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Studies on Aflatoxin, Prenatal Exposure and Its Toxicosis in Adamawa Sate, North East of Nigeria

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Abstract

Of the known mycotoxins, the most important in relation to direct hazard to human health are the aflatoxins produced by a large number of *Aspergillus* spp. To determine the level of exposure of aflatoxin from mother to child and its mode of transfer. 70 pregnant women in the labor ward of The Federal Medical Centre Yola were investigated for their aflatoxin content by using the velasco fluorotoxinmeter which comprised of 89 samples of amniotic fluid, 213 of serum from maternal blood and 211 serums from neonatal cord blood; 57 of those were controls. The aflatoxin values of G₁, G₂, and M₂ above 20 ppb were obtained in 66 samples of the amniotic fluid (74.1%); 133 from venous maternal blood (62.4%) and 142 from neonatal cord blood (67.2%). This results is suggestive that aflatoxin present in maternal blood crosses the transplacental barrier and accumulates in the fetus which further explains the high concentration of aflatoxin in the amniotic fluid and the in - utero exposure to these toxins.

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1. Introduction

Aspergillus is a fungus that essentially belongs to grains storage flora. It grows optimally at 25 °C with a minimum necessary water activity of 0.75. It starts to produce secondary metabolites at 10-12 °C, but the most toxic ones are produced at 25 °C with a water activity of 0.95 [1, 2]. Secondary metabolites named aflatoxins is a group of mycotoxins produced by a large number of *Aspergillus* species, basically by three phylogenetically distinct sections. The main producers are *Aspergillus* (*A.*) *flavus*, and *A. parasiticus*, but it has been demonstrated that *A. nomius*, *A. pseudotamarii*, *A. parvisclerotigenus*, and *A. bombycis* of section Flavi, *A. ochraceoroseus* and *A. rambellii* from section Ochraceorosei and *Emericella* (*E.*) *stellata* and *E. venezuelensis* from Nidulatans section also generate it [3-5]. All of them contaminate a large fraction of the world's food, including maize, rice, sorghum, barley, rye, wheat, peanut, groundnut, soya, cottonseed, and other derivative products made

from these primary feedstuffs in low-income countries [6-8]. Although aflatoxins have been a problem throughout history, until 1960 they have been recognized as significant contaminants within agriculture, because in this year they were initially isolated and identified as the causative toxins in "Turkey-X-disease" after 100,000 turkeys died in England from an acute necrosis of the liver and hyperplasia of the bile duct after consuming groundnuts infected with *A. flavus* [9-11]. In 2004, 4.5 billion of the world's population was exposed to aflatoxins because they are also everywhere. Some essential factors that affect aflatoxin contamination include the climate of the region, the genotype of the crop planted, the soil type, the minimum and maximum daily temperatures, and the daily net evaporation [9]. Moreover, aflatoxin contamination is also promoted by stress or damage to the crop due to drought before harvest, the insect activity, a poor timing of harvest, the heavy rains during and after harvest, and an inadequate drying of the crop before storage. Levels of mycotoxins have

been the subject of intense research efforts [12, 13]. All fungi, including *Aspergillus* produce a large number of metabolites divided arbitrarily into primary and secondary metabolites [14]. Primary metabolites are those which are concerned with the fundamental life processes of the organism while secondary metabolites cover the remaining compounds for which there is no such function. Many of the secondary metabolites are of complex chemical composition and are extremely toxic to other forms of life, e.g. the antibiotics and aflatoxins which is the subject of this work. Of the known mycotoxins, the most important in relation to direct hazard to human health are the aflatoxins [15, 16].

2. Materials and methods

2.1 Sample collection

Serum: 3-5 ml of whole blood samples were collected from pregnant women in the labor ward either before or after delivery. The venous maternal blood was taken using disposable syringes, and collected in universal bottles for centrifugation. 10 ml of cord blood was collected into universal blood containers directly from the umbilical cord immediately after delivery. Serum was collected using a drawn-out pipette after centrifugation with a bench centrifuge at Centrifuging at 5000 revolution per minute for 5 min. The sera were frozen and analyzed later.

