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Original Research

Occurrence and the level of contamination of aflatoxin M1 in raw, pasteurized, and ultra-heat treated buffalo milk consumed in Sohag and Assiut, upper Egypt

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ABSTRACT

Aim: This study evaluated the aflatoxin M1 (AFM1) in raw, pasteurized and ultra-heat treated (UHT) buffalo milk consumed in upper Egypt. **Materials and Methods:** Milk samples were collected from January to June 2013. The competitive enzyme-linked immunosorbent assay was applied. **Results:** All the tested samples were positive for AFM1, which were above the Egyptian regulations limits 0 ng/L. The raw milk showed 93% of samples were above the permitted limit set by the European Commission (EC), whereas 3.3% of samples exceeded US Food and Drug Administration (US FDA) tolerance limit. The pasteurized milk showed 100% and 13.5% of samples exceeded the EC, and US FDA, respectively. UHT milk Brand I and II showed 100% and 86.5% of samples were above EC, and 36.6% and 6.6% of samples exceeded US FDA, respectively. **Conclusion:** These results raise concerns about the milk consumed in Egypt and it must be monitored for AFM1 contamination.

KEY WORDS: Aflatoxin M1, buffalo milk, enzyme-linked immunosorbent assay

INTRODUCTION

The exposure to the contaminant aflatoxin, a mycotoxin, has become a global issue because it is a potent carcinogen and is linked to the development of liver cancer [1]. Aflatoxin is naturally produced by the *Aspergillus* species of fungi, mainly *Aspergillus flavus*, *Aspergillus parasiticus, and Aspergillus nomius* [2]. Aflatoxin M1 (AFM1) is the pre-dominant (4-hydroxy) metabolite of aflatoxin B1 (AFB1), which is ingested by cattle through contaminated feed. Once ingested, AFB1 is rapidly absorbed by the gastrointestinal tract and metabolized to AFM1. The toxin appears in the blood stream 15 min after ingestion, at which point it is secreted into the milk by the mammary gland [3]. The amount of AFM1 found in milk depends on several factors, including (but not limited to) animal breed, lactation period, and mammary infections. Until 6% of AFB1 is secreted into the milk as AFM1 [4]; because this toxin is resistant to heat treatments [4], it is almost entirely retained in pasteurized milk and milk-based products. Moreover, only a minimal decrease in AFM1 content has been verified in ultra-heat treated (UHT) milk after long-term storage [5]. The acute toxicity of AFM1 is similar or slightly less than that of AFB1, but its carcinogenic potential is approximately 10 times less than that of AFB1 [6]. Thus, the International Agency for Research on Cancer has classified AFB1 and AFM1 as class 1 (carcinogenic) and 2b (possibly carcinogenic) carcinogens, respectively [1].

Milk has the greatest potential for introducing AFM1 into the human diet[7] because it is not destroyed by pasteurization and can be transferred to other milk-based products [6]. This increases the occurrence of AFM1 in commercially available dairy products, which are ingested in large quantities by the human population, especially infants and young children. Because regulatory limits throughout the world are influenced by economic factors, they often vary between countries [8]. The establishment of strict maximum residue limits (MRLs) in milk and admissible limits for AFB1 in cattle feed are considered to be sufficient to minimize aflatoxin consumption. Maximum levels in liquid milk range from 50 ng/L in the European Union (EU) [9], Codex Alimentarius Commission [10], and morocco[11] and reach to 500 ng/L in Iran[12] and the USA [13]. Syria's regulatory level falls in the middle of these two values at 200 ng/L [11]. Thus, the maximum permitted level of AFM1 in milk in the EU is among the lowest in the world and is based on the ALARA principle [14]. The EU has set even more restrictive MRLs regarding the presence of AFM1 in baby food [15]. Europe also has strict guidelines with regard to AFB1 limits in feedstuff supplies, with a limit of 5 mg/kg of feed for dairy cattle. This level is currently enforced in the EU countries, the new member states, and EFTA countries [16].

