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ORIGINAL ARTICLE

Evaluation of Aflatoxin M1 and Heavy Metal in Raw Materials and Infant Formula Produced in Pegah Dairy Plants, IRAN

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KEYWORDS

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ABSTRACT: This study was conducted to investigate the presence of lead (Pb), cadmium (Cd) and aflatoxin M1 in raw materials and all types of infant formula produced in Pegah Dairy Plants (PDPs) in Shahrekord, Iran. To this aim, 80 samples of raw cow's milk, whey protein, starch, vitamin and all type of infant formula were collected for 6 months during 2018-2019. Cd and Pb were measured by Graphite Furnace Atomic Absorption Spectrophotometry. Aflatoxin M1 (AFM1) samples and analyzed by ELISA technique. The incidence of contamination of AFM1 in vitamins, raw cow's milk, whey powder, and baby formula collected from Pegah dairy plants were ND, 34, 23 and 8 ppb, respectively. The result indicated that none of aflatoxin M1 in infant formula samples exceeded FDA lead consumption guidelines. In addition, maximum mean lead in maltodextrin, starch, vitamin, raw cow's milk, whey powder, baby oil, infant formula, immediate, whole and skim was 20, 23, ND, 49, 20, 21, 17, 17, 12, and 17 ppb, respectively. Further, the maximum cadmium content was found to be 5.5, 9.5, ND, 14, ND, 6.3, 12.30, 7.6, 9.2 and 5.4 ppb, respectively, where the value of the samples was not above the maximum recommended limits by standard of Iran, European Community (EC) and Codex Alimentarius (CAC). The average of the heavy metal (Cd and Pb) and AFM1 in the maltodextrin, starch, vitamin, raw milk, whey powder and baby oil, and the types of infant formula produced by Pegah dairy Plants (PDPs) Shahrekord was below the recommended levels by Iran standard.

INTRODUCTION

Dairy products, including milk, contain a large number of nutrients like vitamins, proteins and also calcium. The main consumers of these nutrients are children [1]. Although milk may be consumed in its unprocessed form, it also is utilized for preparing yoghurt, cheese, infant formulations and other dairy products [2, 3]. The Aflatoxin M1 which is produced by toxins, mainly by molds such as *Aspergillus flavus* and *Aspergillus parasiticus*, exert adverse effects on living organisms and crops, resulting in economic and health-related issues [4]. Aflatoxins are considered as toxic, teratogenic, mutagenic, immunosuppressive and carcinogenic compounds to animals and humans [5, 6]. Livestock which has received contaminated feed may be found by Aflatoxin M1, as a hydroxylated metabolite of aflatoxin B1 [7]. Further, Aflatoxin M1 (AFM1) has a potency close to one order of value less than the potency of aflatoxin B1 (AFM1) [8]. Therefore, it is important to determine Aflatoxin M1 levels in milk and infant products in order to protect children from its potential hazards [9]. International Agency for Research on Cancer (IARC) introduced aflatoxin B1 as primary and aflatoxin M1 as secondary groups of carcinogenic compounds [6]. According to the European Community

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(EC) and Codex Alimentarius Commission (CAC), the maximum level of AFM1 in milk and milk and dairy products should not exceed 50 ng/L [10,11]. Therefore, measuring AFM1 levels is important in certain samples of milk, and routine-monitoring surveys should be considered in this regard [12]. Furthermore, Aflatoxin M1 exhibits resistance to pasteurization, thermal inactivation, and other food processing procedures [4,13,14]. Thus, it is essential to protect materials and products against contamination by AFM1 in order to produce high quality milk [15]. Chemical and industrial pollutants endanger human life both directly and indirectly. In addition to the existing contaminants, production and processing methods, along with their manipulation can be considered as a factor for entering various metals, which can be useful and harmful in food. Lack of digesting heavy metals in the body is regarded as one of the most important issues. In fact, after entering the body, heavy metals do not repulse the body, but they precipitate and accumulate in some tissues such as fat, muscles, bones and joints, which may cause many diseases in the body [16,17]. Lead and cadmium are a reason for producing neurotoxic, hematologic, and nephrotoxic effects even at low concentration. In addition, neurotoxin and reproductive toxin, due to their persistent unchangeable impacts exert adverse effects particularly on the brains of infants and children [18,19]. In fact, the most recent infant formula study in the world has mainly addressed exposure to lead and cadmium through the typical diet of children about 0 month to 2 year, as well as the need to reduce this exposure for public health [20,21]. However, limited information is available about the presence of these metals among baby foods in Iran. Plants cultivated in land exposed to contaminated water spread heavy metals to the environment and the food consumed by animals and people [22-24]. Furthermore, exposure to such metals exerts an adverse effect on certain organs that can result in cancer, metabolic disorders, heart failure, and fatigue. Besides that, it reduces the learning ability in childhood [25,26]. Thus, the Pb and Cd concentration should monitor in milk and infant formula for public health and product quality [3].

