



Determination of Aflatoxin M₁ in Pasteurized and UHT Milk in West-Azerbaijan Province of Iran

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Article type

Short communication

Abstract

Keywords

Aflatoxin M₁
Milk
Enzyme-Linked Immuno-
sorbent Assay
Iran

Received: 2 Sep 2015

Revised: 26 Nov 2015

Accepted: 15 Dec 2015

Background: Aflatoxin M₁ (AFM₁) as a carcinogenic and toxic compound has considerable significance from public health viewpoint. The aim of this study was to determine the level of AFM₁ in pasteurized and UHT milk in West-Azerbaijan province of Iran.

Methods: A total of 360 commercial milk samples including 220 pasteurized milk and 140 ultra-high-temperature (UHT) milk were purchased randomly from large supermarkets and retail shops in seven different cities of West-Azerbaijan province of Iran. Concentration of AFM₁ in the samples was investigated by enzyme-linked immunosorbent assay (ELISA) method. Statistical analyses were carried out using SPSS version 16.0.

Results: From total of 360 samples, 77.7% (n=280) were contaminated to AFM₁ with a mean of 75.8±9.2 ng/l. Seventy percent (n=154) of pasteurized milk and 54.2% (n=76) of UHT milk, exceeded the limits recommended by European commission (EC) regulations (50 ng/l). Besides, 2.27% of pasteurized milk (n=5) and 2.1% of UHT milk (n=3) exceeded the prescribed limit of institute of standards and industrial research of Iran (ISIRI) and US food and drug administration (FDA) regulations (500 ng/l). Mean content of AFM₁ in contaminated pasteurized and UHT milk samples were 76.2±8.4 and 72.6±7.2 ng/l, respectively with no significant difference ($p>0.05$).

Conclusion: We found high presence of AFM₁ in milk samples which may pose a serious health hazard to local people. Therefore, milk and dairy products should be monitored continuously for this issue. Also, it is necessary to educate dairy farmers about effective ways to reduce aflatoxin contamination.

Introduction

Aflatoxins are a group of structurally related mycotoxin which consist of four naturally occurring compounds including aflatoxin B₁, B₂, G₁ and G₂ which are mainly produced by three species of moulds namely *Aspergillus flavus*, *A. parasiticus* and rarely *A. nomius* (Eslami et al., 2015; Fallah et al., 2009; Heshmati, 2010; Khodadadi et al., 2014; Nemati et al., 2010; Norian et al., 2015).

Aflatoxin B₁ (AFB₁) has strong teratogenic, mutagenic, and carcinogenic effects (Barikbin et al., 2015; Fallah et al., 2009). Aflatoxin M₁ (AFM₁) is a monohydroxylated derivate of AFB₁, which in liver is metabolized by cytochrome P450. It is excreted into the milk of both human and lactating animals (Fallah et al., 2009; Gurbay et al., 2010; Kamkar, 2005; Mason et al., 2015; Montaseri et al.

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2014). A direct relationship has been shown between the amount of AFB₁ consumption and the secreted AFM₁ in milk. It has been shown that in 0.3-6.2% consumed AFB₁ by animals is secreted into milk as AFM₁ (Ayar et al., 2007; Creppy, 2002; Fallah et al., 2009; Unusan, 2006). The content of AFM₁ in milk is not significantly affected by thermal process, pasteurization and ultra-high-temperature (UHT) treatment, or storage of dairy products (Prandini et al., 2009). Although AFM₁ is less carcinogenic and mutagenic than AFB₁ as its parental compound (Battacone et al., 2005), but the international agency for research on cancer (IARC, 2002) has categorized it from group 2 to group 1 according to the recent investigations on its carcinogenicity.

Milk and dairy products constitute an important part of people's diet providing as a good source of calcium and proteins. Therefore, presence of aflatoxin in these products poses an important hygienic risk in human health (Heshmati and Milani, 2010). Considering its public health significance, many countries have set maximum limits for aflatoxin, which vary among countries. European community (EC) has prescribed the maximum acceptable level of AFM₁ in liquid milk as 50 ng/l (EC, 2001). Both, US food and drug administration (FDA, 1996) and institute of standards and industrial research of Iran (ISIRI, 2005) have set the maximum level of 500 ng/l of AFM₁ in liquid milk. This study was aimed to determine the levels of AFM₁ in pasteurized and UHT milk marketed in West-Azerbaijan province as a main dairy producing region in Iran.

