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Probabilistic Health Risk Assessment of Aflatoxin M1 in Pasteurized Milk from Different Cities of Iran

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ABSTRACT

Background: The presence of Aflatoxin M1 (AFM1) in dairy products results from the ingestion of feedstuffs contaminated with aflatoxin B1 by ruminants. The current study aimed to determine the AFM1 concentration in commercial pasteurized cow milk samples obtained from the Iranian market.

Methods: A total of 54 pasteurized cow milk samples, manufactured between January and April 2019, were purchased from different cities in Iran, including Tehran, Isfahan, Sari, Tabriz, Zanjan, Kermanshah, Ahvaz, Shiraz, and Kerman. These samples were analyzed using a competitive enzyme-linked immune-sorbent assay technique. The associated health risk was estimated by the Monte Carlo simulation method. Additionally, the margin of exposure and cancer risk were employed as benchmarks to assess threats to consumer health.

Results: AFM1 was detected in 33 samples (61 %), totally with concentrations ranging from 0.003 to 0.45 µg kg^{-1}. 9.26 % of the samples exceeded the maximum residue level specified by Iran's national standard (0.1 µg kg^{-1}). The average concentration of AFM1 in the 54 pasteurized milk samples collected from different regions was determined to be 0.042 ± 0.072 µg L^{-1}. Nevertheless, all samples remained below the US Food and Drug Administration (USFDA) maximum limit (0.5 µg kg^{-1}). Human health risk assessment showed that about half of the consumers were at risk based on the Margin of Exposure (MOE) assessment.

Conclusion: The results of this survey indicate the usefulness of a monitoring program to supervise the safety of commercially available pasteurized cow milk for consumers.

1. Introduction

Mycotoxins, as the secondary metabolites of fungi, are widely recognized for their adverse effects on human and animal health, including toxicity, carcinogenicity, mutagenicity, immunosuppression, and teratogenicity [1, 2]. Mycotoxins are commonly abundant in foodstuffs such as wheat, corn, maple syrup, peanuts, barley, dried fruit, and

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spices [3]. Among the mycotoxins, aflatoxins are a highly toxic group that can be easily produced due to improper food stora ge after harvest and during production [4]. Aflatoxins B1 (AFB1) is the most dangerous type of aflatoxin, mainly converts to its 4-hydroxy derivative in the liver of lactating cows through rapid absorption and biotransformation with the interference of P-450 cytochrome enzymes, leading to the formation of AFM1 [5, 6]. The presence of AFM1 in milk, which is known as a carcinogenic compound (Group 1) by the International Agency for Research and Cancer (IARC) [7], critically threatens public health, owing to the necessity of consuming cow milk products as a nutritious food for all age groups, especially children [3, 8, 9]. Given the high consumption rate of cow milk and the importance of applying food safety guidelines, different countries have set the maximum allowable limit for AFM1 in milk and dairy [10, 11]. According to the USFDA, the permissible amount of AFM1 in raw, pasteurized, and sterilized milk was established as 0.5 $\mu g\ kg^{-1}$ [8], while the maximum residue levels of AFM1 in raw, pasteurized, sterilized, and flavored milk have been set to 0.1 $\mu g\ kg^{-1}$ by the Iran National Standards Organization (INSO) [12]. The European Union (EU) has announced that the AFM1 level in raw milk, heat-treated milk, and milk used for the manufacture of milk-based products should be below 0.05 $\mu g\ kg^{-1}$ [13]. It should be noted that thermal processes, including sterilization and pasteurization, do not significantly influence the amount of AFM1 in raw milk, nor the preparation and storage of various dairy products. Therefore, it remains in processed milk and milk products such as cheese and yogurt [14]. Monitoring for AFM1 in dairy products has been an ongoing global concern for several decades, the monitoring in dairies has been continually surveyed worldwide, as the contamination of lactating dairy cows with aflatoxins may serve as a critical potential risk for the introduction of AFM1 into the human diet over long periods, which should not be neglected or underestimated [2, 4, 5, 10, 14-21]. This paper was carried out to determine the content of AFM1 in commercially available pasteurized cow milk samples in different cities of Iran and assess the related probabilistic health risks.