The present study aimed to evaluate Aflatoxin M1 and Heavy metal in raw materials and Infant formula produced in Pegah Dairy Plants (PDPs) in Shahrekord in northwest of Iran.

MATERIALS AND METHODS

Sample collection

Infant formulations for infants of 0–6 months (Pegah Dairy Plants, Iran) were randomly selected during 2018-2019. A total of 80 samples of maltodextrin, starch, vitamin, raw cow's milk, whey powder, baby oil, infant formula (immediate, whole and skim) samples were randomly collected during 6 months for analyzing Pb, Cd, and aflatoxin M1. Then, the samples were directly obtained from Pegah Dairy Plants Dairy Plants (PDPs) in Shahrekord before pasteurization. Finally, 30 mL milk was collected and poured in an amber glass flask and kept refrigerated until analysis.

Sample evaluation and ELISA test procedure

Regarding raw cow's milk samples and evaporated milk, 5 mL was incubated at 4°C for 30 min, and then centrifuged for 10 min at 3000 g. The samples of milk serum under the fat layer were collected and directly assayed for AFM1 by a specific ELISA kit (Romer Labs, Singapore). Regarding full-cream milk powder and infant formula, 9.1 g of the powder was dissolved in 100 mL double-distilled water, and the solution was warmed up to about 50°C and homogenized by a magnetic stirrer. Next, the sample was prepared as per the above-mentioned procedure for preparing the sample of raw cow's milk.

The quantity of aflatoxin M1 was measured by ELISA using the Ridascreen® aflatoxin M1 (Romer Labs, Singapore) test kit. The milk samples were centrifuged at 3000 rpm for 10 minutes in 10°C. Then, the upper creamy layer was fully isolated through aspirating. An adequate number of microtiter wells were embedded in the micro well holder for prepared and standard samples. In the next step, 100 µL of the prepared samples and standard solutions were introduced into separate wells and incubated at room temperature for 60 min in the dark. The wells were filled with 250 µL of washing buffer and depleted by absorbent paper. Afterwards, 100 µL enzyme conjugate was introduced and incubated at room temperature for 60 min in the dark. Besides that, 50 µL of chromogen solution and 50 µL of the substrate solution were introduced to each well and stirred well. Then, they were incubated at room temperature for 30 min in the dark. Accordingly, 100 µL of the stop reagent was added to each well and mixed completely. Finally, the absorbance percentages of the calibration curve performed with standards were measured at 450 nm using Multiskan Ascent ELISA Plate Reader (Lab Systems, Vantaa, Finland). The absorption intensity was inversely proportional to AFM1 concentration in the samples [13].

Quality control

In order to validate the results, a standard solution of AFM1 was prepared from Sigma Aldrich Chemicals (A6428). AFM1 free milk samples were spiked with standard solution of AFM1 at the concentrations of 0.01, 0.05, 0.1 and 0.2 µg/L. Finally, AFM1 recovery percentages were recorded in the range of 96.3-98.4% (Table 1) [2017].

