2014

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Aflatoxin M₁ and ochratoxin A in human milk in Ribeirão Preto-SP, Brazil

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ARTICLE INFO

Article history:

Received 17 October 2013

Received in revised form

13 December 2013

Accepted 13 December 2013

Keywords:

Human milk

Aflatoxin M₁

Ochratoxin A

ABSTRACT

The objective of this study was to determine the extent of aflatoxin M₁ (AFM₁) and ochratoxin A (OTA) contamination in human breast milk in the city of Ribeirão Preto, São Paulo State, Brazil. During 2012, 100 samples of human milk were collected at the local Human Milk Bank. The method comprised, immunoaffinity column purification and isolation, liquid chromatography separation and fluorescence detection. The average percentage recoveries of AFM₁ and OTA spiked at 20 and 50 ng/L in control human milk were $78.1 \pm 11.7\%$ and $73.7 \pm 9.6\%$, respectively. The average relative standard deviations of AFM₁ and OTA spiked at the same levels were 11.7 and 9.6% respectively. The limits of detection was 0.3 ng/L for AFM₁ and OTA. The limit of determination was 0.8 ng/L for both mycotoxins. This method was used to analyze 100 human milk samples, of which, two samples were found to contain AFM₁ at level greater than 0.3 ng/L. OTA was detected in 66 samples (66%), wherein 32 were above the limit of detection and 34 were in the range of from 0.8 to 21 ng/L. Results of our study indicate that breast-fed Brazilian infants had only an insignificant exposure to AFM₁ and OTA.

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1. Introduction

Breast milk provides human infants with indisputable short-term health benefits and is widely acknowledged as the most complete form of nutrition for infants, conferring a range of benefits for infants' health, growth, immunity and development (Healthy People 2010, Centers for Disease Control and Prevention, Atlanta, Georgia, USA). The long-term consequences of breast feeding are more difficult to assess, but there are compelling epidemiological data which suggest that breast feeding may protect babies from developing allergies and may result in a lower incidence of obesity in teens and adults (Rautava & Walker, 2009).

Human milk may be a lifesaving therapy for infants and young children with unusual medical conditions. Donor milk has been used successfully to treat a number of medical conditions in infants (Tully, Lockhart-Borman, & Updegrave, 2004).

Mycotoxins, such as aflatoxins and ochratoxin A (OTA), are natural toxins produced as secondary metabolites by moulds.

When lactating mammals consume aflatoxin B₁ (AFB₁) contaminated feed or food, AFB₁ is metabolized to form the monohydroxy derivative, AFM₁, which may be expressed in milk.

The results of a study in which male Fischer rats were separately given AFM₁ and AFB₁ by stomach tube indicated that the AFM₁ hydroxylated derivative has a much lower carcinogenic potency than does the parent substance (Wogan & Pagliarunga, 1974). Another study also reported that AFM₁ was found to be a weak hepatic carcinogen compared to AFB₁ (Cullen, Ruebner, Hsieh, Hyde, & Hsieh, 1987). Although AFM₁ in milk is less hazardous, by far, than the parent compound, a limit of 0.5 parts per billion is applied in the United States and in many other countries, largely because milk tends to constitute a large part of the diet of infants and children.

OTA can also be found in milk. It is a kidney toxin and is carcinogenic in rats and mice and suspected as being the cause of Balkan endemic nephropathy, a disease reported to occur in some individuals in Yugoslavia, Bulgaria, and Romania. OTA has the longest half-life for its elimination of any of the mycotoxins examined. The IARC (1993) has classified ochratoxin A as a possible human carcinogen (category 2B).

Hassan et al. (2006) studied OTA as an environmental risk that caused renal injury in breast-fed Egyptian infants and concluded

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Fig. 1. LC chromatograms of (a) blank, (b) human milk samples naturally contaminated with AFM₁, retention time, 3.8 min and (c) OTA, retention time, 10.4 min.

that there was a significant correlation between a higher OTA level in infants' sera and the degree of microalbuminuria.

Biasucci et al. (2011) study the presence of OTA in cord serum and human milk, a positive relationship between serum OTA and ratio serum/milk OTA was found. The intake of OTA had no effect on the cord blood creatinine level. Muñoz et al. (2010) determined OTA and its metabolite ochratoxin alpha in milk and blood from lactating women, there was no evidence for conjugates of OTA and the data on OTA in breast milk and levels reported in blood from women in Chile are indicative of an efficient lactational transfer of the mycotoxin.

There are many reports of AFM₁ and or OTA contamination in human milk, in Iran (Sadeghi et al., 2009), Brazil (Navas, Sabino, & Rodriguez-Amaya, 2005), Turkey (Gurbay, Sabuncuoglu, et al., 2010; Gurbay, Girgin, et al., 2010), Italy (Galvano et al., 2008), Egypt (Polychronaki et al., 2006), Jordan (Omar, 2012) and Germany (Muñoz, Wollin, Kalhoff, & Degen, 2013).