By the end of 2003, AFM1 regulations were in place in 60 countries, with the majority being in the EU and candidate EU countries. However, additional countries include those in Africa, Asia, and Latin America, which also apply the limit of 50 ng/L [17]. The European Commission (EC) Regulation (no. 1881/2006) set a maximum permissible limit of 0.05 mg/L for AFM1 in raw and heat-treated milk, and milk used to manufacture dairy products [9]. In Egypt, the Ministry of Health established that fluid milk and dairy products should contain no AFM1[18] and although the mycological quality of raw milk in Egypt has been studied extensively, the data on the content of AFM1 in raw or heat-treated milk are not readily available [19]. The goal of the present study was to determine the incidence and concentrations of AFM1 in different types of milk samples (raw, pasteurized, and UHT buffalo milk) marketed and consumed in Upper Egypt. In addition, it was to compare our results against the regulations regarding AFM1 that have been legislated by the EC, US Food and Drug Administration (US FDA), and Egyptian Regulations.

MATERIALS AND METHODS

Sampling

Ninety samples of whole buffalo raw milk, pasteurized, UHT-treated Brand I and II milks were collected (30 samples from each type). Samples were collected from January to June 2013 from units available in retail stores from the cities of Sohag and Assiut. There was no repetition of batches. The sources of UHT milk Brands I and II were the two largest companies responsible for milk trade in the Egyptian market. The sample unit was one original package of each milk type (i.e., 1 L of buffalo raw, pasteurized, and UHT milk). Samples were identified, placed in polypropylene bags, and immediately sent to the laboratory to be analyzed.

Determination of AFM1 Content

Quantitative analysis to evaluate the presence of AFM1 in the different milk samples was performed by competitive enzyme immunoassay using REF AFM1 kits (Immunospec Corporation, California, USA; cat no. E 27-414), which contained microtiter plates coated with antibodies specific to AFM1, an AFM1 standard solution at concentrations of 0, 100, 500, 1000, 5000, and 10000 ng/l, with 0.5 ml each in methanol as ×10 concentrate, peroxidase-conjugated AFM1, substrate/chromogen, and stop solution.

Preparation of Milk Samples

For all the types of milk samples, 5 mL of milk was chilled to 4°C and was centrifuged for 10 min at 3000 g. The fatty layer was removed and 450 μL of the defatted milk was taken off and mixed with 50 μL methanol. This solution was applied directly to the enzyme-linked immunosorbent assay (ELISA) microtiter plate.

Reagent Preparation

Because of the standards are concentrated ×10, they diluted by enclosed standards/sample diluent 1:10. i.e., 50 μL standards + 450 μL diluent (methanol).

Test Procedure

A sufficient number of microtiter wells were inserted into the microwell holder according to the manufacturer's instructions. One hundred microliters of the AFM1 standard solutions and of the samples (100 μL/well) were added in duplicates to the wells and incubated for 30 min at room temperature in the dark. The wells were washed three times with 250 mL washing buffer. After the washing steps, 100 μL of the peroxidase-conjugated AFM1 was added and the mixture was incubated for 15 min at room temperature in the dark. After incubation, the wells were washed again three times with 250 mL washing buffer, and 100 μL of substrate/chromogen was added to each well, followed by gentle shaking of the plate and incubation for 15 min at room temperature in the dark. At the end of the incubation period, 100 μL of the stop solution was added into each well; the plate was then shaken manually for gentle mixing of the contents. Absorbance was measured at 450 nm using an ELISA micro-plate reader.

Evaluation of AFM1 Concentration

The absorption is inversely proportional to the AFM1 concentration in the sample. The detection limit for the method is <10 ng/L, with a recovery rate is 102% for milk. The dilution factor was one. The values were calculated using the RIDA-SOFT Win software. Statistical analysis was performed by Student's t-test using the SPSS software package, version 16 (Chicago, USA).

RESULTS

All the milk samples tested in this study were positive for AFM1 and the incidences and concentrations of each are summarized in Tables 1 and 2.

Raw, Pasteurized and UHT Buffalo Milk (Sohag)

Samples from Sohag had AFM1 concentrations presented in Table 1.

