ORIGINAL RESEARCH

Detection of Aflatoxin M1 in human milk and its effect on growth parameters in exclusively breast fed infants of rural Mathura, India

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ABSTRACT

Introduction: Human milk alone is a complete nutrition for the newborn till 6 months of life. Due to consumption of grains stored in humid and hot rooms and drinking contaminated animal milk, pregnant and lactating mothers can transmit Aflatoxins to the fetus in the womb or to the infant through breast milk. Few studies have reported plausible growth faltering among infants due to exposure to aflatoxins. **Methodology:** A Longitudinal study with sample size of 45 nursing mothers was conducted. AFM1 quantification was done by ELISA. We followed the birth cohort of enrolled infants till 12 weeks to measure growth by length and weight of the infant. **Results:** Through our study we found that all lactating mothers were secreting high levels of AFM1 from their breast milk. Most infants showed below average gain in weight and length during the study period of 12 weeks since enrollment. The growth in length was not different among boys and girls. The gain in weight was not independent of the different categories of growth like average, below average, stationary and declining among infants across the 3 visits of the study period for girls. There existed some relation between poor weight gains across the 3 visits among girls; not boys. **Conclusion:** The infants started growing well but could not catch-up.Despite high exposures, and poor catch-up, growth parameters were not associated with the levels of Aflatoxins.

INTRODUCTION

When pregnant and lactating mothers ingest Aflatoxin B1contaminated food, it is metabolized to its monohydroxy derivative[1], Aflatoxin M1, which can be secreted throughbreast milk. High doses of aflatoxins can cause acute poisoning that may be harmful, especially to the liver. Chronically exposed persons may develop teratogenic, mutagenic and carcinogenic manifestations along with poor immunity[2].

Humanmilkis widely known as a complete form of nutrition for infants, providing a range of benefits for infants' health, growth, immunity and development. As our country aims to reduce under-nutrition significantly by the year 2030, the practice of human milk feeding to infants is encouraged to reach our nutrition goals. Stunting[3] in children indicates chronic malnutrition, but there is evidence that aflatoxin exposureearly[4] in infant's life can affect growth. Though exclusive breast feeding is the most sought strategy for infant's normal growth in the first 6 months of age, it might become a hindrance in baby's growth and wellbeing, if the mother[5] continuously consumes contaminated food. This has been documented in some studies that found an association between toxins in mother's milk and infant growth faltering[6]. Nursing mothers who feed on stale and damp grains, milk, eggs, peanuts and other foods found to contain moulds of aspergillus species may become a source of mycotoxins to their babies[6].

OBJECTIVES

1. To measure the concentration of aflatoxin M1 in human milk samples
2. To measure growth faltering among infants of enrolled mothers
3. To determine the association between aflatoxin M1 exposure and growth parameters among infants of enrolled mothers

