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Research article

Investigation of aflatoxin M1 in infant formula and raw milk in northwestern Syria

Rahoom Nasser, Hussein Alomar *

Department of Pharmacology and Toxicology, Faculty of Pharmacy, Idlib University, Syria

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ABSTRACT

Aflatoxin M1 (AFM1) is one of the most significant chemical contaminants in milk, and may pose health risks to consumers. Due to poor food control in northwestern Syria, it was necessary to conduct an investigational study on the presence of AFM1 in raw milk and infant formula. In this study, 88 samples of milk were collected and divided into two parts: 40 samples of raw cow's milk were collected from cow farms and 48 samples of marketed infant formula were sampled, distributed among 6 different brands. The levels of AFM1 were determined using ELISA technology. The results showed that all raw milk samples were contaminated with AFM1, and 20 (55.55 %) of them exceeded the permissible limit according to the European Commission (EC) standards of 0.05 μg/kg. Most infant formula samples were contaminated with AFM1, and the percentage of samples that exceeded the permissible limit according to European standards was 43 (89 %). A risk assessment was also conducted for the exposure of children to the AFM1 levels obtained in our study, where the risk index was HQ $>$ 1 which indicates the presence of health risks in children consuming infant formula powder. Based on the results obtained, we conclude that the consuming milk in both raw and dried forms can pose health risks to consumers, especially children. Therefore, we recommend implementing AFM1 level testing for imported infant formula in drug control laboratories. Additionally, we suggest monitoring both imported and local feeds to ensure they are free of AFB1.

1. Introduction

Milk is considered an ideal natural food for consumers of all age groups, due to its high nutritional value [1], as it contains numerous macro- and micro-nutrients essential for growth and maintaining a healthy body [2] It contains a high percentage of protein, and is a valuable source of calcium, vitamins and antioxidants, in addition to some fatty substances known to possess anticarcinogenic properties such as butyric acid [3]. However, this milk may be subject to contamination with many pollutants, especially chemical pollutants, the most important of which is the aflatoxin AFM1, which belongs to a family of mycotoxins called aflatoxins. Aflatoxins are secondary metabolites with high toxicity produced by certain types of *Aspergillus fungi* that grow on different fodder and agricultural crops. Aflatoxin B1 is the most common and carcinogenic member of this family being classified as group A carcinogen by the International Agency for Research on Cancer (IARC) [4]. Aflatoxin M1 is another important member of the AF family. It is a hydroxylated derivative of AFB1 that is produced in the liver by the action of cytochrome enzymes CYP450 and then it enters the blood circulation of mammals and is excreted in their milk [5]. AFM1 residues remain resistant when heat milk is processed or fermented, and there is no

* Corresponding author.
E-mail address: hussein.alomar@idlib-university.com (H. Alomar).

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evidence that cold storage, freezing, heat treatment, fermentation or milk drying alter the concentration of aflatoxin. AFM1 is mainly dissolved in the aqueous phase of milk or adsorbed on casein molecules [6]. It is also considered a carcinogenic and mutagenic substance in humans. AFM1 is also excreted in human breast milk and causes effects on infants that include immunosuppression, weight loss, and developmental complications [5].

Due to the war conditions in northwest Syria, there is lack of nutritional control, high incidence of cancer in recent years, and lack of studies to investigate aflatoxin M1 in this resource-limited region. The aim of the present work is to assess the levels of AFM1 in infant formula and raw milk in northwestern Syria and to perform risk assessment for the health of children.

2. Materials & methods

2.1. Samples

Eighty-eight samples of milk were collected in two parts, i.e. 40 samples of raw milk were collected from cow farms located in the Northwestern Syria, over three months (October, November and December 2022). Also, 48 samples of infant formula that were marketed in northwest Syria were sampled out from 6 different brands, and 4 replicates out of a brand of first infant formula of the same batch No, and 4 replicates of follow-on formula of the same batch no, and the brands were coded with symbols next: A, B, C, D, E, F.