The goal of our study was to examine the levels of AFM₁ and OTA in the city of Ribeirão Preto, São Paulo State, Brazil. This report updated data reported 2005 about levels of contamination of these two mycotoxins in human milk samples collected in the Human

Milk Bank “Enfermeira Anália Ribeiro Heck”, of Hospital das Clínicas – USP, in Ribeirão Preto City, São Paulo State. The Human Milk Bank was founded in 1976, provide milk from screened donors that has been used successfully treat a number of medical conditions in infants in the region of Ribeirão Preto City. The human milk is analyzed, pasteurized and storage in freezer at -20°C , until be distributed for the hospital that needs it. The Bank has about 35 donors/week and provides milk for about 40 infants/day.

2. Material and methods

2.1. Materials

A total of 100 human milk samples were collected from the Human Milk Bank of Hospital das Clínicas – USP, Ribeirão Preto City, São Paulo State, Brazil from June 2011 to Aug 2012. Volunteers gave informed written consent to participate in this study, which was approved by the Ethics Committee of Instituto Adolfo Lutz.

2.2. Chemicals and supplies

The chemicals and supplies used in the study were: AFM₁ standard (A6428, Sigma Chemical Company, St Louis, MO, USA); methanol and acetonitrile, LC grade (EM Science, Gibbstown, NJ, USA); immunoaffinity column (IAC), wideboreAflaOchra (Vicam, Watertown, MA, USA).

A stock solution of AFM₁ was prepared in acetonitrile and OTA was prepared in methanol; the concentrations of the solutions were determined according to AOAC International Official Methods 986.16, 971.22 and 970.44 (AOAC, 2010). AFM₁ and OTA working solutions were prepared by diluting appropriate portions of the stock solutions of the mycotoxins with the respective mobile phase to give the following concentrations: 2.5; 5; 10; 20; and 40 ng/L. For spiking solutions, appropriate portions of the stock solutions were diluted with methanol to give concentrations of 107 µg/L for OTA and 100 µg/L for AFM₁.

2.3. Apparatus

Equipment used in this study included spectrophotometer (Hitachi, Tokyo, Japan); vortex mixer (Fanem, São Paulo, Brazil); centrifuge (Fanem, São Paulo, Brazil); and column manifold (Supelco, Bellefonte, PA, USA).

2.4. Analytical procedure

2.4.1. Sample preparation and extraction

2.4.1.1. Human milk samples. The liquid samples were shaken manually for 5 min before being opened to ensure sample homogeneity. Test samples were centrifuged for 20 min; the top layer was aspirated, and 50 mL of the lower layer was diluted with 20 mL hot water (80°C) and saved for IAC purification and toxin isolation.

2.4.1.2. Human milk for recovery study. An appropriate amount of AFM₁ and OTA was added to 50 mL control milk samples (containing AFM₁ and OTA < 0.3 ng/L) to obtain AFM₁ and OTA levels 20 and 50 ng/L, in triplicate. Test portions were diluted in 20 mL hot water (80°C) and saved for IAC analysis.

2.4.2. Immunoaffinity column purification and isolation

Test solutions from Section 2.4.1 were passed through an IAC secured on a column manifold. The column was then washed twice with 10 mL water. AFM₁ and OTA were eluted with methanol. The eluate was evaporated to dryness and the residue was reconstituted in 0.5 mL LC mobile phase.

Table 1

Recovery of aflatoxin M₁ and ochratoxin A in human milk ($n = 3$).

Mycotoxin	Mycotoxin spiked, ng/L	Recovery, %		
		Means	SD	RSDr
AFM ₁	50	73.7	5.6	7.7
	20	82.6	15.9	19.2
	Means	78.1	11.7	13.5
OTA	50	70.6	6.9	9.8
	20	76.9	12.3	16.0
	Means	73.7	9.6	12.9

S.D. = standard deviation, RSDr = relative standard deviation.

2.4.3. Liquid chromatography

LC system included a LC pump (Shimadzu Instruments, Kyoto, Japan) with a fluorescence detector, a Rheodyne L.P. injector with a 50 µL loop (Rheodyne, Cotati, CA, USA) and a Shim-pack CLC-ODS (M), 4.6×250 mm, 5 µm column (Shimadzu, Kyoto, Japan). Mobile phase was a mixture of acetonitrile–water–acetic acid (50 + 50 + 1 v + v), isocratic mode with a flow rate of 0.8 mL/min. The fluorescence detector was programmed from 0 to 5 min an excitation wavelength of 360 nm, emission 430 nm for AFM₁, and for 5–12 min 333 nm and 443 nm for OTA. The injection volume was 50 µL.

3. Results and discussion

Fig. 1 shows LC chromatograms of blank (a) and human milk samples naturally contaminated with AFM₁ (b) and OTA (c). The control milk sample shows no peak near the AFM₁ and OTA retention time (3.8 min for AFM₁ and 10.5 min for OTA). This indicates that the method provides test extracts that are free of interferences for LC analysis.

