milk averaged 42 ± 9.3, 27 ± 2.8, 26 ± 4.1, and 18.5 ± 2.8 μg/kg and 85 ± 7.3, 62 ± 6.1, 46 ± 6.2, and 41 ± 6.5 ppb μg/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' mi(a)croenvironment 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, pivotally 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 μg/kg) and AFM_1 in milk (0.05 μg/kg) (the EU, 2010) and Iran's permissible AFB_1 in feed (5–20 μg/kg) and foods (5–15 μg/kg) and AFM_1 in milk [(0.5 μg/kg, kg is equal to liter in case

Table 1: Cluster random sampling and widespread contamination of aflatoxin B_1 in feedstuffs and aflatoxin M_1 in bulk milk during spring, summer, autumn, and winter in thestudy area (see also Figure 1).

Type of feed/milk	Sampling period	Samples, *n*	≥Iran's permissible level, *n* (%)
Concentrate	April-May	6	4 (66.6)
	July-August	6	3 (50.0)
	November	6	5 (83.3)
	February	6	6 (100.0)
	Total	24	18 (75.0)
Corn silage	April-May	6	4 (66.6)
	July-August	6	3 (50.0)
	November	6	4 (66.6)
	February	6	4 (66.6)
	Total	24	15 (62.5)
TMR	April-May	6	5 (83.3)
	July-August	6	4 (66.6)
	November	6	5 (83.3)
	February	6	6 (100.0)
	Total	24	19 (79.2)
Bulk milk	April-May	49	39 (83.3)
	July-August	51	34 (66.6)
	November	49	39 (83.3)
	February	51	44 (100.0)
	Total	200	156 (78.0)

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® AFM_1, R-Biopharm, 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 $E_{550\,nm} = 2.1 \times 10^4\ M^{-1}\,cm^{-1}$ 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_2O_2 using microtiter plate spectrophotometer at 450 nm (Multiskan Plus Type 314, Labsystems, Helsinki, Finland). The analyses of phagocytosis and killing of *E. coli* and *S. aureus* were done accordingly [2, 17].