The raw milk samples were collected from three regions in Northwest Syria (Idlib, Jisr Al-Shughour and Sarmada) by using sterile plastic containers with a capacity of 100 ml, and they were protected from light during the collection process. They were stored in the freezer at a temperature of -18 °C until analysis.

2.2. Preparation of samples for the ELISA technique

AFM1 levels were determined using the Enzyme Linked Immuno-Sorbent Assay (ELISA) technique being the most qualitative method for the determination of AFM1 according to the European Food Safety Authority (EFSA) [7]. A direct competitive ELISA kit was used to determine AFM1 in raw milk and infant formula for research purposes made by (SEKSM-0005, Solarbio, China) Sensitivity: 0.01 μg/kg. Sample preparation was done according to the kit instructions, the raw milk samples did not need any prior preparation steps except to be at room temperature. 1 g of each sample of infant formula was weighed and placed in a 10 ml plastic tube, then 7 ml of distilled water was added and mixed well for 5 min, then 100 μL of milk were added.

2.3. AFM1 detection by ELISA

The absorbance of the standard solutions and samples was obtained by dividing it by the absorbance value of the standard with concentration 0 and multiplying the result by 100 to obtain the maximum percentage of absorbance. Then the data was entered into the ELISA Calc software to draw the relationship between the percentage absorbance of the titers and their concentration, and a decreasing logarithmic curve was obtained with four parameters: Four-parameter Logistic curve fitting, as shown in Fig. 1. Its equation and R^2 value are as follows:

$$\text{Equation}: y = (A - D) \ / \ [1 + (x/C)\hat{B}] + D$$

$$R^2 = 0.99$$

3. Statistical analysis

The means of variable ± SD were recorded, the data were statistically analyzed using SPSS using nonparametric tests because the

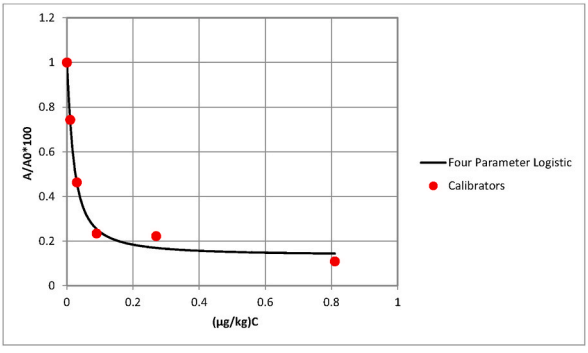

Fig. 1. Standard curve of AFM1 concentrations using ELISA technique.

data did not follow a normal distribution. The Mann-Whitney test was performed to find statistical differences in AFM1 levels between the two groups of infant formula for the first stage and the follow-up milk, and the Kruskal-Wallis test was conducted to test concentration differences between samples by geographical area. A level of p-value <0.05 was considered statistically significant.

3.1. Risk assessment

3.1.1. Risk assessment in infant formula

Exposure to the studied infant formula samples was calculated in infants aged (0–12) months, according to age and normal body weight as expressed by the (CDC) * in order to highlight the difference in exposure to AFM1 and calculate the risk index for cancer (Hazard Quotient HQ) [8].

The estimated daily intake (EDI) for AFM1 was calculated, expressed as one (ng/kg b.w/day), based on the average AFM1 concentration detected in the two types of first infant formula and follow-on milk, and the average daily milk intake (IPF), according to the equation:

$$\text{EDI (ng / kg b.w / day)} = \frac{\text{MCAFM1 (ng/L)} \times \text{IPFI (L/day)}}{\text{BW (kg)}}$$

MC_{AFM1} is the mean concentration of AFM1 in IPF reported in this study.

IPFI is the mean daily infant powdered formula intake.

BW is normal body weight as expressed by the Centers for Disease Control and Prevention (CDC)

The hazard index is calculated as a ratio between the average exposure and the toxicity threshold, and a ratio >1 (HQ) indicates an unacceptable exposure level. To calculate HQ, we used the following equation [8]:

$$\text{Hazard Quotient (HQ)} = \frac{EDI\ (ng/kg\ b.w./day)}{RFD\ (ng/kg\ b.w./day)}$$

3.1.2. Health risk assessment in raw milk

The EDI as well as the HQ were determined according to a risk assessment study conducted in 2022 [9], where the EDI was calculated from the following formula:

$$\text{HI} = \text{EDI (ng / kg b.w. / day)} \setminus \text{TD}_{50}\ (100\ \text{ng / kg bw})$$

where TD_{50} is the dose at which 50 % of experimental animals developed malignant tumors, with a safety factor of 50,000, which was reported as 100 ng/kg [9].