A linear calibration curve (coefficient of correlation, $r^2 = 0.999$) for AFM₁ with linear range from 2.5 to 40 ng/L was obtained using the least-squares regression equation: $y = 21036x$. OTA with the same linear range the linearity r^2 was 0.994 and $y = 19,283x$.

Table 1 summarizes the results of the recovery study for AFM₁ and OTA added to human milk samples. The mean recovery and relative standard deviation (RSDr) at added levels of 20 and 50 ng/L were $78.1 \pm 11.7\%$ for AFM₁ and $73.7 \pm 9.6\%$ for OTA. The limit of detection (LOD) for both mycotoxins was 0.3 ng/L, and was determined by using the average value of blank samples plus 2 standard deviations.

The limit of quantification for both mycotoxins was 0.8 ng/L (approximately 3 times the LOD). The results indicate that the method is adequate for the determination of AFM₁ and OTA in human milk at low levels.

Table 2 shows the results for OTA in human milk samples. In this study 100 human milk samples were analyzed and OTA was detected in 66 samples; among these samples, 32 samples contained OTA above LOD and 34 samples contained OTA ranging from 1 to 21 ng/L, with an average level of 4 ng/L. The occurrence and

Table 2

Ochratoxin A contamination in human milk ($n = 100$).

No. of samples	Range, ng/L
34	< 0.3
32	$0.3 - < 0.8$
29	$0.8 - 5$
2	$6 - 10$
2	$11 - 15$
1	21

incidence of AFM₁ were much less than for OTA, only 2 samples were contaminated with AFM₁ at 0.3 and 0.8 ng/L. One of these 2 samples was also contaminated with OTA at 1.1 ng/L. The levels were much lower than the maximum legal limits in baby food in Brazil, 2 µg/kg for OTA and 0.5 ng/kg for AFM₁, (Ministério da Saúde, 2011).

In 2005 Navas et al. (2005) analyzed for AFM₁ and OTA in 50 samples from a Human Milk Bank in São Paulo City, and found only one was contaminated with AFM₁, at 20 ng/L and two with OTA, at 10 and 20 ng/L. Navas et al. (2005) also indicated that although the incidence and occurrence observed in her study were low, but she recommended that the study be extended to other milk banks of the city of São Paulo. Our human milk study was a follow-up to this recommendation although in a different city of São Paulo State, Ribeirão Preto-SP is locate about 400 km far from São Paulo City, it is a agricultural region.

Comparing the result of AFM₁ concentration in human milk from this work with our previous study in fluid cow milk samples, the concentrations of AFM₁ in cow milk ranged from 8 to 437 ng/L, and 30 of 47 samples were contaminated with the toxin (>1 ng/L) (Iha, Barbosa, Okada, & Trucksess, 2013). The advantages of breast feeding are many. In addition to being nutritious, human milk may contain less mycotoxins and their metabolites; however, if breast feeding is not an option, then it might be safer to feed infants with powdered infant formula. None of the 7 powdered infant formulae designated for babies aged from 0 to 6 months were found to be contaminated with AFM₁.

In Egypt, Polychronaki et al. (2006) analyzed 388 samples and found 36% contaminated with AFM₁, with a median concentration of 13.5 ng/L. Galvano et al. (2008) collected 82 human milk samples from an Italian hospital and detected AFM₁ in 4 samples, 7–140 ng/L. Occurrence and levels of AFM₁ in Brazilian human milk samples were significantly lower than those from other countries. Sadeghi et al. (2009) reported that in Tehran, Iran, 157 of 160 human milk samples were determined to be contaminated with AFM₁ at concentrations of 0.3–26.7 ng/kg. Gurbay, Sabuncuoglu, et al. (2010) studied 75 human milk samples from Ankara, Turkey, and found AFM₁ in all samples at concentration levels of 60.9–300.0 ng/L.

There were also reports of finding OTA in human milk in several countries. Galvano et al. (2008) analyzed 82 human milk samples collected from a hospital in Italy and found OTA in 61% of the samples with OTA levels ranged from 5 to 405 ng/L. Gurbay, Girgin, et al. (2010) found 100% OTA contamination with levels ranging from 620 to 13,111 ng/L in 75 human milk samples collected in Ankara, Turkey. Muñoz et al. (2013) obtained 90 milk samples from mothers in Germany and found more than 50% of the samples contained OTA > 10 ng/L.

In our study we found 66% of the 100 samples contained OTA > 0.3 ng/L with an average of 4 ng/L.

In conclusion, the levels of AFM₁ and OTA in all human milk collected from the city of Ribeirão Preto, São Paulo State, Brazil. During 2012 was very low, much lower than the current Brazil regulatory limits. Therefore, the infant health risk associated with the two mycotoxins in human milk was very low in this area. We therefore did not estimate the exposure assessment. In addition, in

Brazil, breast feeding could result in lower mycotoxin exposure than cow milk feeding.

Acknowledgements

The authors are grateful to FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) for financial support.

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