2. Materials and Methods

2.1 Sampling

A total of 54 commercial samples of pasteurized cow milk (1000 mL milk packet) were randomly obtained from supermarkets in different capital cities of Iran, including Tehran, Isfahan, Sari, Tabriz, Zanjan, Kermanshah, Ahvaz, Shiraz, and Kerman (6 samples from each city). All of the samples were manufactured between January and April 2019 and selected from different brands. They were kept at 4°C until testing for AFM1 concentration and analyzed before their expiry date.

2.2 Reagents

The reagents were mostly available in Ridascreen® aflatoxin M1 test kit (R-Biopharm GmbH., Darmstadt, Germany) that contained a microtiter plate coated by capture antibodies, peroxidase-conjugated aflatoxin, AFM1 standard solutions, substrate/ chromogen with red staining, anti-AFM1 antibody, and stop solution containing 1 N sulphuric acids. Methanol and acetonitrile were analytical grades, supplied from Merck (Darmstadt, Germany).

2.3 Sample Preparation

As instructed by the Ridascreen® test kit, 10 mL of milk samples was centrifuged at 3500 rpm for ten min at 10 °C. In the next step, the upper layer was completely discarded. Afterward, 100 µL of skimmed milk was directly applied to each well for analysis.

2.4 Experiment

According to Atasever *et al.* (2010), AFM1 was determined using ELISA by Ridascreen® aflatoxin M1 test kit, because of cost-effectiveness, reliability, and quickness [7, 15]. An adequate amount of microtiter wells was put into the microwell holder for prepared samples and AFM1 standards. Next, 100 µL of standards and the sample solutions were mixed into the wells. They were softly blended by manually rocking the plate. Incubation was done for 1 h at ambient temperature in the darkroom. The engagement of antibody binding sites is proportional to AFM1 concentration. The obtained solution was poured off the wells and rinsed with distilled water. The washing procedure was repeated twice so that it was ensured that the liquid was completely removed from the wells. After that, 50 µL of chromogen and 50 µL of substrate solution were poured into the wells and manually mixed. Incubation was carried out for 30 min at 25 °C in the darkroom. Then, 100 µL of stop solution was added to each well and mixed thoroughly. Consequently, the solution color was changed to yellow from blue. Finally, the absorbance was evaluated at the 450 nm wavelength against the air blank in the ELISA reader. All experiments were done in triplicate and the mean values were recorded.

2.5 Evaluation of AFM1

Absorbance percentages were put to the calibration curve with various concentrations of Standard solutions (0, 5, 15, 20, 40, and 80 ng L^{-1}). The absorbance values resulting from samples and standards were divided by the absorbance value of the first standard (zero standards) and multiplied by 100. Thus, the zero standard equals 100 %, and we express absorbance values in percentages. There is an inverse proportion between absorption and AFM1. The test preparation record indicates 0.003 and 0.01 µg L^{-1} as the lower detection limit (LOD) and lower quantification limit (LOQ) for milk. Based on the instruction of the Ridascreen kit, in spiked milk (10-80 pg mL^{-1}), the recovery rate is 95 %, with an average coefficient of variation of 15 %. Besides, the RIDAVIN computer program prepared by R-Biopharm was used for evaluating ELISA kits.

2.6 Human health risk analysis

Health risk assessment was conducted for AFM1 via intake

of pasteurized milk. In the first step, dietary exposure assessments were performed using the following formula [22]:

$$Equation\ 1. \qquad DI = \frac{C \times IR}{Body\ weight}$$

Dietary intake (DI) is defined as the AFM1 intake (ng/kg BW/day) via pasteurized milk consumption. C is the concentration of AFM1 in pasteurized milk (ng/kg) followed by log-normal distribution in the crystal ball model. IR is the milk ingestion rate in Iranian consumers (g/day) assumed triangular distribution with three points consisting of 28, 38, and 43 g/day consumption rates [23]. According to the World Health Organization (WHO), body weight for adults was set to uniform distribution in the Monte Carlo simulation with lower and upper bounds of 60 and 70 kg, respectively [24]. For health risk assessment in the current study, both MOE and cancer risk approaches were applied [25]. The MOE analysis used a benchmark dose lower confidence limit of 10 % (BMDL10) set at 400 ng/kg BW/day, as indicated by Equation 2 [26]. Cancer risk assessment, on the other hand, incorporated the cancer potency values provided by the Joint FAO/ WHO Expert Committee on Food Additives and the European Food Safety Authority (EFSA) [26, 27]. Exposure to Aflatoxins can cause extra hepatocellular carcinoma cancer cases per 100,000 in HBsAg - and HBsAg + patients. The potency factor for HBsAg - (PHBV -) and HBsAg + (PHBV +) patients was equal to 0.01 and 0.3, respectively [27]. To estimate cancer probability within the Iranian population, the percentage of the HBsAg + carrier population (0.017) and the HBsAg - non - carrier population (0.983) were taken into account, as outlined in Equation 3 [28].

$$Equation\ 2. \qquad MoE = \frac{BMDL10}{DI}$$

$$Equation\ 3. CR = (\ \%\ PopHBV+) \times (DI) \times (PHBV+) + (1 - \ \%\ PopHBV+) \times (DI) \times (PHBV-)$$

2.7 Statistical Analysis

Data analysis and calculations, including the determination of mean, minimum and maximum values, and standard deviation, were performed using SPSS v.21 (SPSS, IBM, Armonk, NY). The Kolmogorov-Smirnov test, a nonparametric test, was employed for statistical analysis, with a significance level set at $P \leq 0.05$. To compare the groups, a one-way analysis of variance (ANOVA) was conducted, followed by post-hoc Tukey honestly significant difference (HSD) test, with a significance level of $P < 0.05$. Furthermore, linear regression analysis and correlation coefficient calculations were carried out using Excel 2013 (Microsoft, Redmond, WA, USA). For the current risk assessment, the authors used Monte Carlo simulation as a probabilistic method to address uncertainty. The Monte Carlo simulation was conducted in Oracle Crystal Ball software (v. 11.1.2.4, Oracle, Co., USA) [29]. In the simulation, the reputation number was set at 100,000, and the 95th percentile was considered the health risk benchmark in the cumulative probability graph [30]. The occurrence of AFM1 health risk does not arise when the MOE exceeds 10,000. However, if the MOE falls below 1,000, AFM1 occurrence in pasteurized milk can pose a health risk.

3. Results and Discussion

There is a growing body of literature that recognizes the importance of AFM1 assessment in dairy products. Numerous studies have investigated the presence of AFM1 presence in raw, pasteurized, and UHT milk marketed in Iran [10, 14], Pakistan [4], Taiwan [1, 31], Turkey [15], Italy [3, 5], Lebanon [32], India [33, 34], and China [19]. The AFM1 levels of the current research are summarized in Table 1. Samples with AFM1 values above the LOD were considered positive. Based on the AFM1 calibration curve in Figure 1, the correlation coefficient (R^2) between the concentrations and absorbance was linear ($R^2 = 0.93$).

