

Contamination level and exposure assessment to Aflatoxin M1 in Jordanian infant milk formulas

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Abstract

Aflatoxin M1 (AFM1) is a principal hydroxylated-aflatoxin B1 (AFB1) metabolite, and has been classified as possible human carcinogen (group 2B). The aim of this study was to survey the contamination level, estimated daily intake (EDI) and tolerable daily intake % (TDI%) of AFM1 in Jordanian infant milk formulas. A total of 120 samples, 48 starter and 72 follow-on formula samples were collected and analyzed using ELISA technique. Of the 120 surveyed samples, 58 (48.33%) were AFM1-positive and exceeded the EU maximum limit for AFM1 in IMF (25 ng/kg). The average AFM1 concentration was 69.93 and 84.78 ng AFM1/kg, with range of <5 – 89.25 and <5 – 213.84 ng AFM1/kg in starter and follow-on formula, respectively. It is also noteworthy the high EDI of AFM1 by infants (1.557 and 1.551 ng AFM1/kg b.w./day), and the high TDI% values (786.9 and 775.9%). In addition, current study indicated high-extrapolated AFB1 content in the feed; accordingly, raised the need to implement good agricultural and hygienic practices as preventive and controlling measures to decrease AFM1 in milk and IMF through controlling AFB1 in feed at the farm level. Finally, it is obvious that the contamination of IMF by AFM1 is an international problem, and the protection of infants and young children against AFM1 in Jordan requires a fundamental setup of clear legal limits of AFM1 in Jordanian standards and strict monitoring and continual analysis of IMF traded and consumed in Jordan.

Introduction

Mycotoxins are highly toxic secondary metabolites produced by mold genera, such as *Aspergillus*, *Fusarium* and *Penicillium*

(Kumar *et al.*, 2008; Sweeney & Dobson, 1998). Aflatoxins (AFs) are the most carcinogenic mycotoxins metabolites produced mainly by strains of *Aspergillus (A.) flavus* and *A. parasiticus*. Majority of the food isolated AFs are aflatoxin B1, B2, G1, G2, M1, and M2 (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2) (Alshannaq & Yu, 2017; Kumar *et al.*, 2008). AFM1 is a principal hydroxylated-AFB1 metabolite that is bio-transformed by hepatic microsomal cytochrome upon ingestion of AFB1 in feed, and usually excreted in milk (Alshannaq & Yu, 2017).

AFs accumulate in body and cause adverse health effects, including carcinogenic, mutagenic, teratogenic, nephrotoxic, hepatotoxic and immunosuppressive effects, as well as developmental and birth defects (IARC, 2012). AFB1 has been classified as the strongest naturally occurring human carcinogen (group 1), while AFM1 has been classified as possible human carcinogen (group 2B) (IARC, 2012). The main AFM1 target organ in human body is the liver so it is primarily a hepatic disease. Milk is the main risk factor of human exposure to AFM1. Milk is considered as the perfect food for adults and infants, providing the major vital and functional ingredients for human life (Awaisheh *et al.*, 2012, Awaisheh *et al.*, 2013, Rababah *et al.*, 2015). Infants are the foremost milk consumers and the most vulnerable group in population, which make them more susceptible to the adverse effects of AFM1, particularly hepatocellular carcinoma (WHO, 2014). Therefore, the incidence of AFM1 in milk and milk products is a serious health concern. The level of AFM1 in milk depends on the level of AFB1 in feeds. The contamination of milk and milk products by AFM1 is an inevitable process as at least 25% of the world's crops are contaminated with mycotoxins including AFB1, and large amount of these crops are used for dairy cows feeding (FAO, 2003). Moreover, AFM1 is heat stable and can resist various thermal treatments, and is almost entirely retained in pasteurized, powdered and infant milk formula (IMF) (Galvano *et al.*, 2001). Therefore, the incidence of AFM1 in milk and milk products cannot be prevented but can be controlled by restricting the level of AFB1 in feeds.

Internationally, the needs emerged and insisted on the importance of surveying and controlling the levels of AFM1 in various dairy products, with more attention to IMF. Therefore, the EU and Codex Alimentarius legal maximum tolerance limits (MTL) for AFM1 in IMF was 25 ng/kg (EC Regulation, 2006), with a tolerable daily intake (TDI) of 0.2 ng/kg b.w./day (Kuiper-

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Goodman, 1989). However, recent studies reported the increasing incidence AFM1 in IMFs, with many samples exceeding the international MTL (El-Tras *et al.*, 2011; Kabak, 2012; Ghanem, 2009; Sadeghi *et al.*, 2009). These reports drew the attention to the high health and life-threatening risks the infants may expose to upon consuming such contaminated products.