Raw, Pasteurized and UHT Buffalo Milk (Assiut)

Milk samples from Assiut contained AFM1 concentrations were presented in Table 1.

AFM1 Levels in Milk Samples Exceeded Regulatory Limits

The regulatory limits for AFM1 established by the EC/Codex, US FDA, and Egyptian standards are summarized in Table 3.

Comparison of AFM1 Levels in Milk from Sohag and Assiut

Figure 1 shows the statistical differences between the incidence and concentration of AFM1 from the three types of milk from Sohag and Assiut.

Table 1: Occurrence and distribution of aflatoxin M1 in different types of buffalo milk samples from Sohag

Sample	Number of sample	Positive samples			
		Average (ng/L)	Mean±SD (ng/L)	%	Minimum-maximum (ng/L)
Raw milk	15	123.27	64.49±16.8	100	9.93-490.48
Pasteurized milk	15	277.46	226.62±22.2	100	99.58-964.5
UHT milk Brand I	8	127.9	118.8±22.0	100	97.7-562.2
Brand II	8	71.75	84.67±19.8	100	36.44-88.19

UHT: Ultra-heat treated, AFM1: Aflatoxin M1, SD: Standard deviation

Table 2: Occurrence and distribution of aflatoxin M1 in different types of buffalo milk samples from Assiut

Sample	Number of sample	Positive sample			
		Average (ng/L)	Mean±SD (ng/L)	%	Minimum-maximum (ng/L)
Raw milk	15	250.79	130.6±29.9	100	99.38-500
Pasteurized milk	15	226.37	197.1±14.6	100	69.77-468.2
UHT milk Brand I	7	107.68	94.82±48.75	100	51.13-201.20
Brand II	7	185.71	95.96±23.1	100	18.43-646.34

UHT: Ultra-heat treated, AFM1: Aflatoxin M1, SD: Standard deviation

Table 3: Levels of AFM1 (ng/L) in raw, pasteurized and UHT buffalo milk samples exceeding limits established by the EC/Codex, US FDA and Egyptian regulations

Sample category	Positive samples	*n* (%)		
		Exceeding EC	Exceeding US FDA	Exceeding ER
Raw milk	30	28 (93)	1 (3.3)	30 (100)
Pasteurized milk	30	30 (100)	4 (13.5)	30 (100)
UHT m UHT milk Brand I	15	15 (100)	5 (36.6)	15 (100)
Brand II	15	13 (86.5)	1 (6.6)	15 (100)

EC: European Commission, (2006), the limit in milk is 50 ng/L. US FDA: US FDA, (2011), the limit in milk is 500 ng/L. ER: Egyptian regulations, (1990), the limit in milk is 0 ng/L. FDA: Food and Drug Administration, UHT: Ultra-heat treated, AFM1: Aflatoxin M1, SD: Standard deviation

DISCUSSION

Throughout the world, there is much attention focused on the control of AFM1 contamination in milk, which is demonstrated by many factors. Many scientific papers have been published on the development and validation of novel analytical methods for measuring AFM1. In addition, over the last 5 years, several surveys have been conducted regarding the occurrence of AFM1 in dairy products. Finally, the importance of this topic is underscored by the various international organizations involved in monitoring AFM1 levels. The first self-evident observation is that the problem of AFM1 contamination in dairy products is mostly perceived in a specific geographical area, as almost all investigations have been carried out in the Middle East [17].

In the present study, we found that all samples from both cities tested positive for AFM1. The mean concentration of AFM1 in raw buffalo milk from Sohag was 64.49 ± 16.8 ng/L, with an average of 123.27 ng/L. Of all raw milk samples tested, 86.5% contained AFM1 at levels higher than the maximum permissible limit of 50 ng/L set by the EU regulations [9]. In Assiut, the mean concentration of AFM1 in raw buffalo milk was 130.6 ± 29.9 ng/L, with an average of 250.79 ng/L. All samples from Assiut tested above the MRL set by the EU regulations, but only one tested at the 500 ng/L maximum set by the US FDA [20]. Our results demonstrate a higher incidence of AFM1 contamination compared to an earlier study performed by Salem [21]. This study investigated the occurrence of aflatoxins in raw milk from six dairy farms in Assiut-Egypt and found that 59% of samples tested positive for AFM1 (compared to our results showing all Assiut samples contained AFM1). However, our data of the average levels of AFM1 in raw buffalo milk (123 and 250 ng/L) do correlate with previous researches presented earlier in Egypt focused on AFM1 in buffalo milk. These data demonstrated AFM1 contamination at levels similar to what we report here (220 and 228 ng/L) [22,23]. In a recent study by Motawee *et al.* [24], they found 84% of the buffalo milk samples had 150 ng/L of AFM1 level, and only one sample had 270 ng/L in Ismalia, Egypt, and also these results are similar to what we report here.

