

Aflatoxin M1 levels in lactating mothers in two Nigerian cities

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Abstract

Background: In Nigeria, there is paucity of information on exposure to aflatoxin by lactating women and children. Aflatoxin (AFs) are potent carcinogens that are produced as secondary metabolites of strains of *Aspergillus parasiticus* and *Aspergillus flavus* that grow on important food crops such as groundnuts, maize and other oilseeds. Present study reports on the aflatoxin exposure of lactating women in two cities in Southwest Nigeria.

Methods and Findings: One hundred and twenty samples of human breast milk were collected into universal plastic bottles by self-expression from women attending immunization clinic. The samples were collected into iced cooler and stored at 4^o C within 24 hours before extraction and were screened for Aflatoxin M₁(AFM₁) using immunoaffinity column and High performance Liquid Chromatography with fluorescence detection. Aflatoxin M₁ was detected in 17 samples of human breast milk (14.1%). Range for AFM₁ was between 2-187ng/ml. T-test using SSPP version 14.0 at 95% confidence interval of difference gave a value of 0.332 AFM₁. Main diets of Nigerians include maize, cassava, yam, melon, groundnuts, wheat, and bread.

Conclusions: The findings therefore support the possibility of dietary recommendations to women, during pregnancy and lactation, aimed to tentatively reduce the aflatoxins contamination of human milk. AFM₁ contamination of breast milk was not frequent (14.1%), however some positive samples can cause toxicological effects. Finding suggests need for urgent attention to the possible public health implications.

Key words: Public health, Aflatoxin M₁, Human breast milk, Immunoaffinity column, Nigeria, Women.



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Introduction

The contamination of food with aflatoxin is a global one; however, it is more serious in tropical countries of the world where relative humidity is high and temperatures conducive

for the growth and production of aflatoxin by moulds. Aflatoxin (AFs) are potent carcinogens that are produced as secondary metabolites of strains of *Aspergillus parasiticus* and *Aspergillus flavus* that grow on important food crops such as groundnuts, maize and other oilseeds [1-3].

Breast milk is considered to be the best food for the infant [4-5] and the importance of breast feeding for normal growth and development has become increasingly recognized. However, in addition to its nutritionally and immunologically beneficial components, human milk may contain trace amounts of a wide range of contaminants. The occurrence of mycotoxins in human, animal and milk formula is probably one of the most serious public health concerns since milk is an important food for adults and unique nutrient for infants [6-7]. The latter are extremely vulnerable because their diet is much less varied and depends on mothers' dietary habits. It has been reported that in human milk variable amount of mycotoxins ingested by the mother can be accumulated in an intact metabolized form [8-10]. Approximately 95% of AFB₁ metabolites excreted in milk are in the form of AFM₁, though AFM₂, AFG₁ and AFB₁ have been reported. Although AFM₁ is considered a detoxification product, there is evidence of a putative AFM₁ carcinogen role as reported by Peers, D'Mello and Macdonald, Codex Committee on Food Additives and Contaminants [11-13].

In spite of the recognized need for frequent surveys of food items for mycotoxins, few studies are available on the occurrence of aflatoxins in biological fluids. In addition to being potent carcinogens, aflatoxins may contribute to early child growth retardation and particularly strong associations between aflatoxin and child growth [14-19] have been reported around the weaning stage in Beninese infants [20]. A study conducted in Gambian children reported that secretory IgA in saliva may be reduced by dietary levels of AF [17]. The immune status of Ghanaian adults has been reported to be affected by aflatoxin exposure [21].

Milk is the main nutrient for the first six months of life as has been entrenched in the Nigerian Health Programme. There is the possibility that these children who are the future of the nation are exposed to doses of aflatoxins. Infants are more susceptible to the adverse effects of aflatoxins. It has been stated that African countries should be devoted to monitoring milk and milk products other than those from cows as previously shown [17]. Presently there is lacuna of data on aflatoxin M₁ levels in human breast milk in Nigeria. Therefore it is important to monitor aflatoxins levels in human breast milk in Nigeria since it is a risk of serious health hazard to mother, foetus and infants.