Amniotic fluid: Amniotic fluid was collected in a sterile bed pan by either artificial or natural rupture of the membrane before birth. If membrane ruptures before patient reaches labor ward, residual fluid is collected after birth. If badly stained with meconium it is discarded. Hence amniotic fluid was not got from all the mothers, especially in cases of old still birth. Amniotic fluid in sterile bed pan was then poured into McCartney bottles and kept frozen till analysis.

Aflatoxin G₁, G₂ and M₂ mixed standards (RIVM Bithoven, Netherlands), chloroform-BDH (Analar Chem. Ltd. Poole, England) trichloroacetic acid (BDH), Alumina Woelm Pharma (GMBH Co. Eschevege), florisil - BDH (about 60 - 100 U.S. mesh), sea sand Fisher scientific Co. N.J. U.S.A.) silicar gel - carlo erbamilano (Omm 0.05 -s- 20), methanol (M&M Ltd. England) was purchased.

2.2 Instruments

Oven (B and T. A scarce Co.), velasco fluorotoxin meter (VFM) (Neotec Instrument Inc. USA), simple vacuum pump, desk centrifuge (MSB) and water bath were used.

The method used was that by Onyemelukwe and Ogbadu [17], with a modification which involved the introduction of aflatoxin chloroform extraction step. This was then utilized for the preparation of samples for analysis.

To 1 ml of serum or fluid sample 9 ml of 10% trichloroacetic acid was added to precipitate serum or amniotic fluid proteins. The filtrate was transferred to a separating funnel (100 ml), and the aflatoxin was extracted with 20 ml chloroform by shaking vigorously for 5 min. The lower chloroform layer was collected in a beaker and evaporated to dryness using a water bath.

The beaker was allowed to cool and the residue dissolved with 20 ml chloroform methanol (96+14) by gently swirling the beaker. The solution was then transferred to a vial. It was stored away from dust and light wrapped with aluminum foil when not immediately analyzed.

2.3 Preparation of microcolumn for velasco fluorotoxin meter analysis

One end of the column was plugged not too tightly with glass wool 2-3 mm in depth. At the top of the column was placed a small funnel fitted with plastic tip. 5-7 mm in depth of sand was added using the scoop. A layer of florisil was then added, of depth not more than 5-7 mm. Another layer of sand 5-7 mm was again added. Next was added, the silica gel about 15 mm in depth. Finally a layer of neutral alumina was added about 15 mm in depth.

2.4 Development of aflatoxin on microcolumn

The prepared micro column was wetted with chloroform. This was done by lowering the bottom of the column into a vial containing chloroform, which was allowed to seep up through the various layers. By using a syringe, 1 ml of the sample solutions previously prepared or standard aflatoxin solutions – 50 ng per ml from the vial (or bijou bottles) was transferred into the wetted columns and allowed to drain. The drain time for 1ml of solution in the columns was about 5 min. Pressure or vacuum was applied to reduce the drain time. A simple and effective vacuum arrangement was to place the column in the test tube, with the side arm, and to attach the small rubber bulb on the side arm as a source of vacuum, by varying the amount of pressure exerted on the bulb before attaching to the side arm the flow rate was varied. This vacuum technique became easier to control after a few runs. It was necessary to avoid very fast flow rates (i.e. 1 ml drained in less than 2 min, with attendant loss of aflatoxin). After the solution had

drained, 1 ml of chloroform - methanol (96+4) was added to the column and allowed to drain. While the column was still wet it was placed in the velasco flurotoxinmeter (VFM) instrument and the reading taken.

2.5 Calibration of AFM

The VFM was calibrated using the standard micro columns developed and a blank column. The operational procedure for the calibration was as follows: The VFM was plugged into an electric source and the power switched moved to the on position. The lamp start push-bottom was depressed and held in this position for approximately 20 seconds then released. The lamp operation was verified by ensuring that the bluish-white glow from the hole on the rear access cover of the VFM was on. When the lamp did not start, the starting operation was repeated.

One hour was allowed for the warm-up and stabilization of the instrument. Then the blank micro column was inserted into the sample holder and the micro column cap placed over the exposed portion of the micro column. The VFM was adjusted to read zero using the ZERO control knobs, and the blank micro column was removed.