MATERIAL AND METHODS

1. **Study Design:** A longitudinal study with conducted by defining the exposure variable as Aflatoxin M1 levelsin breast milk fed to infants. Outcome variables were weight gain and length gain, in exposed compared to not exposed infants of the enrolled lactating mothers.
2. **Study population:** We selected the residents of 4 villages in the rural field practice area of medical college located in Mathura, U.P, India. These were village, Randhera, Ladpur, Khursi and Behrawali of district Chatta, Mathura.
3. **Sampling method:** All lactating mothers with child birth 0-3 months prior to data collection were selected from the rural field practice area of medical college. We had chosen 4 villages purposively, and enrolled all 0-3 month old infants as the birth cohort.
4. **Sample size:**45 lactating mothers with their infants were enrolled. No multiple birth infants were selected, as twins[7] may not grow as rapidly as singletons.
5. **Inclusion criteria:** Lactating mothers who were exclusively breast feeding the infants less than 12 weeks old were included.
6. **Exclusion criteria:**Infants more than 12 weeks old, infants with known congenital anomalies, co-morbidities and sick infants were excluded, mothers unwilling to participate.
7. **Time period:** 1stDecember to 31st March 2022
8. **Study Tool:** After taking informed consent, the lactating mothers were interviewedand a format was filled by the MSW of rural health training center of KD Medical College, Hospital and Research Centre.
9. **Method of data collection:**
10. **Enrolment of mothers and their infants in the study-** Accredited Social Health Activists (ASHAs) of the villages were instructed to provide a list of women who deliveredhealthy babies not more than 3 months of age to begin with. Their birth weight was noted from MCH cards issued by the health center. The Medical Social Worker (MSW) and a Public Health Nurse,of our college were guided to collect breast milk samples of about 20 ml from the enrolled mother's milk in sterile containers, packed in cold boxes and reached to the Microbiology lab of our college. The samples were stored in a refrigerator of the laboratory, and test was administered for 10-15 samples everyday. Breast milk was collected by either of the breasts, after expressing manually for 3-5 minutes.
11. The rationale behind choosing 0-6 months old exclusively breast fed infants was to decipher the association between AFM1 in mother's milk as the only factor in this birth cohort and growth parameters as no other food was being given to the infant. Infant's length was measured in cm and weight in kg at every visit.An infantometer and baby weighing scale were used for this purpose. The PHN carried out the data collection (weight, length measurement and breast milk sample collection). Our MSW filled a guided format with all the information required in the study. He was also trained to code samples of human milk before storage and transportation along with the maintenance of cold chain.
12. b. Follow-up: The infant was followed up after 6 weeks and 12 weeks ofthe first visit. The researchers noted the age of the infants in weeksfor accurately planningthe follow-ups. Infant's weight and length were measured at each visit.
13. c. Procedure of testing: A Competitive ELISA AFM1 testing kit was purchased from Elabscience,through a vendor, Progenbiolab, Kirti Nagar, New Delhi-15. Human milk samples were collected and sent to the Microbiology laboratory of KD Medical College, Mathura, India for ELISA test. A trained laboratory technician and a microbiologist carried out the procedure. Around 10-15 samples were tested in one lot.
14. **Data analysis:**Data was entered in Epi Info 7. Descriptive statistics were represented in the form of frequency tables to describe the growth parameters in the birth cohort. Birth weight cut-off was taken from the one used inNational Family Health Survey-4 (NFHS-4) data[8]. LBW infants were noted below 2500 grams corresponding to below -2 SD of WHO z-scores according to Multi Centre Growth Reference Study[9] (MGRS). Infant's weight and length for age were classified using z-score tables according to MGRS standards.Those below -3 SD weight or length were termed as severely underweight or severely stuntedrespectively and those between -3 to -2 SD were considered moderately underweight or stunted. -2 to +2 SD were considered to have normal growth parameters.
15. Further, infant's weight gain was classified according to MGRS growth velocity tables for 1 month and 2 monthly gains. As a standard for catch-up growth, 50th percentile[10] was considered to be average. Any measure below this level was classified as below average growth and if there was no increment in weight for age, we called it stationary growth. A measured reduction in weight was noted as declining weight for age. Growth increments in weight were assessed at 4

weeks interval (2nd visit) and 8 weeks interval (3rd visit) using 1 month and 2 month growth increments respectively; from percentile tables[10].

16. As for length gain, we measured gain from the first to third visit because there was 3 months' growth velocity table available for length for age. A cut off ofbelow 25th percentile decided poor growth in length according to Indian standards[10,11]. Above 25th percentile was classified as averagegrowth in length,some growth but below 25th percentile was considered below average.
17. Chi-square test of Independence was used to understand whether the catch-up weight gain had some relationship across the visits to the infants. Chi-square was also used to test the difference in length gain among boys and girls in the 3 months of study period. Wherever any observed frequency was 0, we added 5 to each component of the Chi-square table for statistical analysis. P-value of less than 0.05 was considered as a significant difference across distributions assuming they followed Gaussian characteristics at significance level of 95%.
18. To calculate exposure, breast milk concentration of Aflatoxin M1 in ng/l was multiplied with frequency of breast feeding in a day and average intake of BM which was assumed to be at least 100ml per feed, in liters (0.1 L). This was divided by the body weight of infant noted at the first visit. This measure of exposure is proxy for daily intake per kg per day. We calculated exposure levels only at the first visit. There was no intervention or health education imparted to the lactating mothers to change their dietary pattern, and we assumed that this exposure remains constant because of persistent exposure from the mother's diet.

