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Evaluation of Aflatoxin M1 Residues in Traditional Iranian Cheese (Koupeh Cheese) samples by ELISA

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ABSTRACT

Background and Objectives: Local cheese made from raw milk is one of the most commonly consumed dairy products in the world. Mycotoxin contamination of foodstuff and its transmission to consumers are extremely important public health issues. The purpose of this survey was to determine the level of aflatoxin M1 (AFM1) residues in Koupeh cheese, a traditional fermented Iranian cheese produced in spring and summer.

Methods: We randomly collected 48 local cheese samples produced in Mahabad (northwest of Iran) during spring and summer. The level of AFM1 was measured by enzyme-linked immunosorbant assay using commercial kits and a microplate reader.

Results: All samples contained measurable amounts of AFM1. Cow milk cheese samples contained higher level of AFM1 compared to sheep milk cheese samples. The level of AFM1 in the samples from both animals was lower in summer. There was no significant difference between the mean level of AFM1 in summer and spring. Moreover, 33.3% of cow milk cheese samples collected in spring and 16.6% of the samples collected in summer contained toxin levels higher than the maximum allowed concentration set by the European Commission (250 ng/Kg) and by the Institute of Standards and Industrial Research of Iran (200 ng/Kg).

Conclusion: The results of this study show that the level of AFM1in Koupeh cheese is influenced by the livestock type and production season, in a way that the level of contamination is higher in spring.

Keywords: Cheese, Cultured Milk Products, Aflatoxin M1, ELISA.

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INTRODUCTION

Milk and dairy products are main parts of human diet. A close relationship has been reported between consumption of dairy products and health status, efficiency, IQ, and the regulation of metabolic activities (1). The quality of milk and dairy products is important and relies mainly on the health of livestock and their feed (2). Various studies on animal feed have shown that the contamination of animal feed with molds (especially *Aspergillus* species) leads to production and transfer of aflatoxin to milk and its products (1, 2). Livestock can be exposed to mycotoxins such as aflatoxin through consumption of the feeds contaminated with mycotoxin-producing molds during growth, harvest, and storage. When lactating animals such as cattle consume aflatoxin B1 (AFB1)-contaminated feed, the toxin is metabolized to form the monohydroxy derivative, aflatoxin M1 (AFM1), which is excreted in their milk (3). The presence of mycotoxins in food and animal feed depends on various factors such as geographical area, season, humidity, temperature, and storage conditions. The presence of AFM1 in milk and dairy products can cause toxicity and carcinogenicity. Aflatoxins are as large group of mycotoxins produced by *Aspergillus* species such as *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* (4, 5). AFM1 is the hydroxylated metabolite of AFB1 (6) excreted in milk through mammary glands of human and lactating animals (7). About 0.3-6.2% of AFB1 is metabolized into AFM1 and then excreted in milk depending on factors including animal genetics, seasonal variation, milking process, and environmental conditions (8). The nutritional value of dairy products is strongly influenced by the quality of raw milk.
In Iran, cheese production has become popular in the recent years and according to statistics, about 20% of milk produced in the country is converted to cheese in the dairy industry. Moreover, the share of traditional cheese production is about 80% (9). The International Agency for Research on Cancer (IARC) has identified AFB1 as a carcinogen (10). Studies have shown that the presence of AFM1 in milk and dairy products is health threatening, especially in the populations where dairy products are the main part of the daily diet (7). Therefore, in this study, we determined the level of AFM1 residues in Koupeh cheese, a traditional fermented Iranian cheese produced in spring and summer.