Despite the high risks of mycotoxins on human health, particularly infant's health, surprisingly, official Jordanian standards of IMFs lack clear legal limits of AFM1 in these formulas, which implies that these formulas are not officially and routinely tested for AFM1. Upon extensive search, only one small-scale study by Omar (2016) was found in Jordan, where results of 20 samples showed that the level of AFM1 in 85% of IMF samples was higher than MTL, with average of 120.26 ng/kg and range from 16.55 to 288.68 ng/kg. Despite the important evidence these results might prevail about the health risks the infants in Jordan might be exposed to due to AFM1 contamination, the limited number of surveyed samples in this study is a serious limiting factor and could not give an actual pic-

ture about the contamination level of AFM1 in IMF in Jordan. In addition, this study did not assess the exposure risk assessment of Jordanian infants to AFM1 in IMF. For these reasons, the aim of this work was to assess the risk of exposure of Jordanian infants to AFM1 through determination the levels of AFM1 in expanded samples size of the leading brands of IMF marketed in Jordan using ELISA.

Materials and Methods

Samples collection

Total of 120 powdered IMF samples representing the most traded IMF brands in Jordan (n=15; 3 different batch numbers from each brand), were randomly collected from different markets and pharmacies in Jordan during March and September 2016. Collected samples were from local and imported sources, and classified as starter formula (given to infants during the first 6 months of age, 48 samples) and follow-on formula (given to infants from the 6th month on and for young children, 72 samples) (Table 1). Samples were stored in plastic bags at -20°C until the analysis.

Samples preparation

A portion of 10g from IMF samples was dissolved in 100 ml double distilled water, warmed up to 50°C and stirred on a stirrer hot plate, and centrifuged at 3500 g for 10 min at 10°C. The upper fat layer was skimmed, and milk serum was directly assayed for AFM1 using a specific ELISA kit.

Analysis of AFM1 by competitive enzyme linked immunosorbent assay (ELISA)

Determination of AFM1 was carried out using Ridascreen Aflatoxin M1 competitive ELISA kit (Art No. R1121) from R-biopharm (Derm-Stadt, Germany), with detection limit of 5 ppt. Before use, the kit was kept at room temperature (20-25°C) for 1 h. For the preparation of standard curve, AFM1 standard solutions of 0, 5, 10, 20, 40, and 80 ppt were provided with the kit. Samples analysis was carried out according to the manufacturer's instructions. Briefly: AFM1-microtiter plate was pipetted with

100 µL of antibody solution into each well. The plate was gently mixed by manual shaking and incubated for 15 min at 25°C, wells were then washed twice by 250 µL of washing buffer and tapped upside down vigorously against absorbent paper. A 100 µL of the standard solutions or prepared samples was added in duplicate to separate wells, gently mixed and incubated in dark for 30 min at 25°C. Wells were washed out again as above described. A 100 µL of conjugate solution was added into wells, gently mixed and incubated in dark for 15 min at 25°C, then washed out twice again. A 100 µL of substrate/chromogen solution was then added to each well, mixed gently and incubated in dark for 15 min at 25°C, followed by adding 100 µL of the stop solution (H₂SO₄) to each well. The absorbance was measured at 450 nm in Bioteck-XLD800 Multi Scan Plate Reader (Bioteck, VA, USA), and the absorption intensity was inversely proportional to samples AFM1 contents. AFM1 sheet supplied with the kit was used to generate a standard curve and to calculate the concentration of AFM1 in the samples.

Infants estimated daily intake (EDI) of AFM1

The infants EDI of AFM1 was calculated from the daily intake of starter and follow-on formula, the average AFM1 level in IMF samples, and mean body weight (b.w.) for infants (Bogalho *et al.*, 2018). The two daily IMF consumption estimates were considered: 150 mL/kg b. wt. (for babies of 6 months age with 6.5 kg b.w.) and 1 L (for infants of 12 months age with 8.2 kg b.w.). According to the IMF manufacturers, 4.5 g IMF was required to prepare 30 mL of ready milk formula. The EDI was calculated according to Bogalho *et al.* (2018):

$$\text{EDI (ng/kg b.w./day)} = [\text{AFM1}] \times [\text{milk consumption}]/[\text{b.w.}]$$

The percentage of TDI from consumption of IMF was calculated as follows:

$$\text{TDI \%} = \text{EDI/TDI} \times 100$$

where TDI was derived from formula of Kuiper-Goodman (1990).