19. **Exposure at first visit = (AFM1 concentration in ng/L X BFF X 0.1 L)/Body weight at first visit in kgs**
20. A linear regression model was applied to find the association of length and weight gain at 4 weeks (2st visit) and 8 weeks (3rd visit), with predictors like Aflatoxin M1 exposure at 1st visit, birth weight, length at first visit, birth order and Age in months at each visit.
21. **Budget:** The researchers self-funded the cost of collection, transportation as well as analysis of samples collected from lactating mothers in the rural settings and transported to the microbiology laboratory of KD Medical College, Mathura.

RESULTS

BIRTH CHARACTERISTICS OF THE COHORT

Out of 45 infants born in October to December 2021, 48.9% were girls and 51.1% boys. Majority of the infants in this study were in the age group of 2-3 months (42.2%) at first visit, followed by 1-2 months of age (33.3%) and the least were in the age group of less than 1 month (24.4%). Birth order 1 (35.6%) was most common in this cohort, followed by 3 or more (33.3%). 8.9 % infants were born with very low birth weight, 11.1% were low birth weight and 80 % were of normal birth weight.

EXPOSURE LEVELS

Aflatoxin M1 was detected in all milk samples, rendering all infants exposed. Mean Aflatoxin M1 concentration in human milk at the first visit was 1794.67 ng/l.These infants were exclusively breast fed. Estimated daily intake (EDI) of Aflatoxin M1 exposure from mother's milk calculated from the first visit among infants was found to be 390.75 ng/kg bodyweight. Range of exposure was from 48 ng/kg/day to 943.1 ng/kg/day.

GROWTH PARAMETERS OF THE COHORT

According to Table 1, the proportion of normal weighted (-2 to +2 SD) girl infants was only 13.6%, catch up growth at 4 weeks brought the proportion of normal weight to 40.9%, but there was weight faltering at 12 weeks, the proportion became 13.6% again. 59.1% infants showed a trend of severely underweight (≤-3SD) category at 12 weeks' time, beginning with 45.5 % in the first visit. Likewise even boys showed growth faltering at twelve weeks, with severely underweight were 56.5%, and moderately underweight were 26.1%. More infants were deteriorating in the weight for age parameter from moderate to severe in 12 weeks' time. However chi-square test applied for Independence suggested that there was no relationship between weight for age frequencies under different categories during the visits across the intervals of measurement at first visit, 4 weeks and 12 weeks thereafter. The measurements are independent of each other.

Table 1: Distribution showing weight and length for age according to WHO Z-score tables

Gender	1st visit (%)	4 weeks of 1st visit (%)	8 weeks of 2nd visit (%)	Chi-square statistic (p-value)
Girls weight for age				
Normal	03 (13.6)	09 (40.9)	03 (13.6)	7.46 (0.113)
-3 to -2 SD	09 (40.9)	06 (27.3)	06 (27.3)	
≤-3 SD	10 (45.5)	07 (31.8)	13 (59.1)	
Total	22 (100)	22 (100)	22 (100)	
Boys weight for age				
Normal	05 (21.7)	08 (34.8)	04 (17.4)	

-3 to -2 SD	09 (39.1)	03 (13.0)	06 (26.1)	5.29 (0.26)
≤-3 SD	09 (39.1)	12 (52.2)	13 (56.5)	
Total	23 (100)	23 (100)	23 (100)	
Girls length for age				
Normal	12 (54.5)	11 (0.5)	08 (36.4)	1.59 (0.81)
-3 to -2 SD	03 (13.6)	03 (13.6)	04 (18.2)	
≤-3 SD	07 (31.8)	08 (36.4)	10 (45.5)	
Total	22 (100)	22 (100)	22 (100)	
Boys length for age				
Normal	12 (52.2)	13 (56.5)	09 (39.1)	1.68 (0.79)
-3 to -2 SD	03 (13.0)	02 (08.7)	03 (13.0)	
≤-3 SD	08 (34.8)	08 (34.8)	11 (47.8)	
Total	23 (100)	23 (100)	23 (100)	

As shown in Table 1, there exists no association between length for age parameters for both girls and boys under each category (normal, stunted, and severely stunted) across different visits. The chi-square value suggests that the entities are independent of each other. Even though born normal (54.5%), the length for age proportion reduced to Severe Stunting (≤3 SD); 45.5% at the end of 12 weeks. Boys showed similar trends, proportion of severe stunting was 34.8%, at the first visit, remained the same in the second visit but faltered in the third visit to 47.8% severe stunting at 12 weeks of follow-up.