A wetted standard aflatoxin micro-column (50ng per ml) was inserted into the sample holder and the micro column cap placed in position. The Calibration control knob was used to adjust the VFM to read 20ppb. By alternatively using the

blank and standard micro columns repeatedly (about twice each) the zero and 20 ppb readings were obtained. The 20 ppb setting was the average of two standard micro column readings. The aflatoxin standards used were G₁, G₂ and M₂.

3. Results and Discussion

The results outlined are those obtained from initial analysis of aflatoxins in amniotic fluid and serum from both venous maternal blood and neonatal cord blood by the AFM (Table 1, 2). Table 3 shows that from the total sample of 570 analyzed 375 (67.5%) was positive for aflatoxin those taken from the amniotic fluid a total of 89 and 66 (74.1) was positive, maternal and neonatal cord blood showed a prevalence of 133 (62.4%) and 142 (67.2%) respectively from a total of 213 and 211 of samples analyzed.

A consideration of the difference in the percentage of positive detection of aflatoxin between the controls (59.6%) and other serum samples and amniotic fluid (74.1%, 62.4% and 67.2 %) which further implies that the controls either did not ingest high levels of aflatoxin or that it had been eliminated these from the sera through the activity of their microsomal mixed function oxidase system (MFO), which has the capacity to metabolize aflatoxin [14-16]. This fact therefore could account for the reduction of aflatoxin to the low levels observed in the controls. The metabolic implication is that the consequently toxicity, if such levels exceed the metabolic capacity of the liver.

A comparison of the percentage positive aflatoxin detection levels in Zimbabwe (31.42%); Sudan (23.2%) and Nigeria (83.64%) suggest a higher level in Nigeria than in the other countries. One of the factors contributing to this difference could be based on agriculture - farming system staple foods, storage and processing of foods (climatic differences could also have influence on these differences.

The biochemical effects of aflatoxin have been attributed to the interaction of the compounds with DNA, RNA and proteins, also to the inhibition of protein and nucleic acid synthesis [18, 19].

The impairment of nucleic acid synthesis should alter protein synthesis for example, the incorporation of ¹⁴C -leucine into rat liver slices was inhibited when aflatoxin B1 was added to the system [11, 19]. When tissue cultures of human liver were incubated loss of RNA from the cytoplasm and an apparent loss of chromatin from the nucleus [4, 5].

Table 1. Percentage prevalence of aflatoxin across the various patient's samples

Samples	Number tested	Number positive
Control	57	34(59.6%)
Amniotic	89	66(74.1%)
maternal blood	213	133 (62.4%)
neonatal cord blood	211	142 (67.2%)
Total	570	375 (65.7%)

Table 2. The standard deviation and mean of amniotic fluid

Groups amniotic fluid	G ₁	G ₂	M ₂
Total	1298.54	1220	1674.5
Mean	18.98175	24	21.54268
Std. Deviation	16.16768	240	14.30764
Range	0.5-59	0.5-51.5	0.5-59

Presence of this high percentage of aflatoxin above 20 ppb as shown in the results (appendix) of the sera and amniotic fluid, if not eliminated from the liver, might lead to accumulation and

The findings in this investigation there is suggestive evidence that these pregnant women were exposed to aflatoxins in their diets during gestation period and that these substances can be detected in the serum of both maternal blood and neonatal cord blood and amniotic fluid, in a higher population of these women.

Circumstantial evidence of the role of aflatoxins in human disease includes epidemiological correlation between aflatoxins in food and hepatoma in Africa and Thailand [8], severe liver disease in India after ingestion of aflatoxin-contaminated foods [1, 16], and epidemiological and clinical correlations between eye's syndrome in children and dietary exposure to aflatoxin in Thailand [20, 21].