Materials and methods

Samples

A total of 360 commercial milk samples (220 pasteurized milk and 140 UHT milk) from 17 different brands were purchased randomly from large supermarkets and retail shops in seven different cities of West-Azerbaijan province in North-West of Iran from January to February 2012. All samples were stored at 4 °C and tested for presence of AFM₁ before their expiry date.

Sample preparation

Each sample was centrifuged at 3500 g for 10 min at 10 °C. After that, upper creamy layer was removed by a Pasteur pipette and then the lower phase was provided for the next quantitative test.

ELISA assay

The concentrations of AFM₁ were determined using the enzyme linked immunosorbent assay (ELISA). A RIDASCREEN[®] competitive enzyme immunoassay kit

(R-Biopharm, Darmstadt, Germany) based on antigen-antibody reaction was used for the quantitative analysis of AFM₁. Briefly, 100 µl standard solution and defatted milk samples were added into each well of micro-titer plate and then incubated in the dark for 40 min at room temperature. In the next step, the liquid was removed and each well was washed twice with 250 µl washing buffer. After that, 100 µl diluted enzyme conjugate was separately added to each well and incubated in dark for about 15 min at room temperature. Each well was washed twice with washing buffer. Subsequently, 100 µl substrate containing 50 µl urea peroxidase and 50 µl tetramethylbenzidine as chromogen was added to each well and mixed thoroughly and incubated in the dark at room temperature for 15 min. In the last stage, 100 µl of the stop reagent (1 N H₂SO₄) was added into each well and the optical densities (OD) were measured at 450 nm using a ELX800 microplate reader (Bio-Tek Instruments, Winooski, Vermont, USA) within 15 min. Five ng/l was considered as the lowest detection limit in milk, based on the manufacturer's guideline. Validation of ELISA was carried out by determination of recoveries and the mean variation coefficient (CV) for milk samples spiked with different concentrations of AFM₁ (10, 50, 150, 250, and 500 ng/l). The mean recovery score in spiked milk samples was 96.2% with a CV of 8.5%. According to the manufacturer's guideline, the recovery rate in spiked milk was 95% with a CV of 14%.

Statistical analyses

Statistical analyses were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL). Data were analyzed by ANOVA and reported as mean±SE. The levels were considered significantly different at *p* values less than 0.05.

Results and discussion

Out of 360 milk samples, 77.7% (n=280) found to be contaminated to AFM₁ with a mean of 75.8±9.2 ng/l (Table 1). AFM₁ was found above measurable level (5 ng/l) in 85% (n=187) and 66.4% (n=93) of pasteurized and UHT milk samples, respectively (Table 2). In 63.8% of the total samples, 70% (n=154) of pasteurized milk and 54.2% (n=76) of UHT milk, AFM₁ level exceeded the limits recommended by EC regulations. On the other hand, the levels of AFM₁ in eight of the total samples, 2.27% (n=5) of pasteurized milk and 2.1% (n=3) of UHT milk, were exceeded the prescribed limit of US and ISIRI regulations.

As shown in Table 3, AFM₁ levels in milk samples in the present study were higher than most of the other previous similar researches performed in other regions of

Iran. In some previous mentioned investigations, a direct relationship has been shown between season and contamination rate of AFM₁ in milk. For example, Nemati et al. (2010) revealed that the mean level of AFM₁ in milk samples was significantly higher in winter compared to summer (56.3 versus 17.4 ng/l) in Ardabil province of Iran, suggesting a strong relationship between season and AFM₁ residue. This phenomenon may be attributed to relation between type of feed intake and the AFM₁ levels in milk resulting from intake of high AFB₁ residue in concentrated feed and silage in winter compared to low risk of AFB₁ intake during summer when the green pasture composed the major part of feeds of lactating animals, i.e. cattle, sheep and goat. In our work, sampling was carried out from January to February which this

duration time is coincided with winter in Iran. So, such high AFM₁ contamination rate in this study may be related to sampling season. On the other hand, in contrast to most arid and semi-arid parts of Iran, the North-West areas of this country in which West-Azerbaijan province is located, has a cold weather, explaining this finding.