MATERIAL AND METHODS

This observational and cross sectional study was performed on 48 randomly collected traditional cheese samples from the city of Mahabad (Iran) during spring and summer. The samples (100g each) were kept in freezer at -18 °C. Presence of AFM1 in the samples was evaluated by enzyme-linked immunosorbent assay (ELISA). ELISA kits (sensitivity of 5 ng/L) used in this study were purchased from R-Biopharm Inc., Germany. In this test, cross-reaction with AFM1 was 100% and no cross-reactivity was observed with AFB1, B2, G1 and G2. Moreover, the aflatoxin recycling rate has been reported to be 95% with 15% error rate. First, the samples were thawed and placed in the bottom of a refrigerator for 12 hours before the test. After homogenization, 2g of each cheese sample were weighed accurately and then added to a 50 ml balloon containing 40 ml of dichloromethane. The contents were stirred for 15 minutes and the suspension was filtered using syringe filters. Then, 10 ml of the filtrate was evaporated at 60 °C and the sediment was dissolved in a mixture of 0.5 ml methanol with 0.5 ml phosphate buffer and 1 ml heptane. The mixture was centrifuged at 2700 RPM for 15 minutes at $10^{\circ C}$. Supernatant (heptane layer) was removed completely and 100 µl of sub-phase (methanol layer) was diluted with 400 µl of phosphate buffer. Later, 100 µl of standard solution and the prepared cheese samples were added to a microplate. After placing the microplate at 20-25 °C for an hour, the content of the microplate was removed and all wells were washed with 250 µl of special wash buffer. Next, 100 µl of aflatoxin solution conjugated to the enzymes was added to the wells and the microplate was placed in an oven at 25-20 °C for an hour. The contents of the wells were removed completely and the wells were washed twice with 250 µl of the wash buffer. Then, 50 µl of substrate and 50 µl of chromogen were added to each well and the microplate was incubated in the dark at 20-25 °C for 30 minutes. Finally, the reaction was inhibited by adding 100 µl of stop solution. Absorbance of each sample at 450 nm was read with BioTek ELISA Reader (USA). The

results were analyzed using SAS (version 9.1) and Microsoft Excel 2007.

RESULTS

The results showed that all samples contained measurable amounts of AFM1. The cheese produced from cow's milk had higher concentrations of AFM1 compared to the cheese produced from sheep's milk (Table 1). The level of AFM1 contamination in both animal species was higher in the spring. The highest concentration of AFM1 was detected in the cheese produced from cow's milk in the spring. However, the lowest amount of AFM1 was found in the sheep milk cheese produced in the summer (45.86 ng/Kg). However, the lowest mean level of AFM1 was related to the cow milk cheese produced in the summer.There was no statistically significant difference between the mean level of aflatoxin found in cow and sheep milk cheese ($P>0.05$). The results also showed that the mean concentration of aflatoxin in spring and summer was significantly different ($P<0.05$).

The highest mean level of AFM1 was found in the samples collected in the spring (186.66 ng/Kg), while the lowest mean AFM1 level was related to the samples collected in the summer (136.06 ng/Kg) (Figure 1). We also found that the highest level of AFM1 in the cheese samples from both animals was related to the spring, indicating that season might be a determining factor for the level of aflatoxin contamination of dairy products (Figure 1).

Table 1- Mean level and range of AFM1 contamination in cheese samples produced from cow and sheep milk

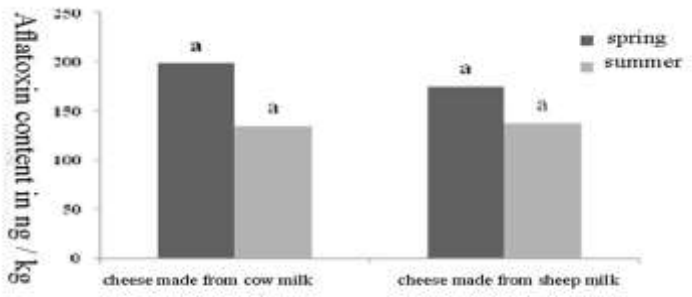
Livestock	Season	Number of samples	Concentration of AFM1 (ng/Kg)	
			Range (ng/Kg)	Mean ± Standard deviation
Cow	Spring	12	92.3-348.3	198.24±91.55
	Summer	12	51.9-280.1	134.75±69.2
Sheep	Spring	12	70.1-382.3	175.07±86.33
	Summer	12	45.86-264.3	137.3±61.7
Total		48		