Materials and Methods

Study population and sample collection

Breast milk samples were collected from 120 Nigerian women that agreed to participate in the study (participation rate 98%) during the month of June, 2008. These women were

from two hospitals in two cities in South Western Nigeria (Ibadan and Abeokuta). This is a tropical rain forest zone with high humidity and temperatures which promote mould growth and aflatoxin production. The people are famous for consumption of foods such as maize, yam chips, groundnuts, groundnut oil, melon, cassava-based foods, beans, rice, and wheat bread. Breast milk samples were collected (5ml) for aflatoxin determination into universal bottles and stored at 4°C prior to extraction within 24hours.

Ethical considerations

The study was approved by the Ethics committee of the Ministry of Health, Oyo state of Nigeria. All subjects were made aware of the content of the study and if they agreed to participate breast milks were collected from willing mothers manually.

Collection of dietary, socioeconomic, demographic and clinical data

The intake of dietary sources of aflatoxins was assessed by asking women about their diet and eating habits focusing mainly on foods more likely to be sources of aflatoxins [3,22]. The focused foods were cereals, maize, yam chips, groundnut oil, melon and nuts used for soups, beverages (bournvita, ovaltine, and chocovita) containing cocoa, dried fish, dried meat and milk.

Validation and Reproducibility of measurement

Validation of measurement was done by using Hipp Baby formula bought from supermarket in Munich, Germany. Seventy grams of powdered milk were weighed into a 1-litre beaker and 450ml of Millipore water added. The mixture was stirred vigorously with a stirrer for 5 minutes. The temperature of the milk was raised to 50°C to ensure proper dispersion of milk fat. There after milk temperatures were lowered to room temperature. Four centrifuge tubes containing milk were put in the cold room (4°C) for 15min. The solidified fat were scooped off and the milk samples filtered using ashless filter circles (MN 640W, diameter 150mn) (Macherey-Nagel, Germany). Seventy milliliters of defatted Hipp milk were measured into 100ml of volumetric flask. Three flasks containing Hipp defatted milk was spiked with 25ng/ml AFM₁ and two samples served as controls. AFM₁ was extracted as described below. The average recovery rates were calculated.

Breast milk aflatoxin extraction

Breast milk was collected into universal plastic bottles by self-expression. The samples were collected into iced cooler and stored at 4°C within 24hours before extraction. Extraction was done following the method of Lamplugh [23].

Briefly 1-ml of hexane was added to each sample and centrifuged (3000 x g, 5min) and the hexane layer removed. This was repeated twice for each sample. Thereafter, 1-ml of chloroform was added to the defatted milk and centrifuged (4,000 x g, 10min). The chloroform layer was collected into plastic bottles. This was repeated four times and chloroform layer pooled together. The extracts were kept in a dark cupboard overnight to dry. The dried samples were taken to Germany for High Performance Liquid Chromatography (HPLC) analysis.

Analysis. Chemicals and solvents used were HPLC grade or equivalent. All water used was distilled and for HPLC, passed through a Milli-Q purification system (Millipore, UK) and acetonitrile used was HPLC grade (Merck) .

Immunoaffinity columns (RIDA aflatoxin column, R-BIO-PHARM, Darmstadt) were used for clean-up of the sample extracts. The immunoaffinity column (IAC) was brought to room temperature, plugged onto Luer-attachment of a vacuum pressure facility. A solvent reservoir column was attached to the top of the IAC and 10 mls of Millipore water were added to dried extract and vortexed before transferring into the reservoir. The sample was allowed to flow through the IAC at a rate of 1ml min⁻¹. Slight vacuum pressure was applied. The column was washed twice with 10 mls each of Millipore water at a flow rate of 1ml min⁻¹. The reservoir was removed and the IAC was dried by applying slight pressure. Aflatoxin M₁ was collected into glass bottles previously treated with 2N H₂SO₄. The solvent used for eluting was 1.25-ml methanol/ acetonitrile (20:30, v/v). The eluate was dried in a water bath at 40°C and 80kPa. The dried extract was re-dissolved with 1.25-ml of acetonitrile: water (25:75, v/v). The solution used for determination of aflatoxin M₁.