Table 2: Distribution showing weight and length gain among infants

	First follow-up at 4 weeks 1 month interval	**Second follow-up at 8 weeks after 2 months interval**	**Chi-square statistic (p-value)**
Girls weight gain			
Average	13 (59.1)	01 (04.5)	8.92 (0.03)
Below average	09 (40.9)	17 (77.3)	
Stationary	00 (0)	00 (0)	
Declining	00 (0)	04 (18.2)	
Total	22 (100)	22 (100)	
Boys weight gain			
Average	09 (39.1)	00 (0)	6.24 (0.1)
Below average	11 (47.8)	20 (86.9)	
Stationary	01 (04.3)	01 (04.3)	
Declining	02 (08.7)	02 (08.7)	
Total	23 (100)	23 (100)	
Girls and Boys length gain at 12 weeks interval (first and third follow-up interval)			
Girls Average	07 (31.8%)		0.17 (0.67)
Girls Below Average	15 (68.2%)		
Boys Average	06 (26.1%)		
Boys Below Average	17 (73.9%)		

Table 2 depicts that there exists a significant relationship among girls with regards to weight gain at 4 weeks after the first visit and 8 weeks after the second visit, within the 12 weeks' follow up period. The chi-square test statistic suggests a lack of independence between frequencies across different categories describing growth viz. Average, below average, stationary and declining. Among girls, none were stationary or declining in weight for age attribute in the second visit, while 18.2 % girls showed decline in weight for age (4 girls) at 3rd visit. Majority of the girls were below average yet gaining weight in the 3rd visit. Only 1 girl showed average gain in weight at 12 weeks.

Among boys, in the first interval, majority gain in weight for age was below average (47.8%) while none in the second interval were average weight gainers. 4.3 % showed stationary weight across the two intervals and 8.7% showed declining weight in both intervals of measurement. Like in girls, most boys showed below average weight gain in the 3rd visit.

According to Table 2, the difference in length gain for age across all categories (average, below average) between both boys and girls during the 12 weeks study period visits was not significant. We evaluated a 3

months' average 25thpercentile increment in length for both boys and girls due to non-availability of data on birth length. Below average growth was indicated in the majority (68.2%) of girls. Likewise, among boys the length increment in 3 months was below average (73.9%).

Table 3: Linear regression model for increment in growth of infants due to Aflatoxin M1 exposure and other predictors

Predictors of weight gain	Weight gain at 4 weeks (1)		Weight gain at 12 weeks (2)	
	F-Test	p-value	F-test	p-value
AFM1 EDI at first visit	1.27	0.27	0.46	0.5
Age in months at 4 weeks (1)/ 12 weeks (2)	0.08	0.78	0.13	0.72
Birth Weight	1.89	0.18	0.67	0.42
Birth order	1.35	0.25	0.002	0.96
Predictors of length gain	**Length gain at 4 weeks (3)**		**Length gain at 12 weeks (4)**	
	F-test	p-value	F-test	p-value
AFM1 EDI at first visit	0.04	0.85	0.003	0.95
Age in months at 4 weeks (3)/ 12 weeks (4)	3.26	0.07	0.09	0.76
Length at first visit	5.76	0.02	0.54	0.47
Birth order	2.36	0.13	0.07	0.79

We also found that stationary trend in length gain increased from 18.2% in the second visit to 40.9% in the third.

ASSOCIATION BETWEEN AFLATOXIN M1 EXPOSURE AND GROWTH PARAMETERS AMONG INFANTS

The third study objective was to find the exposure of AFM1 through mother's milk (EDIat the first visit) dependent weight and length increment at 4 weeks and 12 weeks thereafter.We included 3 other predictors for our linear regression modelwhich could affect the association of growth along with the primary predictor (EDI). Birth weight,birth order and the age of the infant at 2nd visit and 3rd visitwere chosen for predicting weight gain. Length at first visit, birth order and age of the infant at 2nd and 3rd visits corresponding to the interval of visits, were the predictors chosen for the gain in length. As shown in Table 3, the p-value associated with F-statistic was found to be >0.05. Hence there existed no relationship between predictors themselves and with the outcome variables. However, there was a significant association between infant's length at first visit and consequent gain at 4 weeks' interval.