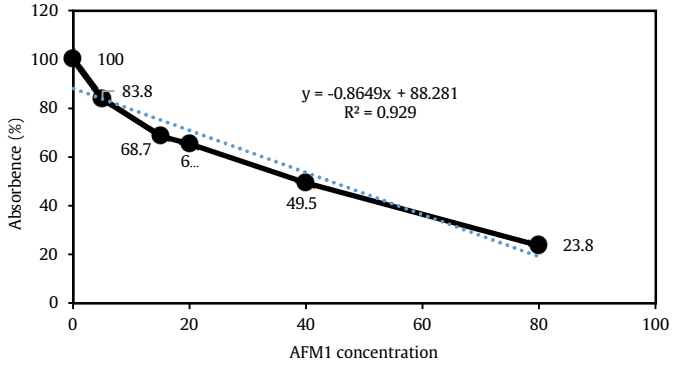

Figure 1. AFM1 Calibration Curve

The results of this study revealed AFM1 contamination in 33 (61 %) of the pasteurized milk samples. According to the INSO, 49 samples (91 %) indicated the presence of AFM1 below the legal limit (0.1 µg kg^{-1}). However, the AFM1 concentration in 5 samples (9.26 %) was higher than the permissible values set by INSO. Considering the USFDA regulations, AFM1 values in all samples did not exceed the allowable limits. The average concentration of AFM1 was 0.042 ± 0.072 µg L^{-1}, ranging from 0.003 to 0.45 µg L^{-1}. The highest contamination value was related to the sample obtained from Tabriz city (with an average of 0.09 ± 0.18 µg L^{-1}), while the lowest contamination belonged to the samples from the cities of Sari, Tehran, and Ahvaz (Not Detected). According to the statistical analysis, average AFM1 concentrations in pasteurized milk samples purchased from different regions showed no significant differences ($P > 0.05$). A study conducted by Fallah (2010) showed that AFM1 was found in 71.5 % (63 samples) of 116 pasteurized milk samples and 62.3 % of 109 UHT milk samples marketed in central Iranian regions. The mean concentration of pasteurized milk samples was 0.0528 µg L^{-1}, and the range was 0.0058-0.528 µg L^{-1} [14]. In Another study by Fallah *et al.* (2015) in Qazvin province of Iran, the results demonstrated the detection of

AFM1 in 204 raw milk samples (80.3 %), at a range of 0.011 to 0.321 μg L^{-1}, which was in line with our study. None of the samples exceeded the USFDA limit of 0.5 μg kg^{-1} for AFM1 in milk. This study also indicated a significantly higher AFM1 level in samples gained in winter compared to samples taken in summer, due to seasonal variability [16]. The results obtained from the study by Rahimi *et al.* (2012) [35] showed that there was AFM1 contamination in 60 pasteurized milk samples (40 %), with the range and average of 0.011-0.094 μg L^{-1} and 0.034 ± 0.019 μg L^{-1}, respectively, which was so close to the current results. In China, Li *et al.* (2017) reported that all samples of pasteurized milk were below the EU limit (0.05 μg kg^{-1}), while AFM1 levels were detected in 11.9 % of UHT milk samples above the EU limit [17]. In Argentina, Lopez *et al.* (2003) investigated the AFM1 prevalence in 77 various types of milk samples. AFM1 contamination was detected in 18 samples (23 %) at 0.010-0.030 μg L^{-1} [18], which is lower than the current study. In another work by Campone *et al.* (2017) in Italy, the maximum AFM1 contamination level discovered in pasteurized milk was 0.045 μg kg^{-1} [5], which is inconsistent with our work, demonstrating the great quality of raw milk applied for pasteurization and sterilization in the mentioned country.

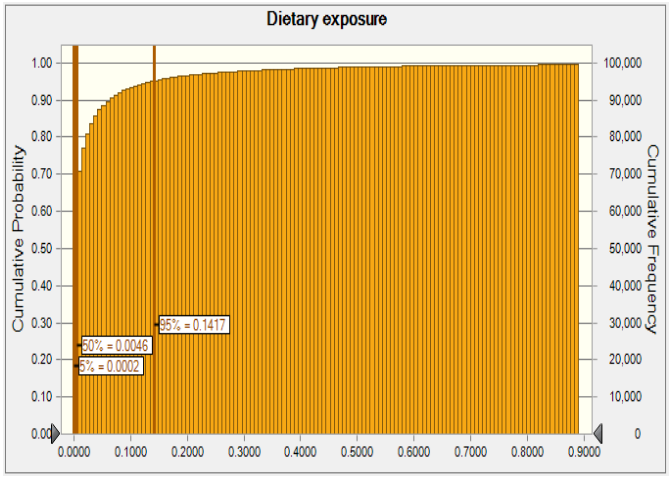

Figure 2. Exposure assessment of the Iranian population by pasteurized milk consumption contaminated with AFM1