HPLC determination of aflatoxins

The HPLC system consisted of a LDC, Milton Roy, Consta Metric 1 pump and a Lichrosorb RP-18 (Merck Hibar) column (particle size of 5 µm, length, 125 mm, inside diameter, 4mm). The pump pressure was 60MPa. The Injector was automatic type (Rheotype, Gilson Abimed Model 231). The detector had a fluorescence spectrometer (Shimadzu RF 535, gamma excitation, 365 nm, gamma emission, 44nm). The flow rate was 1ml min⁻¹ and the injection volume was 50µl. The mobile phase was water/acetonitrile (75:25).

Standard solutions

Aflatoxins M₁ was obtained from Sigma-Aldrich (St. Louis, MO, USA). The commercial stock solution of AFM₁ was 1000ng/ml. The spike solution was made by diluting the

stock solution 1:40 approximately 25ng/ml using HPLC grade acetonitrile/water. Out of the diluted stock solution, 140 µl were added to 70ml of defatted Hip baby milk.

Calibration curve was prepared by diluting 2µg/l of AFM₁ in a 1:500 dilution. The concentrations of standards were prepared to give the following 1.5µg/l, 1.0µg/l and 0.25µg/l. The stock solutions were stored at 4°C when not in use. For recovery experiments, uncontaminated baby formula milk was spiked with AFM₁ at 2.5ng/ml.

Statistical analysis

Comparison between means was evaluated by student t-test and analysis of variance. A value of p< 0.05 was considered significant.

Results

Average recoveries for reproducibility measurements ranged from 89 to 103%. The reproducibility of measurement is shown in **Table 1**. The calibration curves showed good linearity.

AFM₁ was found in 17 samples (14.1%) as shown in **Table 2**. The contamination level of AFM₁ ranged from 2-187 ng/ml. HPLC scans of standard aflatoxin M₁ are shown together with aflatoxins in breast milk samples of women (**figures 1, 2 and 3**). T-test using SSPP version 14.0 at 95% confidence interval of difference gave 0.332 AFM₁. Data obtained from subjects indicated all consumed basic diets such as maize, yam, cassava, groundnut, melon and bread. Consumption of basic food staples might have contributed to levels of aflatoxins detected in subjects, however individual ability in relation to state of health to effectively detoxify these toxins may have played significant role in the number of positive women.

Table 1. Validation and reproducibility of measurements using Hipp baby formula.

Sample(µg/g)	Ret. Time min	Area	Mv min	Height	Mv
Amount µg	%	Recovery.			
Std AFM1 2.0	5.48	221323	878118	2.0001	100
Std AFM1 1.0	5.49	110008	43988	1.0003	100
Std AFM1 0.5	5.47	54896	219905	0.4985	99.7
Std AFM1 0.25	5.48	27419	111156	0.2504	100.2
Spiked milk 2.5	5.57	55298	246355	2.23	90
Spiked milk 2.5	5.57	56604	256889	2.33	93
Spiked milk 2.5	5.57	59533	271803	2.58	103

Table 2. Aflatoxin M₁ contamination in human breast milk samples.

Frequency distribution of samples Contamination						
<10	10-50	50-100	100-500	500-1000	>1000	
Range Average +ve						
AFM ₁ (ng/ml)	5	8	1	3	-	2-187 44 17.