DISCUSSION

The results from our study revealed that Aflatoxin M1 exposure to infants through breast feeding is 100% or in other words, all infants were exposed to AFM1 in the birth cohort enrolled in this study. Dimensions like dwelling, Socio Economic Status, eating habits of mothers, were similar among the study population. Our findings were similar to a study by Salas12 et al, in Mexico where they also found AFM1 in all (100%) of human milk samples. The range of AFM1 concentrations was from 5 to 66.23 ng/L, much less in comparison to our research; 895.1 ng/l. Another study by Abdulrazzaq13 et al reported AFM1 in human milk within the range 210-4060 ng/L, located in United Arab Emirates, similar to our research.

Based on a study conducted by Mehta[14] et al, AFM1 was detected in 41% breast milk samples. They also mentioned that there are currently no threshold limits for aflatoxins in breast milk samples for Indian population. The reason being, the breast milk toxin levels has nothing to do with commercial purpose, so its levels are not regulated. Range of AFM1 concentrations in the study was found to be 3.9 to 1200 ng/l which was similar to our study.

Moreover, Mehta[14] et al reported 18% stunting and 16% underweight for age (below -2 SD) among infants 2 to 4 months old. In contrast, our study reported much higher growth faltering in different follow-ups of infants below 6 months of age. Growth faltering was prominent but the statistics applied did not predict a relationship between growth parameters. The former study measured not only human milk samples, they also investigated other animal milk samples and foods frequently consumed by infants and their mothers post weaning. They calculated the average estimated daily intake of AFM1 by infants to be 3.5ng/kg/day through breast milk. In our study the exposure at first visit was much higher; 390.75 ng/kg/day.

Ishikawa[1] et al, found very low levels of AFM1 in breast milk samples (5.3 %) with concentration less than 0.003 ng/g. Whereas our study revealed minimum exposure of 48 ng/kg, and mean level of concentration in breast milk was 1794.67 ng/L.

In a study by Eshete[2] et al, conducted in Southern Ethiopia, among 360 breast milk samples tested, 64.4% had detectable AFM1 and 5.3% exceeded the 0.025 parts per billion (25 ng/L) limit set by the European Union for infant milk. In comparison, our research showed greater concentrations of AFM1 in human milk samples.

According to Mitchell[15] NJ et al the chronic aflatoxin exposure in their cohort (children up to 36 months of age in Nepal) was not significantly associated with anthropometric z-scores, growth trajectories, age, or feeding status, based on the available time points to assess aflatoxin exposure. Likewise, in our study also there was no significant association between predictors and growth outcomes across the three visits to the infants.

NFHS[8]-4 indicated the prevalence of LBW in India was 16.4%, underweight for age children were 35.7% and 38.4% stunted.According to NFHS[16]-5 data, 35.5% under-5 children were stunted, (rural 37.3%) and 32.1% were underweight. In contrast, the prevalence of LBW was high; 20% in our birth cohort. Our research reported alarmingly high prevalence of underweight and stunting across the study period of 12 weeks among infants below 6 months of age compared to the national statistics for under-5 children.

CONCLUSION

High concentration of AFM1 in human milk samples was found. Despite such levels our findings demonstrate a non-significant association of Aflatoxin M1 exposure and growth faltering. Though catch-up growth was good in the first follow-up, the infants did not continue growing as good as in the second follow-up. There exists some relationship among categories of weight gain across visits. But we cannot link growth faltering seen in the second intervalto aflatoxin M1 exposure.

LIMITATIONS

Detailed information about eating habits of the mother, even during pregnancy and its effect on the infant was a missing link. We need to attribute growth differences to factors like constitutionally small infant, air pollution, mother's tobacco exposure, some harmful chemicals and other mycotoxins secreted through breast milk.

We need to estimate the levels of AFM1in the infant's blood and urine to get more accurate results to quantify exposure. Breast milk samples were taken only once, therefore persistent exposure was not measured in subsequent visits.

RECOMMENDATIONS

Lactating and pregnant mothers must consume fresh food.

Grains should be stored in damp proof containers.

Lactoferrin in human milk coats intestines and prevents aflatoxin absorption; therefore, breast milk is the recommended form of nutrition to infants, even though there exists a risk of poor growth of infants.

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