4. Results

4.1. AFM1 levels in infant formula

The research results showed that most samples of infant formula (97 %) marketed in northwest Syria are contaminated with AFM1. The concentrations of AFM1 in infant formula ranged from an undetectable concentration to 0.24 μg/kg, with an average concentration of 0.06 ± 0.04 μg/kg. In addition, 89 % of this samples exceeded the permissible limit in Europe for infant formula is 0.025 μg/kg, while no sample exceeded the permissible limit of 0.5 μg/kg in America. The results of the Mann-Whitney test did not show any statistical differences for the levels of AFM1 in the two types of milk in the first infant formula and the follow-on formula ($p = 0.45$) as shown in Fig. 2.

Also, we did not find any significant differences of the levels of AFM1 in the first infant formula depending on the type of company (Kruskal-Wallis test, ($p = 0.36$) Fig. 3.

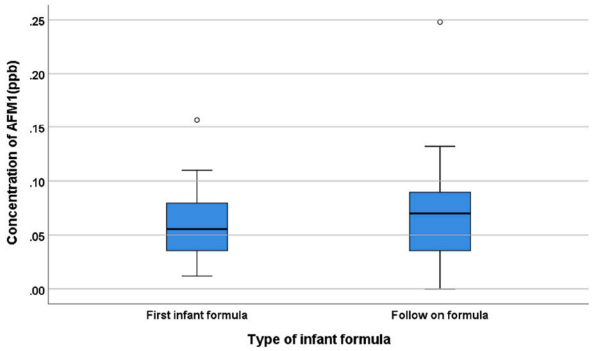

Fig. 2. Comparison of AFM1 levels in first infant formula and follow-on formula.

Moreover, we did not observe any significant differences in the levels of AFM1 in the follow-on milk depending on the type of company (Kruskal-Wallis, ($p = 0.17$) Fig. 4.

Is there a comparison of the AFM1 ranges between first infant formula and follow-on formula for each brand? Our experimental results did not show any significant differences in five of the studied brands (A, B, D, E, F) ($p > 0.05$), while significant differences were noted in the levels of the AFM1 marker between the first stage milk and the follow-up milk in only brand C ($p = 0.02$), Fig. 5.

Exposure and risks to AFM1 were characterized among male and female infants aged (0–12) months based on the daily intake of infant formula at different ages, and the EDI and HQ were calculated, as shown in Table (1).

The HQ > 1 at all ages and in males and females. Infants aged (0–6) months were the most susceptible to AFM1 in IPF. Also, the HQ values in infants up to the age of 6 months were higher than those with their ages ranging from 6 to 12 months.

4.2. AFM1 levels in raw milk

The research results showed that all raw milk samples were contaminated with AFM1, and the concentrations ranged between (ND -1.96) μg/kg, with an average concentration of 0.18, with a standard deviation of 0.35 μg/kg (see Table 2). Half of the samples (20) were above the permissible limit in raw milk, according to European standards, which is 0.05 μg/kg. While only 3 samples (8.33 %) exceeded the permissible limit according to American standards, which is 0.5 μg/kg. We did not find any significant differences in AFM1 levels in raw milk between three regions studied ($p = 0.06$), presented in Fig. 6.

The EDI for 5-year-old children was calculated using the average values of AFM1 concentration in contaminated samples in the three areas studied, and based on daily milk consumption data, where the average milk consumption for 5-year-old children was estimated at 400 ml per day and their average weight was estimated at 19 kg. HQ < 1 Therefore, five-year-old children are not at risk of developing cancer as a result of consuming raw milk contaminated with AFM1 Table (3).