Table 1. The concentrations of AFM1 (μg L^{-1}) in pasteurized milk samples collected from different areas

Area sampling	N	Positive samples	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Sari	6	0	0.003 [a]	0.000	0.000	0.003	0.003
Tabriz	6	5	0.091 [a]	0.176	0.072	0.003	0.450
Tehran	6	0	0.003 [a]	0.000	0.000	0.003	0.003
Kermanshah	6	6	0.030 [a]	0.009	0.004	0.020	0.040
Esfahan	6	5	0.030 [a]	0.014	0.006	0.003	0.041
Zanjan	6	6	0.103 [a]	0.065	0.026	0.020	0.172
Ahvaz	6	0	0.003 [a]	0.000	0.000	0.003	0.003
Shiraz	6	5	0.029 [a]	0.022	0.009	0.003	0.060
Kerman	6	6	0.088 [a]	0.059	0.024	0.027	0.190

The Monte Carlo simulation approach was used for the prediction of dietary exposure to AFM1 in the Iranian population. Figure 2 exhibits the forecasted levels (ng/kg BW/day) for the 5th, 50th, and 95th percentiles. As seen in Figure 2, high pasteurized milk consumers (95th) have about 700-fold higher AFM1 intake compared to low consumers (5th) and accordingly about 30-fold higher than the median of the studied population. Based on probabilistic human health risk assessment, the percentile 5th, 50th, and 95th percentiles of the MOE for AFM1 are shown in Figure 3. A MOE value below 10,000, based on the BMDL taken from animal studies, is considered a decision point indicating high risk to public health. According to Figure 3, the MOE value is consistently below 10,000. Accordingly, the additional cancer cases per 100,000 individuals per year were calculated, and the result is presented in Figure 4 displaying the 5th, 50th, and 95th percentiles. The results showed that AFM1 intake through pasteurized milk in Iran for percentile 95th of consumers will raise the risk of liver cancer by about 0.16 in 10 million populations annually, highlighting that AFM1 is less toxic than AFB1 and either lower in food matrices.

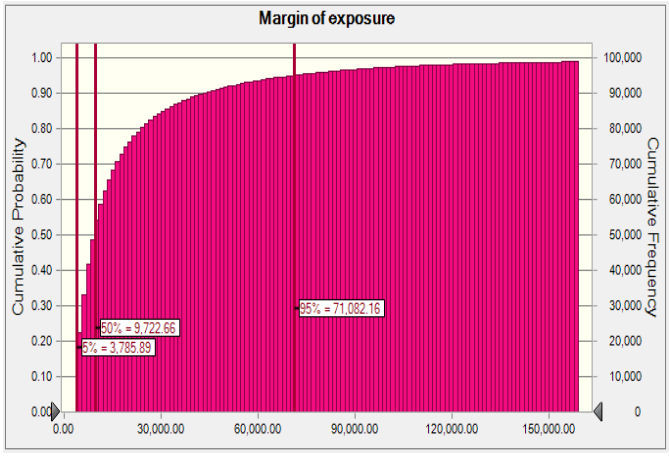

Figure 3. Simulated MOE for assessing the health risk related to AFM1 intake through pasteurized milk consumption in the Iranian population

In a study conducted by Rahmani *et al.* (2018) on AFM1 risk assessment in Iran, Turkey, and Lebanon for pasteurized milk no significant health risk for adult consumers was found which is consistent with our investigations about Iranian adult pasteurized milk consumers. However, Rahmani *et al.* (2018) also observed that the Hazard Index (HI) percentile of 95 % in children is over 1 [20]. Similarly, in a research conducted in Greece (2013), the researchers aimed to evaluate the AFM1 risk associated with various milk types consumed by children. The findings revealed a negligible Hazard Index (HI < 1), thereby affirming the safety of milk consumption in this population group [21]. Furthermore, a risk assessment study specifically focusing on Iranian infant consumers of milk powder estimated the cancer risk associated with AFM1 to be approximately 1 case per 109 individuals [12].