In addition to raw milk, we assayed for AFM1 in pasteurized milk samples from both cities. The pasteurized milk samples from Sohag had a mean AFM1 concentration of 226.62 ± 22.2 ng/L, with an average of 277.46 ng/L. All samples tested were above the MRL set by the EC[9] and 13.5% had levels higher than that set by the US FDA [20]. The pasteurized milk samples

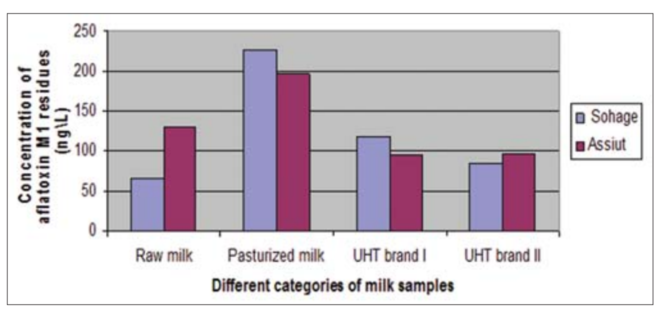

Figure 1: Comparison between different milk samples categories from Sohage and Assiut

from Assiut had a mean concentration of 197.1 ± 14.6 ng/L, while the average was 226.37 ng/L. As with the Sohag samples, all Assiut samples were above the MRL set by the EC [9], but none were above that set by the US FDA [20]. A previous survey conducted on pasteurized milk in Syrian market by Ghanem and Orfi [25]. They found the range of contamination was relatively higher in pasteurized milk than in raw cow and sheep's milk. In addition, this survey demonstrated that 80% of AFM1-contaminated pasteurized cow's milk samples exceeded the European tolerance limit, with a range of contamination between 89 and 765 ng/L. Similarly, a study in morocco found that 89% of pasteurized milk samples contained AFM1 with an average concentration of 186 ng/L [26], which is similar to our pasteurized milk sample results. Although the data for these countries are readily available, the results of the current work are the first available on pasteurized milk in Egypt.

Because aflatoxins are resistant to heat treatment, it was important to include UHT milk samples in this study. We determined that the UHT milk samples from Sohag showed mean AFM1 contamination levels of 118.8 ± 22.0 and 84.67 ± 19.8 ng/L for Brands I and II, respectively (average concentrations were 127.9 and 71.75 ng/L, respectively). All Brand I UHT milk samples and 87% of Brand II samples were contaminated at levels above the MRL set by EC [9]. With regard to the MRL set by the US FDA [20], 27.5% of Brand I samples exceeded it, but none of Brand II samples were above MRL set by the US FDA [20]. The mean AFM1 concentrations of Brands I and II UHT milk samples from Assiut were 94.82 ± 48.75 and 95.96 ± 23.1 ng/L, respectively (averages were 107.68 and 185.71 ng/L, respectively). All Brand I milk samples tested above the MRL set by the EC [9]; however, none were above that set by the US FDA [20]. Of the Brand II samples tested, 85.7% were above the MRL set by the EC [9] and 14.28% were above the US FDA [20] guidelines. Several studies worldwide have assayed for the presence of AFM1 in UHT milk. In Turkey, Unusan [27] found that 58.1% of samples were positive for AFM1 with a mean concentration of 108.2 ng/L (average < 10 to 543.6 ng/L). Samples from Pakistan showed an average AFM1 concentration range of 29.3 to 102.8 ng/L [28]. A study conducted in Brazil showed that 83.3% of milk samples contained AFM1 at a mean concentration of 58 ± 44 ng/L (range, 10 to > 200 ng/L) [29]. In Kafr El-Sheikh, Egypt, 70% of UHT samples were contaminated with AFM1 at a mean concentration of 23.1 ± 4.7 ng/L (range, 6-85 ng/L) [30].