These reports have confirmed observations of the vulnerability of the liver to injury by aflatoxins. Apart from the evidence concerning hepatoma, clinical evidence of aflatoxin poisoning in man is based mainly on acute pathology precipitated by short exposure to aflatoxins [20, 22, 23]. The two findings which call for some explanation are the growth retardation and the enhancement of the maternal liver injury both resulting from aflatoxin administration in late pregnancy. In their experiment they also reported that reduction of maternal food intake is not an adequate explanation for the fetal stunting as complete absence of dietary protein in the last 6 days of pregnancy and causes only moderate fetal growth and retardation in the rat [3, 24].

Other possibilities considered included direct action of aflatoxin on the placenta or fetus or impairment of fetal nutrition secondary to the effect of the toxin on the mother. There was no evidence of placental damage in any of the pregnancy and in particular, they did not find the hemorrhages at the utero-placental junction reported by other investigators in this field.

Fetal tissues are most susceptible to the action of toxic agents or environmental alterations during the period of organogenesis (days 6 - 12 in the rat). Substances causing fetal growth retardation in late pregnancy would be expected to have a more severe effect in early pregnancy with resultant fetal mitotic drugs, x - irradiation [14, 18, 24] and uterine ischemia [6, 7, 11]. A Butler's and Wigglesworth's experiment does not suggest a direct action of aflatoxin on the fetus as administration in early pregnancy failed to cause either fetal death or malformation.

Several findings support the hypothesis: that the fetal growth retardation in their experiments was secondary to the effect of aflatoxin on the mother, only when the rat was conditioned during

the time of most rapid fetal weight gain (days 17-21) was fetal growth retarded. Doses of aflatoxin which failed to produce recognizable lesions in the maternal liver also failed to cause fetal stunting; where liver lesions were produced the severity of fetal stunting appeared proportional to the extent of the maternal liver injury. The single rat dosed in early pregnancy which had stunted fetuses showed evidence of persisting liver damage with widespread severe fatty infiltration [1, 16, 25-27].

There is no evidence as to the mechanism by which maternal liver injury might impair fetal growth. It is, however, known that the fetus obtains its protein requirements in the form of amino-acids and it would seem reasonable to speculate that damage to the protein synthetic mechanisms of the maternal liver might well alter the amino acid composition of the maternal plasma to the disadvantage of the fetus [1, 20]. Experiments using carbon tetrachloride as a hepatotoxic agent have not caused fetal growth retardation (Wigglesworth unpublished observation) but recovery from carbon tetrachloride liver necrosis is very much more rapid than that following aflatoxin administration.

Table 3. Mycotoxins, G₁, G₂ and M₂ contamination in maternal and neonatal cord blood

Groups Blood Group	G ₁	G ₂	M ₂
Total	1977.5	18844.1	1945.4
Mean	16.34298	15.11557	15.9459
Std. deviation	13.25794	10.45532	12.01587
Range neonatal cord blood	0.5-68.5	1-77	0.5-46
Total	1688.5	1770	1786.8
Mean	14.18908	14.75	14.6459
Std. Deviation	13.5273	14.15259	14.14678
Range	0.5-67	0.5-71	0.5-69

4. Conclusion

The phenomenon whereby aflatoxin possesses carcinogenic and toxigenic and biochemical activities affecting the genetic apparatus in one way or the other may be obscure, but nevertheless, the fact that aflatoxins are naturally occurring environmental pollutants found intimately associated with human food and animal feed should be of great concern due to the hazards they pose to human and animal health.

The effects of these factors on the human host are generally mutually reinforcing. Most of the adverse ecologic factors which are probably of significant importance in the etiology and pathogenesis of certain disease, flourish best in the permissive situation of extensive malnutrition.

The possibility of removing the potential carcinogen from the environment of a selected population is certainly difficult, but the progressive urbanization which is occurring in developing countries, may afford an opportunity for determining the effect of urbanization on aflatoxicosis, changes in food habits and sources of dietary staples.

In Nigeria as it is obtainable in other developing countries where fungal infestation and aflatoxin contamination of foods is very common, it is essential that the potential hazards of the problem can be recognized. The food industry and research agencies should make every effort to maintain and improve on the efficiency of the agricultural practices, adopt effective sun or mechanical drying and sound storage of foods. These would appear to offer the most effective method in the prevention of aflatoxin contamination.

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