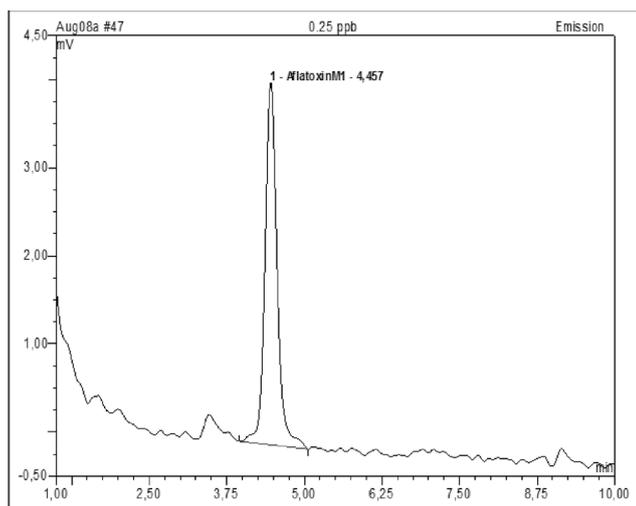


Fig. 1. HPLC scan of standard aflatoxin M₁ HPLC chromatogram. Mobile phase water:acetonitrile, 25:75 v/v; flow rate: 1 ml min⁻¹; column: Lichrosorb RP-18 (Merck Hibar) column, fluorescence spectrophotometer (Shimadzu RF 535, gamma excitation, 365 nm, gamma emission, 444 nm)

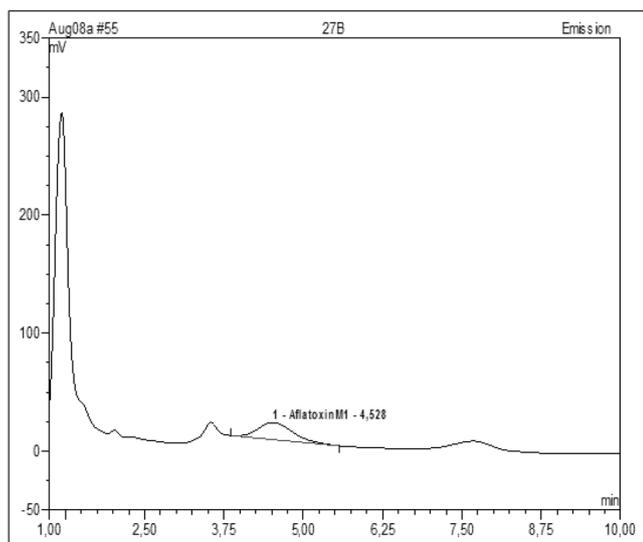


Fig 2. HPLC chromatogram of quantified aflatoxin M₁ in sample 27B equivalent to 187ng/ml. Mobile phase water:acetonitrile, 25:75 v/v; flow rate: 1 ml min⁻¹; column: Lichrosorb RP-18 (Merck Hibar) column, fluorescence spectrophotometer (Shimadzu RF 535, gamma excitation, 365 nm, gamma emission, 444 nm)

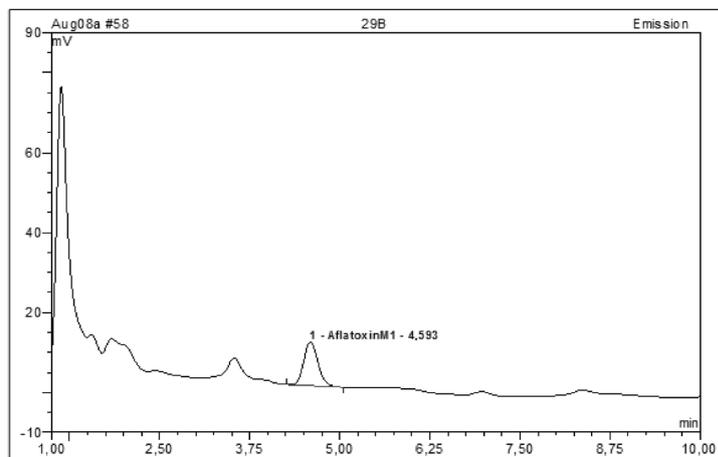


Fig 3. HPLC chromatogram of quantified aflatoxin M₁ in sample 29B equivalent to 136 ng/ml. Mobile phase water: acetonitrile, 25:75 v/v; flow rate: 1 ml min⁻¹; column: Lichrosorb RP-18 (Merck Hibar) column, fluorescence spectrophotometer (Shimadzu RF 535, gamma excitation, 365 nm, gamma emission, 444 nm)