On comparing the results of our study to those of others conducted in Egypt, we noted a higher incidence of AFM1 in the UHT milk samples analyzed herein. Some studies reported very low contamination levels and a higher incidence of AFM1-negative samples [19,31,32]. In contrast, others found a much higher incidence of AFM1-positive samples and, generally, a much higher level of contamination. This divergence was seen even when studies used the same analytical method(s) and analyzed samples from the same country as the reports discussed above [5,33-35]. The variations in AFM1 levels between these studies could be attributed to geography, country, season, environmental conditions, sub-standard agricultural systems, low availability of green fodder, excessive use of concentrated feed, and aflatoxin contamination of feed and grain during storage [12,36-38]. The milk samples investigated in the current study were collected from the Upper Egyptian cities of Assiut and Sohag, which are located in southern Egypt. These cities experience extremely high temperatures, extended dry seasons, and low availability of green fodder. Thus, farmers in these regions often depend on the use of feedstuffs that have been inappropriately stored, which leads to aflatoxin contamination. All these factors and, in particular, feeding animals AFB1-contaminated rations, led to the increased incidence of high AFM1 concentrations in milk in Upper Egypt. This is important considering a recent study that suggested that the most effective way to control AFM1 in the milk supply is to reduce AFB1 contamination of raw materials and supplementary feedstuffs for dairy cattle [12,37]. Considering the fact that pasteurized and UHT milk are important components of the human diet, especially for children, the AFM1 levels in these products are of concern due to their effect on human health. The legal limits for AFM1 in milk have been established by national regulatory authorities in the USA and EU, which cap these limits at 500 ng/L and 50 ng/L, respectively [9]. In addition, most countries have set maximum admissible levels of AFB1 in feed [16], which vary from 50 ng/kg (EU) to 500 ng/kg (US FDA) [9,20]. However, some countries enforce a zero-tolerance strategy (Romania and Egypt) in order to maximally protect consumer health, even at the expense of milk producers [17]. In Egypt, the Ministry of Health established that fluid milk and dairy products should be free of AFM1 [18].

Previous studies conducted in Egypt showed an infrequent occurrence of AFM1 contamination when compared to that in our study [19,22-24,32]. This may be attributed to differences in location, as most of the previous studies were conducted within the delta region. This area lies in northern Egypt, where fertile soil and green fodders are available as feed supplement to dairy cattle nearly year-round. This is in contrast to the Upper Egypt location of our study, where the dry seasons allow farmers to provide their animals green fodders only during a few winter months. Thus, the main feed supplements are grains and concentrated feed that were stored inappropriately. These factors may explain the similar results we obtained for milk samples from Sohag and Assiut, as the samples' AFM1 levels showed minimal significant differences. These areas have the same geographical, seasonal, and environmental conditions along with similar supplementary feedstuffs for dairy cattle. Our data combined with those from previous studies demonstrate the important aspects that must be considered with regard to AFM1 contamination levels in Egypt. Ingesting dairy products contaminated with this toxin poses a serious danger for public health, which is especially true for children, as they have the maximum consumption of milk from all age groups. Therefore, it is necessary to routinely monitor AFM1 levels in dairy products as a quality control measure to protect public health.

CONCLUSION

The results of this study suggest that there is a serious public health hazard under Egyptian regulation in Assiut and Sohag due to AFM1-contaminated milk. All samples tested were positive for AFM1 and the concentrations were high compared with those obtained in studies previously conducted in Egypt. In addition, many samples were far above the legal limits set by other national organizations. Thus, the quality and safety of milk in Egypt, especially in Upper Egypt, must be strictly monitored to protect public health.

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