Table 1. ELISA technique efficiency verification by spiking various levels of aflatoxin M1 in milk

Spiked AFM1 (pg/L)	Observed value (pg/L)	Recovery (%)	Coefficient of variance (%)
10	9.21	97.4	0.33
50	47.56	98.1	0.27
100	98.45	97.3	0.59
200	197.63	98.2	0.43

Pb and Cd analyses

The Cd and Pb were measured in accordance with Iranian National Standard Determination of Food Pb and Cd Content. The samples were then dried and ashed at 450 °C. Afterwards, 6 M HCl (1 + 1) was introduced, and the resulting solution was heated to dryness. The residue was dissolved in 0.1 M HNO_3, and then Cd and Pb were measured using a graphite furnace atomic absorption spectrometer (Varian-Spectrophotometer AA 600). To investigate the precision of the analytical method, multi-element standard solution (Merck) at different concentrations of Cd and Pb (0.2, 1, 10, 50 and 100 µg/kg) was applied for calibration. The standard curve was run at concentrations of 5, 10, and 30 µg/kg for Pb and 0.5, 1.0, and 1.5 µg/kg for Cd. Limit of detection (LOD) and limit of quantification (LOQ) for Pb measurement were 3 and 9 µg/kg, and for Cd 0.4 and 1.2 µg/kg, respectively. Analysis was performed in duplicate for all samples [28,29].

Statistical analysis

All of statistical analyses were performed using the software *SPSS* Statistics (version 19). ANOVA was used to determine the differences among the means. A probability level of $p<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Regarding the different levels of AFM1, there is a serious health problem, especially for those children who are the main consumer of milk. In this study, a total of 80 raw cow's milk, vitamin, whey protein, and infant formula were analyzed using the competitive enzyme-linked immunosorbent assay (ELISA) test. Table 2 shows AFM1 existence and amounts in raw milk, whey protein, vitamin, and infant formula samples produced in Pagah Dairy Plants (PDPs) during six months. The level of AFM1 contamination in the vitamin used in producing infant formula was less than detectable. In wheat powder, the mean aflatoxin M1 levels was between ND -23 ppb with the mean of 7 ppb , and ranged from 16-33 ppb with the mean of 22.44 ppb in the raw cow's milk. The level of aflatoxin in infant formula was about ND- 8 ppb and the mean was 0.444 ppb. The lowest and highest contamination was observed in vitamin (not detectable) and raw cow's milk, respectively. Based on the results in this study, aflatoxin M1 was found in raw milk, vitamin, whey protein, while infant formula samples were in the range of the permissible level as accepted by Iranian food standards [30,31] and EC and CAC assigned a limit of 50 ng/kg AFM1 in milk and 25 ng/kg for baby milk products. The insufficient amount of aflatoxin M1 in infant formula can be an urgent health issue. With regards to the risk of genotoxic carcinogenicity of aflatoxin M1, it is essential to prevent its occurrence in milk and its consumption by infant [32].

The results are in line with those reported by previous research, which demonstrates the occurrence of AFM1 in dairy products including milk. Hussain and Anwar (2008) [4] study in Pakistan on AFM1 contamination in milk from five dairy species showed that 15.8% of buffalo milk samples were contaminated while no contamination with AFM1 was observed in the studied camel milk samples. In another study, Kamkar et al. (2011) [6] in Ardabil investigated the occurrence of AFM1 in raw milk samples using ELISA test. Their result showed that average aflatoxin M1 concentration was 40.01ng/L, and 14.75% of the samples contained aflatoxin M1 at concentrations higher than the maximum recommended limits by ISIRI, EC and CAC. Omar et al. (2016) evaluated the contamination of aflatoxin M1 (AFM1) in milk product by using the competitive ELISA [33]. All of the studied samples contained varied levels of AFM1 ranging from 9.71 ng/kg to 288.68 ng/kg. The concentration of AFM1 in 66% of fresh milk samples was higher than the maximum tolerance limit accepted by the European Union (50 ng/kg). Twelve percent of AFM1 contaminated pasteurized cow milk samples contained a level higher than the European tolerance limit (14.60-216.78 ng/kg). Moreover, the concentration of AFM1 was higher than the maximum tolerance limit in 85% of infant formula samples authorized by the US and the EU (25 ng/kg). In another study, Kamkar et al. (2011) used 111 samples of raw milk in Sarab, Iran [6]. The concentration of AFM1 at 85 cases (76/6%) ranged between 0/015 mg and 0/128 mg. Further, Maktabi et al. (2011) used ELISA technique and found that levels of aflatoxin M1 contamination were observed in 100% of the samples in Ahvaz country on south of Iran [34]. Rastogi et al. (2004) studied the amount of AFM1 in milk and in children milk products in India by applying competitive ELISA technique and reported that 87.3% were infected among the 87 samples [35]. Furthermore, almost 99% infected samples exceeded the EU and CAC authorized range (50 ng/dl). In another study, Gurbay used from HPLC method in which 27 samples, 59.3% were contaminated with aflatoxin M1 and only one infected sample exceeded the EU.