5. Discussion

Results of the present study were compared with those of other studies made. Firstly in infant formula a study in Lebanon in 2019, in which 84 samples of infant formula from 42 brands were collected and analyzed using ELISA technology as well, the results showed that 74 (88 %) of the samples were contaminated with AFM1, and (31 %) of them exceeded the permissible limit according to European standards [10]. In Jordan, the results of a study conducted in 2016 to determine the levels of AFM1 in infant formula, revealed that 85 % of the samples were above the permissible limit in Europe and the concentrations varied from 0.016 to 0.28 μg/kg [11]. Those results are close to the results obtained in our research. However, the results of a study completed in Mexico in 2020 recorded lower levels of AFM1 than the levels recorded in our study, that is, 20 % of the samples were contaminated with AFM1 at concentrations ranging between 0.04 and 0.45 μg/kg, with an average concentration of 0.04 ± 0.09 μg/kg [12]. By comparing the results of our study with the results of a study conducted in Pakistan, in which about half of the samples, 53.84 %, were contaminated with AFM1, while 30.76 % exceeded the permissible limits according to European standards [13]. Also, in a study conducted in the city of Ankara in Turkey in 2014, the percentage of contaminated samples (38.1 %) was less than the percentage obtained in our research, as well as the average aflatoxin concentration of 0.008 μg/kg [14]. In a study in Iran, 29 samples of infant formula from 6 different brands were collected and analyzed using HPLC technology to determine the levels of AFM1 and showed that 3.4 % of the samples were contaminated with an average concentration of 0.007 μg/kg, which is below the maximum permissible limit according to European standards [15].

In a previous Syrian study in 2009, about 95 % of raw cow's milk samples were contaminated with AFM1, and 59 % of them exceeded European standards, while those that exceeded American standards represented 21 % [16].

As for assessing the risks in children as a result of consuming infant formula, our results were similar to the results of the risk assessment study conducted in Lebanon 2022, but the values we obtained were higher than those recorded in the Lebanon study, as their EDI ranged between (0.37–0.78) and (0.40–0.87) (ng/kg b.w./day) in males and females, respectively [8]. While the EDI values in a Turkish study in 2012 were lower than the values we obtained as well, they were 0.08 (ng/kg b.w./Day) in the first infant formula

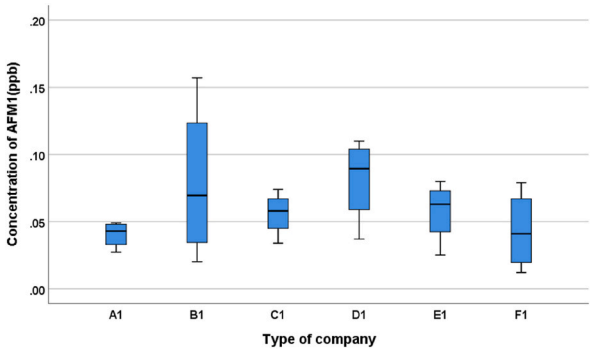

Fig. 3. Comparison of AFM1 levels in the first infant formula by brand type.

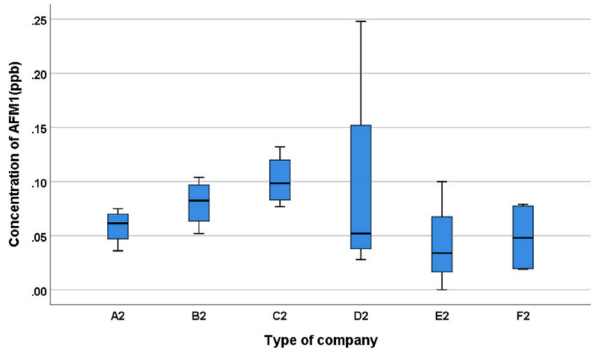

Fig. 4. Comparison of AFM1 levels in follow-on formula **by brand**.