Table 2-AFM1 level in traditional cheese samples depending on the season and type of animal

Livestock type	Seasons	Number of samples	Range (ng/Kg)	Higher than the European standard (250 ng/Kg)	
				Number of samples	%
Cow	Spring	12	92.3-348.3	4	33.33
Sheep	Spring	12	70.1-382.3	2	16.6
Cow	Summer	12	51.9-280.1	1	8.33
Sheep	Summer	12	45.86-264.3	2	8.33

Table 2 represents the results of the statistical analysis of AFM1 levels in the samples based on the season and type of animal.

Figure 1- Comparison of the mean concentration of AFM1 in cheese produced from cow and sheep milk in different seasons



DISCUSSION

Several studies have been conducted about the presence and level of aflatoxin in milk and dairy products. The results of such studies indicate that the prevalence and concentration of contamination is high and risky in some cases but lower than the maximum allowed limit in some other cases. In a study carried out by Falah et al. on 116 white cheese and 94 raw cheese samples, aflatoxin was detected in 161 samples with contamination range of 52.1-785.4 ng/Kg, while contamination level in 24.24% of the samples was higher than the maximum allowed limit set by the European Commission regulation (250 ng/Kg) (7). It should be noted that the maximum allowed limit for presence of AFM1 in cheese has been set at 200 ng/Kg by the Institute of Standards and Industrial Research of Iran (11). In another study conducted by Tickson in Turkey, the amount of aflatoxin in 92 butter and 100 raw cheese samples was studied with ELISA and the results showed that all butter samples and 99% of the cheese samples were contaminated with the toxin. In addition, the level of aflatoxin in 28% of butter samples and 18% of cheese samples was higher than the allowed limit set by the *Turkish Food Codex* (250 ng/Kg) (12). In a study by Mokhtarian and Mohsenzadeh in Gonabad (Iran), it was found that 58% of milk samples contained aflatoxin concentrations higher than the standard limit (13). In Turkey, Yarglio *et al*. evaluated aflatoxin levels in 600 white cheese samples using ELISA. The mentioned study reported that 5% of the samples contained 100-800 ng/Kg aflatoxins and 1% of the samples were contaminated with aflatoxin concentrations higher than the allowed limit set by the *Turkish Food Codex* (14). In a study by Neisi et al., concentration of AFB1 in buffalo feed samples ranged from 0.77 to 64.85 µg/Kg. In addition, the concentration of aflatoxin in 21 samples was higher than the allowed limit (25 µg/Kg) (16). The incongruity in the results of the studies could be associated with several factors including type of cheese production, level of animal feed contamination, quality of milk used for cheese production, transport and storage conditions, and the methods used for the analysis of aflatoxin content (16). The AFM1 content of milk produced in different areas is also influenced by geographical and seasonal factors. For instance, it has been reported that the level of contamination in summer is significantly lower than that in winter (16). In our study, there was a significant difference between the level of aflatoxin in spring and summer, which might indicate a relationship with livestock feeding. Considering the lake of a significant difference in the amount of AFM1 between the cheese samples produced from milk of different livestock, it can be concluded that the level of aflatoxin in the milk could be associated with the feeding of lactating animals in the study area.

Feed type, cultivation, preservation method, temperature and humidity can exacerbate contamination of milk, but the exact effect or mechanism of action of these factors is not clear (17).

CONCLUSION

Based on the results of our study and previous studies in Iran, it should be noted that the level of contamination of local cheese with AFM1 is undesirable. Lack of timely and effective measures for control of aflatoxin contamination in dairy products could lead to serious health consequences. Since AFM1 is the result of the animal's metabolism, it is suggested to conduct more accurate studies on the AFB1 contamination of animal feed. Furthermore, in order to reduce the health risks associated with aflatoxin contamination, modifications should be made regarding the maximum allowed limit of aflatoxin in foodstuffs.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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