Calculation of extrapolated values of aflatoxin B1 concentration in feeds

A linear relationship was proposed between the concentration of AFB1 in feeds consumed by animals like cows and AFM1 in milk and only 1.6% of ingested AFB1 is bio transformed to AFM1 by the dairy cattle (Pettersson, 1998). According to Price *et al.* (1985) the expected concentrations of AFB1 in animal feeds can be back calculated from the concentrations of AFM1 of IMF samples. Therefore, the values of AFB1 contamination in dairy animal feeds were back calculated by the formula given below:

$$\text{AFB1 (}\mu\text{g/kg)} = [\text{AFM1 (ng/kg)} \times 100] / 1.6 / 1000.$$

Results and Discussion

Contamination level of AFM1 in infant starter and follow-on milk formula

The current study is the first ever large-scale study surveying the AFM1 level in IMF in Jordan. Contamination level of AFM1 in 120 IMF samples (48 starters and 72 follow-on) was screened. Results revealed that 58 of 120 samples (48.33%) were contaminated with AFM1 above the international MTL (25 ng/kg). The average of AFM1 was 74.26 ng/kg and ranged from <5 - 213.84 ng/kg. In detail, 24 samples (50%) and 34 samples (47.23%) of the starter and follow-on formulas were contaminated with AFM1 above MTL, with average of 69.93 and 84.78 ng/kg, and range of <5 - 89.25 and <5 - 213.84 ng/kg, respectively (Table 1).

In agreement but higher than our findings, the only study found in Jordan (Omar, 2016) reported 85% incidence rate and 120.26 ng/kg average contamination level of AFM1 in IMF. This higher incidence rate and contamination level could be due to the small samples size surveyed, 20 samples. However, several international studies revealed same high incidence and contamination level of AFM1 in IMF. For example, in Syria the incidence rate of AFM1 in milk used for infants was 80% of tested samples with range of 20 - 765 ng/l (Ghanem, 2009).

Table 1. Numbers (%) of AFM1 negative, non-violative-positive and violative-positive in milk samples.

Milk type	No. samples	Negative (%)	Non-violative positive	Violative positive	Average ng/kg	Min – Max ng/kg
Starter (0-6 months)	48	24 (50)	0	24 (50)	69.93±5.66	<5 - 89.25
Follow up (6-36 months)	72	38 (52.77)	0	34 (47.22)	84.78±9.34	<5 - 213.84
Total	120	62 (51.67)	0	58 (48.33)	74.26±7.54	<5 - 213.84

Negative samples: samples with no detected aflatoxin; Non-violative-positive sample: samples with detected aflatoxin below 25 ng/kg; and Violative-positive: samples with aflatoxin exceeding 25 ng/kg.

Another study in Italy reported that AFM1 was found in 49 (53%) dry infant milk samples, with range of <1 - 79.6 ng/kg with mean level of 32.2 ng/kg (Galvano *et al.*, 2001). In a Korean study, the incidence of AFM1 in IMF was 85% of the samples, with average of 46 ng/kg (Kim *et al.*, 2000). Moreover, Baydar *et al.* (2007) found that 36.5 % of 63 IMF samples contained AFM1 between 0.06 - 0.32 ppb in Turkey. However, it is quite obvious and crucial that the contamination of IMF with AFM1 is an international problem, and the protection of infants and young children from AFM1 requires fundamental national and international cooperation and far-reaching revisions of national and international regulations and approaches of surveillance, toxicity and risk assessment of AFM1 (Juan *et al.*, 2014).