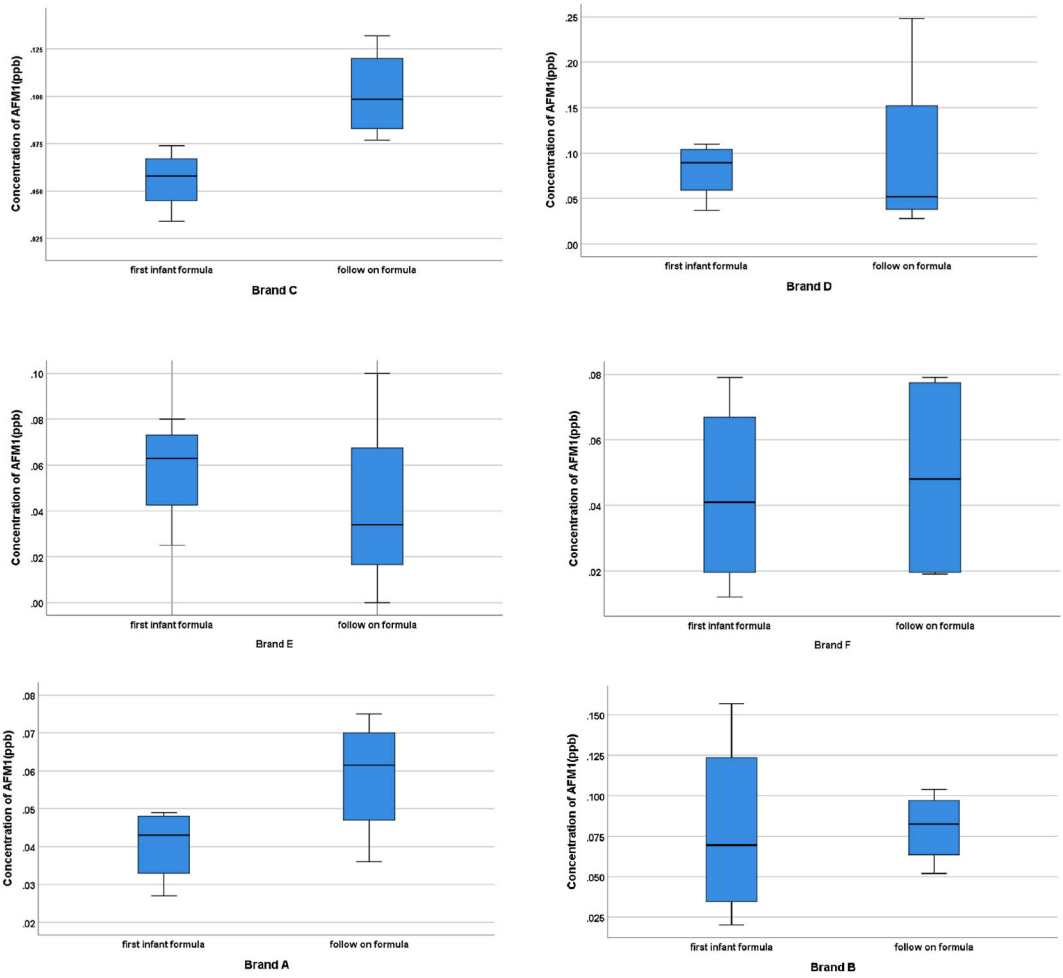

Fig. 5. Comparison of AFM1 levels between first infant and follow-on formula for each brand.

and 0.028 ng/kg b.w./Day in the follow-on formula [17]. The EDI values obtained in a Mexican study in 2020 were higher than our values, ranging from (1.56–14) ng/kg b.w./Day, and the HQ values indicated a risk of cancer [12].

For raw milk, a Turkish study conducted in 2019 on 35 raw milk samples to determine AFM1 levels, all raw milk samples were contaminated with AFM1 similar to what was seen as in our study, but the percentage of samples that exceeded European standards was only 14.28 % [18]. In Iran in 2015, in a study conducted on 254 raw milk samples, the percentage of samples contaminated with AFM1 was (80.3 %), which is less than the percentage of samples contaminated in our research, while the percentage of samples that exceeded European standards was (56.7 %), which is close to the percentage obtained in our research [19]. In a study conducted in

Table 1
Results of exposure assessment of AFM1 in infant formula.