In this study, mean content of AFM₁ in contaminated pasteurized and UHT milk samples were 76.2±8.4 and 72.6±7.2 ng/l, respectively with no significant ($p>0.05$) difference. Similar level of AFM₁ in both pasteurized and UHT milk samples is probably due to this fact that raw bulk milk obtained from lactating animal farms and introduced to Iranian dairy companies for manufacturing of both pasteurized and UHT milk, are originally the same.

Table 1: Concentrations of AFM₁ in commercial liquid milk in West-Azerbaijan province of Iran

Milk product	Sample size	Positive samples No. (%)	Concentration (ng/l)				
			Total samples (Mean±SE)	Positive samples (Mean±SE, Min–Max)		Exceed legal limit N (%) (ISIRI, FDA ^a , EC ^b)	
pasteurized milk	220	187 (85)	54.9±7.1	76.2±8.4	5.4–512.2	5 (2.27)	154 (70)
UHT milk	140	93 (66.4)	41.6±8.0	72.6±7.2	5.8–508.4	3 (2.1)	76 (54.2)
total	360	280 (77.7)	50.4±9.7	75.8±9.2	5.4–510.8	8 (2.2)	230 (63.8)

^a institute of standards and industrial research of Iran (ISIRI) and food and drug administration (FDA) limits for AFM₁ in milk are 500 ng/l

^b European commission (EC) limit for AFM₁ in milk is 50 ng/l

Table 2: Occurrence of AFM₁ in milk samples in West-Azerbaijan province of Iran based to difference toxin ranges

Range of AFM ₁ concentration (ng/l)	Pasteurized milk, N (%)	UHT milk, N (%)	Total, N (%)
<5 ^a	33 (15)	47 (33.5)	80 (22.2)
5-50	33 (15)	17 (12.1)	50 (13.8)
51-150	89 (40.4)	51 (36.4)	161 (44.7)
151-250	42 (19)	13 (9.2)	36 (10)
251-500	18 (8.1)	9 (6.4)	25 (6.9)
>500	5 (2.27)	3 (2.1)	8 (2.2)

^a Distribution of negative samples

Table 3: Published studies in databases about AFM₁ level in milk samples in other regions of Iran

Location	Milk type	Sample size	Samples (%) with AFM ₁ level of >50 ng/l	References
Iran (Gazvin)	raw	170	33.52	Norian et al. (2015)
Iran (Gazvin)	raw	254	56.7	Fallah et al. (2015)
Iran (Tabriz)	pasteurized	50	62	Movassagh Ghazani (2009)
Iran (Sarab)	raw	111	40	Kamkar (2005)
Iran (Ardabil)	raw, sterilized and pasteurized	90	33.3	Nemati et al. (2010)
Iran (Tehran)	UHT	210	33.3	Heshmati and Milani (2010)
Iran (Urmia)	raw and pasteurized	144	6.25	Tajik et al. (2007)
Iran (central part)	pasteurized and UHT	225	22.2	Fallah (2010)
Iran (Shiraz)	pasteurized	624	17.8	Alborzi et al. (2006)
Iran (Ahwaz)	raw	311	12.5	Rahimi et al. (2010)

Conclusion

This study showed high incidence of AFM₁ contamination in pasteurized and UHT milk samples from West-Azerbaijan province of Iran. Thus, consumption of these products could have a serious public health hazard. Monitoring and reduction of AFB₁ levels in farm animal feedstuffs are strongly recommended. The local public health authorities should educate the farmers, dairy companies and consumers about the potential health consequences of aflatoxins. Also, the milk and dairy products must be routinely monitored and the products with high levels of AFM₁ must not be considered for human consumption.

Conflicts of interest

There is no conflict of interest.

Acknowledgments

The authors would like to thank Urmia University Research Council for funding this study.

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