Exposure assessment of Jordanian infants to AFM1 from IMF

Up to our best knowledge, this is the first ever study to assess the EDI of AFM1 by Jordanian infants. Unexpectedly, the current results revealed a high EDI and TDI % values of AFM1 for both age groups (6 and 12 months) (Table 2). EDI results were 1.573 and 1.551 ng/kg b.w./day for 6- and 12-months infants age, respectively. The current EDI values exceeded the international TDI values (0.2 ng/kg b.w./day) by 7.869 and 7.759 times for both age groups, respectively. In comparison to international studies, our results were lower and higher than the results of several studies. Lower than our results, Henry *et al.* (2001) showed that the maximum EDI was 0.48 and 0.55 ng AFM1/kg.bw/day for infants in Catalonia and France, respectively. Ishikawa *et al.* (2016) reported lower EDI values (0.078 - 0.306 ng AFM1/kg

b.w./day) than ours in IMF in Brazil. A study by Oliveira *et al.* (1997) showed higher EDI than our results, where EDI value was 3.7 ng AFM1/kg b.w./day for 4 months infant (6 kg weight), representing 22 ng daily intake of AFM1. However, several other studies reported a variable EDI results in different countries (Spain, Argentina, and Thailand), with values of 0.16 - 3.70 ng AFM1/kg b.w./day (Cano-Sancho *et al.*, 2010, Alonso *et al.*, 2010, Ruangwiset *et al.*, 2011). For Jordan, there are no data available about the EDI values and exposure risks to AFM1 by Jordanian infants. The important issue emerges here is the high-risk possibility of infant's exposure to AFM1 from IMF in Jordan and the world.

Extrapolated concentration of aflatoxin B1 in animal feeds

AFM1 is heat stable ($\leq 120^\circ\text{C}$) and cannot be removed or eliminated by chemical or physical treatments, thus monitoring of AFB1 levels in animal feeds is the only effective measure to control contamination of AFM1 in milk and thus IMF (JECFA, 2001). The maximum allowed contents of AFB1 in complete feeds has been established at 5 μg AFB1/kg feed for dairy animals such as cattle, sheep and goats (EC, 2003). Our results in Table 3 showed that the range of feed extrapolated AFB1 content of milk of starter formula was <0.313-5.578 μg AFB1/kg, whereas that of follow-on formula was <0.313-13.365 μg AFB1/kg. The average range of feed extrapolated AFB1 content of both formula types was <0.313-13.365 μg AFB1/kg. Current results indicated that AFB1 extrapolated content in feed is marginal, and this extrapolated content could pose a serious health problem over the long time of consuming milk by infants. In comparison to our

results, Omar (2016) reported high-extrapolated AFB1 values for fresh and pasteurized cow's milk, the results ranged from 0.6 to 8.1 and 0.9 to 13.5 μg AFB1/kg, respectively. Current findings advise that the best measure to control AFM1 in IMF should start at the farm level to ensure the safety of milk used in IMF processing through controlling the contamination level of AFB1 in feeds by applying the good agricultural and good hygienic practices at the farm level.

Conclusions

The present results reported the first study of AFM1 level and EDI in IMF in Jordan. Of the 120 surveyed samples, 58 (48.33%) were AFM1-positive and exceeded the EU maximum limit. The maximum AFM1 values detected was 89.25 and 213.84 ng AFM1/kg of starter and follow-on formula, respectively. It is also noteworthy the high EDI of AFM1 by infants (1.557 and 1.551 ng AFM1/kg b.w./day), and the high TDI% values (786.9 and 775.9%). The findings of the current study indicated high-extrapolated AFB1 content in feed and accordingly raised the need to reinforce application of good agricultural and good hygienic practices as preventive and control measures to decrease AFM1 in milk and IMF through controlling AFB1 in feed at the farm level. Moreover, it is very important to setup clear legal limits of AFM1 in IMF in Jordanian standards.

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Table 2. Estimated Daily Intake (EDI) and tolerable daily intake% (TDI%) of AFM1 by infants in Jordan.

Age	Milk intake (weight of powder milk)	Average b.w. (kg)	EDI (ng/kg b.w./Day)	%TDI
6 months	975 mL (146.23 g)	6.5	1.573±0.154	786.9
12 months	1000 mL (150 g)	8.2	1.551±0.171	775.9

Table 3. Extrapolated values of Aflatoxins B1 concentration in animal feeds in naturally contaminated IMF.

Milk type	AFM1 (ng/kg)	AFB1 ($\mu\text{g}/\text{kg}$)
Starter (0-6 months)	<5 - 89.25	<0.313 - 5.578
Follow up (6-36 months)	<5 - 213.84	<0.313 - 13.365
Total	<5 - 213.84	<0.313 - 13.365

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