Age(months)	IPF(kg\day)	Average weigh(kg)		EDI (ng\kg bw\day)		HQ	
		Male	Female	Male	Female	Male	Female
0–2	0.13	5.3	5.2	1.45	1.48	7.25	**7.4**
2-4	0.14	6.5	5.9	1.27	1.41	6.35	**7.05**
4–5	0.14	7.5	6.9	1.1	1.2	5.5	**6**
5–6	0.13	8.5	7.7	0.91	1	4.55	**5.04**
6	0.14	8.5	7.7	1.15	1.27	5.75	**6.35**
7≤	0.1	10.1	9.2	0.71	0.79	3.55	**3.95**

Table 2
AFM1 levels in raw milk.

Region	N	Range	Mean ± SD (μg/kg)	Median	Above 0.05 μg/kg	Above 0.5 μg/kg
A	14	0.01–0.83	0.14 ± 0.21	0.04	6 (16.6 %)	1 (2.7 %)
B	14	nd - 0.68	0.11 ± 0.17	0.05	7 (19.4 %)	1 (2.7 %)
C	8	0.04–1.96	0.36 ± 0.64	0.15	7 (19.4 %)	1 (2.7 %)
Total	36	nd - 1.96	0.18 ± 0.35	0.08	20 (55.5 %)	3 (8.3 %)

Nd: No detection.

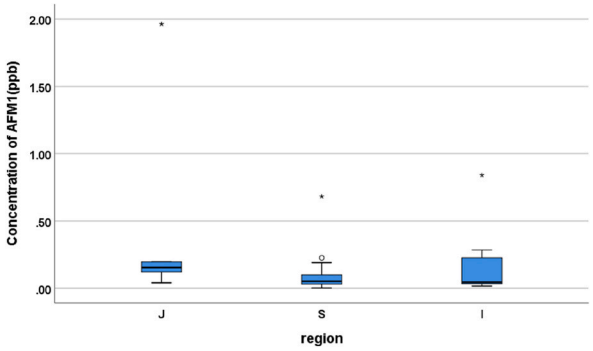

Fig. 6. Concentrations of AFM1 in raw milk in the three regions studied.

Table 3
EDI and HQ values for 5-year-old children.

Region	C (ng\kg)	EDI (ng/kg b.w/Day)	HQ
A	149	3.22	0.032
B	114	2.46	0.024
C	367	7.94	0.079

Egypt, 40 % of the samples were contaminated with AFM1, and the concentrations ranged between (0.008–0.085 μg/kg), and about half of the samples (58 %) exceeded the permissible limit in Europe, almost as in our study [20]. As for Saudi Arabia, in a study in 2017, the percentage of raw milk samples contaminated with AFM1 was 95 %, very close to the percentage we obtained in our research, and the concentrations ranged between (0.09–0.65 μg/kg), with an average of 0.04 μg/kg, which is less than the average that we recorded in our study [21]. In Greece, 46.5 % of the samples were positive for AFM1 contamination, i.e. half the percentage obtained in our research, and only two samples exceeded the limit allowed by European standards [3].

For the risk assessment of AFM1 in raw milk for 5-year-old children, our study's findings were similar to those of a study conducted in Nigeria, which also examined three different geographical regions. The HQ value was <1 as in our study, and the HQ values ranged in the three regions studied for 5-year-old children Between (0.04–0.06) [9].

6. Conclusion

The finding of this study revealed that most of the infant formula samples analyzed were contaminated with AFM1, while all raw milk samples were contained AFM1. This suggests that the feed given to cows in the study areas is likely contaminated with AFB1. The

Consumption of raw milk and AFM1-contaminated infant formula poses significant health risks, particularly for children in north-western Syria, as indicated by HQ values greater than 1. Therefore, it is crucial to implement strict control and continuous monitoring of AFM1 levels in raw milk and infant formula. Enhanced manufacturing standards should be enforced, and breastfeeding should be strongly promoted through awareness campaigns. Additionally, maintaining low AFB1 levels in cow feed is essential to minimize AFM1 contamination.

CRediT authorship contribution statement

Rahoom Nasser: Writing – original draft, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Hussein Alomar:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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