Discussion

Children are highly susceptible population group when exposed to environmental toxicants for a variety of reasons, including lower detoxification capacity, rapid growth, and higher intakes of air, food and water per kg body weight [24]. Therefore, early childhood exposure to carcinogenic aflatoxins may be critical determinants of immediate and later health effects. AFM₁ in breast milk is a marker of dietary exposure of mother to AFB₁ and consequently aflatoxin exposure to breast-feeding infants as previously shown [8]. In this study, 14.2% of mothers were positive for AFM₁ in breast milk although some values were on the high side. Three mothers had AFM₁ values above the stipulated EU level of 50 ng/ml in baby food. In this study, the mean aflatoxin in milk was 44 ng/ml for AFM₁.

The results in this study show that some lactating women in Ibadan and Abeokuta cities in Nigeria are exposed to low to moderate levels of aflatoxin M₁. Daily intake of aflatoxin M₁ by the infants will vary depending on the diet of the mother who possesses it or via the breast milk.

Though aflatoxin or its metabolites may be stored in fat reserves the levels and contribution, if any, to breast milk levels at given stages of lactation are not known. Secondly, the fat content and total milk volume vary within a feed and

between feeds, and this may impact on the levels of aflatoxins observed. Thirdly, the average volume an infant will consume a day will vary. Despite these variations one can make some estimate of aflatoxin M₁ intake in positive mothers with detectable AFM₁. Assuming an average daily milk volume of 500ml, the average intake of AFM₁ by the infant can be estimated to be 22.0 µg/day and the range will be 1.0 -93.5 µg/day. This value is slightly moderate compared to low values obtained by other scientists somewhere around the globe as previously cited. This difference might be due to the fact that samples of breast milk were collected from women in the tropics where aflatoxin contamination of food is highest in the whole world. Some of these children may also be introduced to wean foods that are likely to be contaminated [24]. Thus the aflatoxin burden in children is increased.

In Nigeria, the sources of aflatoxin exposure are well-defined as previously shown [3]. A major contributing factor is poor post harvest management of the harvested crops. In small farmer communities, the crops harvested are usually left to dry over plastic or synthetic sheets, which may promote *Aspergillus* growth and toxin production as previously shown [15]. Most of the crops are consumed locally by either the household or close communities. Worldwide, two of the major sources of aflatoxin exposure are groundnuts and corn. These two crops are mainly consumed in Nigeria in several forms as boiled corn, roasted corn with groundnut and groundnut oil.

In developing countries, growth retardation is often associated with the quantity and/or poor quality of foods, in addition to multiple infectious hazards as previously shown [4]. It is not unlikely that aflatoxins present in human breast milk may play contributory role in growth of children in Nigeria. High levels of aflatoxin-albumin adducts have been associated with retarded growth in Beninese infants as previously shown [20, 26]. Egyptian infants were reported to have stunted growth with modest maternal breast milk levels of AFM₁ as previously shown [17]. Aflatoxins are genotoxic, carcinogenic substances and a threshold value below which the risk for human health is equal to zero does not exist. The joint FAO/WHO Expert Committee on Food Additives (JEFCA) does not establish a tolerable daily intake (TDI), but strongly recommended that the level of aflatoxin should be as low as possible. Therefore, the toxicological significance of the presence of aflatoxin in maternal milk should not be overlooked. Consequently a reduction of the mothers' exposure to aflatoxins and thus a reduction of children's exposure through breast milk are desirable. Where the source of contamination is clearly defined such as in Nigeria, post harvest treatment such as drying and storage of risk foods is an effective method, use of local spices [25-28] effective packaging and fermentation of grains [29-30].

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