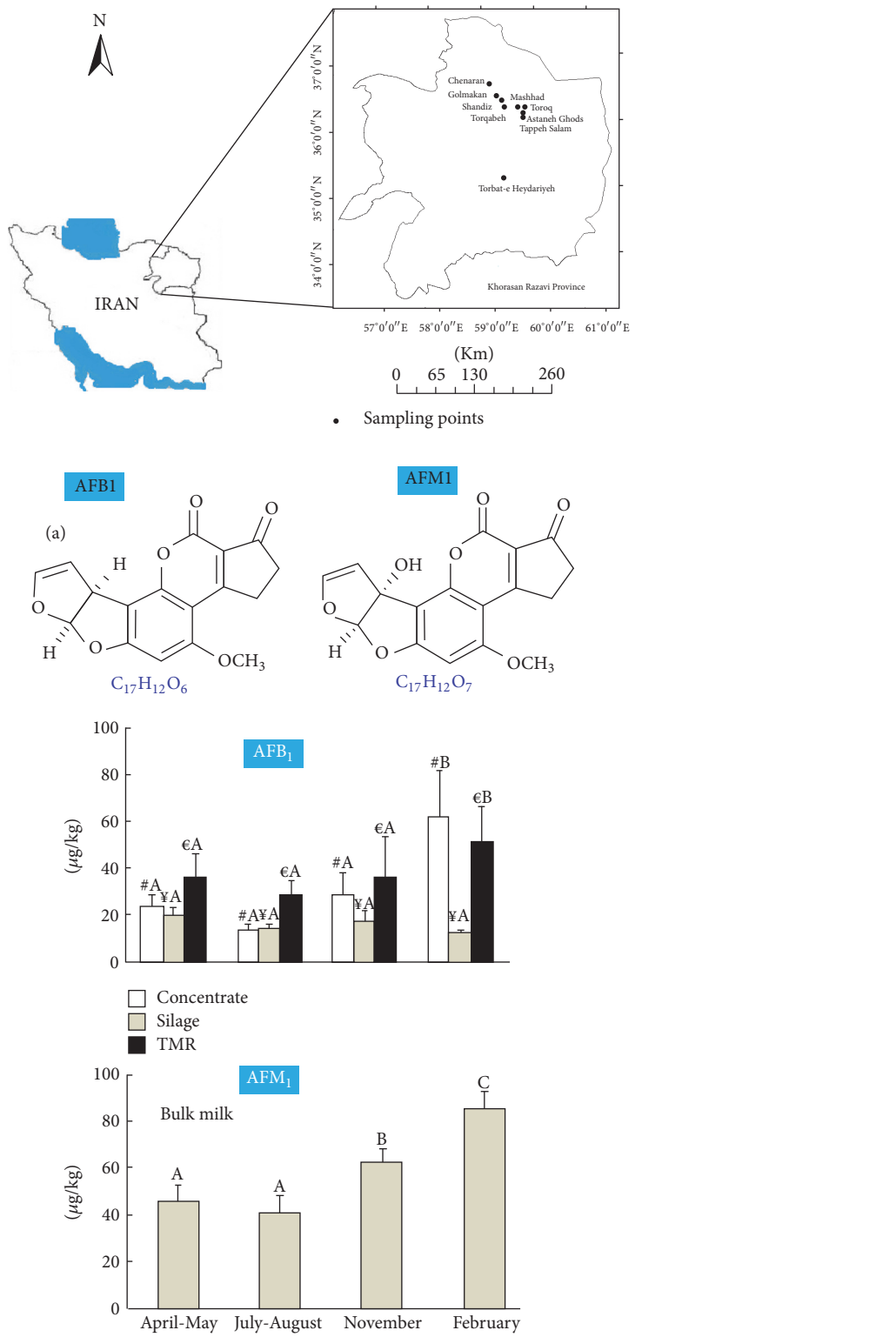

Figure 1: Upper panel, map of the study area showing many intensively large scaled industrial cattle farming in part of semiarid northeastern Iran, where feed and milk samples were seasonally obtained. Lower panel (a), chemical structure of aflatoxins (AFs) B_1 ($C_{17}H_{12}O_6$) and M_1 ($C_{17}H_{12}O_7$) routinely found in animal feed and milk. AFM_1 is derivative of hydroxy AFB_1 and predominant secondary metabolite of AFB_1. One oxygen atom more in AFM_1 leads to a polar lipophobic toxin. Middle, aflatoxin B_1 levels in different types of cattle feed ($n = 72$ feedstuffs), and lower, aflatoxin M_1 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).

Statistical analysis was performed using SPSS version 19 and SAS Version 9.1 (SAS Institute Inc., Cary, NC) software. The Chi-square and Fisher exact tests were used to assess the possible differences in AFs spread in feeds and milk. To compare the measured parameters of two groups, analysis of variance was used; all data were presented as means ± SEM and hypothesis testing was done at the 5% significance level.

3. Results

Of 72 feed samples, more than 52 (average 72.2%) were contaminated with far above permissible AFB_1 in feed (Table 1), from which the levels of AFB_1 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). AFB_1 level in feedstuffs ranged from 4 to 127 μg/kg with average of 28 μg/kg. Among feedstuffs TMR showed maximal level of AFB_1 (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, 27 ± 2.8, 26 ± 4.1, and 18.5 ± 2.8 μg/kg, respectively (Figure 1B). Similarly, bulk milk AFM_1 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 AFB_1- and AFM_1-treated group (Figure 2(b), upper panel insert). Furthermore, while low levels of mix of AFB_1 and AFM_1 showed little effects on blood PMN necrosis (Figure 2(c)), it decreased both the production of O_2^- ($P < 0.01$) 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 AFB_1 and AFM_1 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 AFB_1 and 5 ng/kg for milk AFM_1) 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 AFB_1 production [6, 7, 10], due mainly to weakening anaerobic bacterial mediated lactic acid production, increasing mi(a)croenvironment pH, thereby boosting aflatoxigenic 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 AFB_1 and total AFs (B_1, B_2, G_1, and G_2) 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 AFB_1 for feedstuffs and foods 5–20 μg/kg and 5–15 μg/kg, respectively (Anonymous, 2002); also, maximal tolerable for milk AFM_1 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 AFB_1 and AFM_1, 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 AFB_1 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 (invisible) 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 aflatoxigenic molds and AFB_1 [9, 10]. Though a tiny amount of feed AFB_1 appears in milk of AFB_1-consumed dairy cattle, AFB_1 simply transmit into milk as AFM_1 with high cancer risks for consumers, especially neonates and children, who consume far more milk and dairy products. AFM_1 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 AFB_1 and AFM_1 quantification, (2) our current understanding on the metabolism and toxicodynamics of AFB_1, 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