Table 3 indicates the results of lead and cadmium levels in maltodextrin, starch, vitamin, raw cow's milk, whey powder, and baby oil. As shown, the amount of cadmium and lead contained in the raw material of the Peghas plant is in the standard range. According to Iran's standard, lead and cadmium in maltodextrin should be at 0.5 and 0.05 ppm, and the level of lead and cadmium in maltodextrin should be 4.91 and 18.97 ppb, respectively. Based on the result, the concentrations were observed in starch mean of lead and cadmium 5.3 and 21.58 ppb, respectively. However, Pb and cd concentrations were not observed in vitamins and cheese powders. Cadmium content in baby oil and lead content was 2.67 and 18.22 ppb, respectively, which were less than the recommended standard (Codex STAN 193) for baby oil, which 0.2 ppm. In addition, the amount of cadmium and lead in raw milk was 10.8 and 12.52 ppb, respectively. Further, no significant difference was observed among the groups of formulas with respect to the proportion of samples with detectable levels of these elements.

Table 4 represents the distribution of the lead and cadmium contamination interest across the type of infant formula, instant milk, skim milk and whole milk in Pegah Dairy Plants (PDPs) by utilizing 80 samples. In general, the levels of cadmium and lead in the infant formula samples were considered rather low among the 80 tested products, and all of the concentrations of the heavy metal in infant formula (immediate, whole and skim) were within the recommended range established for lead and cadmium (0.02 ppm) by the Iran standard and FDA codex.

The mean levels of cadmium and lead in infant formula samples were 4.97, and 12.57 ppb, respectively, while it was 13.97 and 5.56ppb in immediate infant formula, respectively. The mean of lead and cadmium in the whole infant formula showed 10.10 and 2.88 ppb, while it was 14.53 and 8.01 ppb, respectively, in skim infant formula. In addition, no correlation was reported between the value of elements and using thermal treatment content or different formula of infant.

The results of the present study are consistent with some other studies. For example, De Castro et al. (2010) investigated the cadmium and lead contamination in infant formula in Brazil and found cadmium lower than the permissible range and level, while the average concentration of lead was relative to local and WHO recommendations of 0.2 mg/kg [36]. Further, In study of Tajkarimi et al. (2008) from 14 dairy plants were sampled in various provinces of Iran and the remaining lead mean was 2.4 ± 1.4 µg/kg in Shoosh country, Khuzestan province (Iran) [37]. Najarnezhad and Akbarabadi (2013) in a study they reported Lead (Pb) and cadmium (Cd) in raw cow's milk in Khorasan-Razavi province, Iran, and they reported the values of Pb was 12.9±6.0 µg/kg and for Cd was 0.3±0.3 µg/kg [38]. Gardener et al. (2019) examined Pb and Cd levels in a large sample size selected by convenience sampling from among US baby foods. The difference in the amounts of Pb and Cd in milk may be related to air pollution resulting from industrial activities in these areas [39]. The difference between the results of this study and others can be related to the sources of heavy metal which contaminated mines, factories, soil type, and seasonal precipitation in different region [6]. The manufacturing process, biochemical composition, and concentration of dairy products can affect the concentration of heavy metals in these products. Finally, lead had a high affinity for casein, and products containing high levels of this protein may include high concentrations of these elements [40]. Moreover, the results of Karimi et al.'s study showed that the potential effect of geographical conditions of dust phenomenon is clear in the presence of heavy metals in raw milk and is one of the reasons for milk contamination with heavy metals [41].