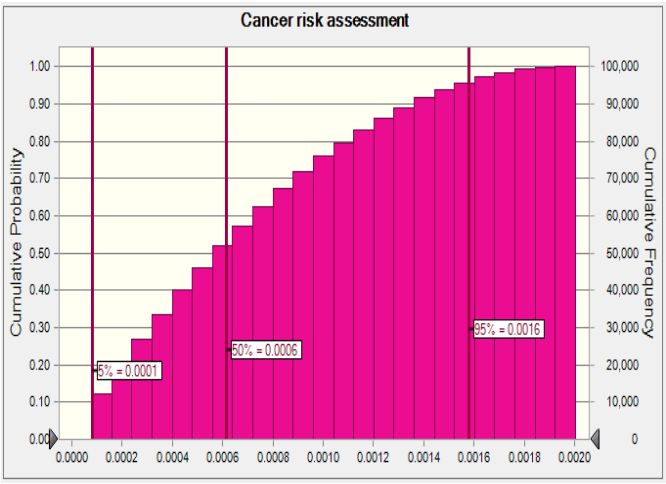

Figure 4. Simulated cancer risk of AFM1 by Monte Carlo algorithm for the Iranian population

Differences in the AFM1 amount in milk and dairies purchased from different provinces significantly depend on geographical area, hygiene of animal husbandries, and storage conditions of cattle feed [16]. It is suggested that the principles of Good Veterinary Practices (GVP), Good Agricultural Practices (GAP), and Hazard Analysis and Critical Control Points (HACCP) systems, play a pivotal role in controlling the pre-harvest and post-harvest steps of dairy cow feed and the processing of dairy products. These practices can help reduce fungal growth, minimize AFB1 formation, and ensure the desired quality of milk without AFM1 contamination. It is noteworthy that the total daily intake of aflatoxin from other foodstuffs, particularly nuts and flour, should also be considered as a critical risk factor for human health. Animal husbandry is the most important point that the lack of hygienic control may lead to contamination of raw milk and health hazards. Concerning the high contamination of pasteurized milk in Tabriz and Kerman cities, cattle ranching should be monitored regularly to ensure the low fungal contamination of feed and consequently high quality of raw milk.

4. Conclusion

To protect consumer health, food safety authorities must implement robust monitoring programs and sustain vigilant surveillance. These initiatives should encompass comprehensive oversight of livestock feed, warehouse conditions, and fodder storage. In our present study, we utilized a Monte Carlo simulation model to conduct a health risk assessment associated with AFM1 through the consumption of pasteurized cow milk. Our findings indicate statistically significant regional variations in AFM1 levels within the country, highlighting that the concentration of AFM1 in milk differs significantly between specific regions. Also, our findings reveal an estimated annual incidence of 0.16 cases of liver cancer per 10 million individuals in Iran, underscoring the significance of this health risk. Notably, our study focused exclusively on pasteurized cow milk, where AFM1 concentrations are comparatively lower than AFB1 levels and are associated with reduced toxicological implications. Consequently, it is paramount that regular monitoring of all aflatoxins within our dietary intake is conducted. Furthermore, we advocate for comprehensive cumulative risk assessments about AFM1 across various dairy products. Such assessments are essential to identify potential risks posed to consumers who frequently consume these products. This approach is pivotal in ensuring the continued safety and well-being of the public.

Authors' Contributions

Vahid Mofid, Anosheh Rahmani: Conceptualization; Supervision; Writing-reviewing and Editing. Leila Peivasteh-Roudsari, Hadis Karami, Marziyeh Karami: Methodology; writing original draft. Behrouz Tajdar-Oranj, Adel Mirza Alizadeh: Software and Data analysis; Reviewing and Editing.

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Conflicts of Interest

There was no conflict of interest among the authors.

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Ethical considerations

There were no ethical considerations to be considered in this research.

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