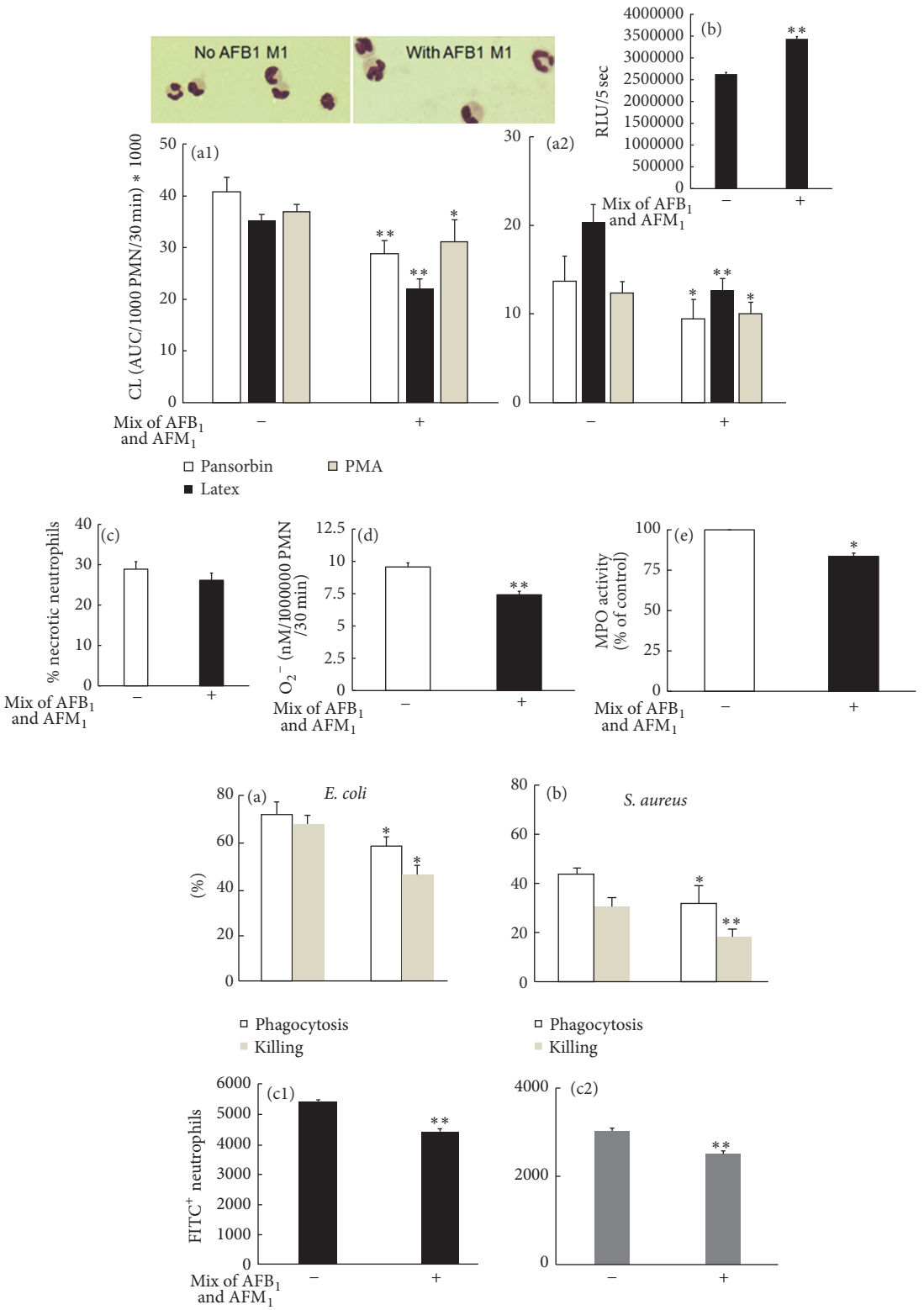

FIGURE 2: Upper panel, luminol-dependant chemiluminescence (CL) in blood (a1) and milk (a2) neutrophils incubated with(out) 20 ng/ml of mix of aflatoxins (AFs) B_1 and M_1 for 12 h and stimulated with PMA, latex beads, and opsonized Pansorbin; values are expressed as the area under the curve (AUC) of 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), O_2^- production (d), and myeloperoxidase (MPO) activity (e) in neutrophils. Lower panel, mix of aflatoxins (AFs) B_1 and M_1 weakens phagocytotic activity and killing capacity of blood neutrophils. Effect of 20 ng/ml of mix of AFB_1 and AFM_1 (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_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_1 in contaminated TMR goes to the blood with a tenth (mainly converted to AFM_1) to milk [20, 22], the assumed AFB_1 and AFM_1 in blood/milk can accumulatively reach above our *ex vivo* tested levels.

The inhibiting effect of AFs on the killing capacity of PMN for *E. coli* and *S. aureus* was manifested mainly by reduction of MPO activity and intracellular ROS production; this further confirms the negative effects of mix of AFB_1 and AFM_1 on the innate immunity; that is why AFB_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_1 and AFM_1 behaves in the phagosome and lysosome, where extremely large amounts of oxidants and granule constituents, through MPO-H_2O_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_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_1 and AFM_1 detection and their ex vivo effect on peripheral granulocytes. Alarmingly, maximal immunotoxic levels of AFB_1 and AFM_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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