The results of the study of Ghajarbeygi et al showed that the amounts of AFM1 in the whole positive samples were much lower the US legal limit (0.5 ppb), but AFM1 was higher than the EU legal limit (0.05) in 30% of the raw milk samples and 5% of the samples had an AFM1 content of higher than the Iranian legal limit (0.1 ppb) [42]. In one study, AFM1 was reported in 47.91% of the samples to exist at a mean concentration of 39.45 ± 18.40 ng/l. The maximum average concentration of aflatoxin M1 was reported for traditional dairy farm samples (43.9 ± 9.5 ng/l). AFM1 concentration was higher than the highest tolerance limit authorized by EU/CAC in 21.5% of raw cow milk samples and 11.9% pasteurized milk samples [43]. Antioxidant properties and medicinal plants can be used to reduce or eliminate any kind of contamination in food or treat various diseases due to the presence of effective plant substances. Also, aromatic plants can be used to improve the properties of food and medicinal plants [44-48].

Table 2. Distribution AFM1 levels (ppb) in ingredient and infant formula samples produced in Pegah Dairy Plants in Shahrekord, Iran

Sample	Number of Sample	Mean± SD	Minimum	Maximum
Vitamin	80	ND±0	ND	ND
Raw cow's milk	80	12.44±1.87	ND	16
Whey protein	80	7±2.62	ND	23
Infant formula	80	0.444±0.044	ND	8

ND: Non detect

Table 3. Cadmium and Lead content in infant formula based on ppb in Pegah Dairy Plants in Shahrekord, Iran

Sample	Number of Sample	Mean± SD		Minimum		Maximum	
		Cd	Pb	Cd	Pb	Cd	Pb
Maltodextrin	80	18.97±1.01	4.91±0.327	16	4.2	20	5.5
Starch	80	21.58±1.74	5.03±0.489	18.5	4.4	23	5.9
Vitamin	80	ND	ND	ND	ND	ND	ND
Raw cow's milk	80	12.52±1.78	10.8±2.83	3	4.9	49	14
Whey protein	80	15.78±5.14	ND	5	ND	20	ND
Baby oil	80	18.22	2.67	16	3.6	21	2

Table 4. Cadmium and lead content in infant formula based on ppb in Pegah Dairy Plants in Shahrekord, Iran

Sample	Number of Sample	Mean ± SD		Minimum		Maximum	
		Cd	Pb	Cd	Pb	Cd	Pb
Infant formula	80	4.97± 3.08	12.57±2.89	1.5	8	12.3	17
Immediate	80	5.56±0.51	13.97±4.096	5	1.5	6.7	17
Whole	80	2.88±0.808	10.10±1.15	6.5	8.5	9.20	12
Skim	80	8.01±0.697	14.53±1.59	2	10	4.5	17

CONCLUSIONS

Milk contaminated with aflatoxins and heavy metals (Cd and Pb) should be emphasized since it can create some potentially dangerous diseases. The risk of this toxin to children's health has been confirmed by a large number of researchers. The results of the present study indicated that aflatoxin M1 levels in various ingredients and infant formula are lower than the values accepted by Institute of Standards and Industrial Research of Iran (ISIRI). The mean of the heavy metal (Cd and Pb) in the raw materials such as maltodextrin, starch, vitamin, raw milk, whey powder, and baby oil, and the types of infant formula produced by Pegah dairy Plants (PDPs) Shahrekord including baby milk powder, instant, complete, and skim were reported as below the recommended levels. Thus, these materials should be evaluated in different regions of the country and should be able to reduce the contamination of these products due to the numerous complications of these metals in infants.

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Conflicts of Interest

The authors declare no potential conflict of interest.

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