

Spermatotoxic Effect of Aflatoxin M1 on The Sperm Cell Quality of Adult Male Wistar Rats

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The effect of aflatoxin on reproductive health takes a systematic approach as it indicates that a significant number of people living in the third world regions are exposed to these toxins due to the poor storage of grains. To expose the effects of aflatoxin M1 a hydroxylated metabolite of aflatoxin M1 Twenty four Adult male Wistar rats were acclimatized and broken down into four groups, Test Group one (Two weeks exposure), Group Two (four weeks exposure), Group Three (Six weeks exposure) and Group Four (Control Group). The Test Group 1, 2 and 3 were orally administered 7.2g/kg (LD50) of Aflatoxin M1 for the desired weeks of exposure and the sperm cell quality was assessed to determine the effect of Aflatoxin M1 on sperm cell quality in Adult male wistar rat. The predetermined level of sperm cell quality is its ability to be motile as the motility of the sperm cell determines its viability. Group three had the highest presence of sluggishly immotile cells with very low progressive motility, followed by group, two and one, 37.5 ± 9.97 , 55.00 ± 9.91 , and 71.67 ± 3.07 as against the control groups 85.8 ± 2.17 with the highest significance at the group three which showed an extended exposure of aflatoxin m1 ($p=0.05$). This study links infertility and aflatoxin consumption, as the higher the doses the more spermatotoxicity is built which invariably could lead to sterility and azoospermia.

Keywords: Cell Quality; Wistar Rats; Aflatoxin**Introduction**

Aspergillus is a known fungus which is mostly found as a flora in grain storage, it has its optimal growth at 25°C with very minimal water activity which is about 0.75. Its secondary metabolites are produced at 10-12°C, the most toxic metabolites have their optimal temperatures at 25 °C with water activity of about 0.95 [1]. These secondary metabolites are known as 'Aflatoxin' (AF) belongs to a group of mycotoxins which are produced in large numbers by the wide family of *Aspergillus* species, although there are basically three phylogenetic distinct sections. The main producers of this toxins are, *Aspergillus flavus* and its counterpart *Aspergillus parasiticus* but much more distinctive there have been toxin production by *Aspergillus nomius*, *Aspergillus parvisclerotigenus*, *Aspergillus bombycis* and *Aspergillus pseudotamarii*, of section Flavi, *Aspergillus rambellii* and *Aspergillus ochraceoroseus* from *Ochraceorosei* and *Emericella astellata* and *E. venezuelensis* [2]. They are known

to contaminate a broad section of the global food which includes, maize, rice, sorghum, barley, rye, wheat, groundnut, peanut, soya, cottonseed and most other product which are processed from these primary feedstuffs in low income countries [3-6]. Aflatoxin have been known to cause health problems throughout history. 1960 unveiled the significance of its contamination within the agricultural sector in that year the toxin was isolated and identified as the cause of "Turkey X-disease" after the death of over 100,000 turkeys which died from liver necrosis and hyperplasia in England, this occurred after the poultry birds had consumed groundnuts infected with *Aspergillus flavus* [7-10]. Also documented and estimated by Williams., *et al.* in 2004 that about 4.5 billion of the world's population are exposed to the toxic aflatoxins owing to the fact that they are commonly found everywhere. These essential factors that contribute to aflatoxin wide contamination of food stuff include the climate of the region, the crop planted and its genotype, the soil

type and the maximum, minimum temperatures coupled with the net daily evaporation [11]. Aflatoxin M1 (AFM1) which is a hydroxylated metabolic derivative of AFB1, AFM2 is a metabolic derivative of AFB2; both are gotten from the metabolism of some animals, and are usually found in breast milk and urine [12,13].

It has been reported that aflatoxins, once ingested (because of their low molecular weight), are rapidly adsorbed in the gastro-intestinal tract through a non-described passive mechanism, and then quickly appear as metabolites in blood after just 15 minutes and in milk as soon as 12 hours post-feeding [14-16]. Recent studies also suggest that the B aflatoxins may cause neural tube defects in populations that consume maize as a staple food (Wild and Gong, 2010). Aflatoxin B1 and its derivative are also known to cause secondary infertility both in males and female rabbits and rats [17,18].

Furthermore, due to this important worldwide issue, some organizations and institutions have been purposing a great number of practical primary and secondary prevention strategies, especially for developing countries, in order to reduce the risks given by this public problem, but they could be beneficial if political wills and financial investments are applied to what remains a largely ignored worldwide health matter.

The aim of this study is to determine the effect of Aflatoxin M1 effects on Sperm cell quality in adult male Wister rats, its toxicokinetics and spermatotoxicity.

Materials and Method

Study site

The University of Benin Animal house in The Department of Anatomy was used as the study site where the male adult Wister rats were acclimatized and orally administered the fungal toxin.

Study animals.

Twenty Four Male Wister (albino) rats 350-470g of reproductive age were obtained. These test animals were kept at room temperature ($25 \pm 2^\circ\text{C}$) under a 12L:12D cycle and were provided with water and rat standard diet (Altromin GmbH, Large) ad libitum. The experimental studies were conducted in accordance with the ethics of the usage of laboratory animals (Helferich, *et al.* 1989).

Experimental design

A randomized block design was used in this analysis. Rats were broken down into groups and subgroups with the treatment plan across the groups. Each group signified two weeks of exposure per day of aflatoxin M1 of 7.2mg/kg per body weight. Twenty

four Adult male Wister rats were acclimatized and broken down into four groups, Test Group one (Two weeks exposure), Group Two (four weeks exposure), Group Three (Six weeks exposure) and Group Four (Control Group). The Test Group 1, 2 and 3 were orally administered 7.2g/kg (LD50) of Aflatoxin M1 for the desired weeks of exposure and the sperm cell quality was assessed to determine the effect of Aflatoxin M1 on sperm cell quality in Adult male Wister rats.

Treatment of male rats

Aflatoxin M1 (Sigma) was given orally with a dosage of 7.2mg/kg per day for 42 days (6 weeks) in a volume of 0.14ml vehicle (de-mineralized water containing 0.2% per 100g body mass). Fertility of the male Wister rats was assessed after dietary Aflatoxin M1 by looking at the sperm cell quality.

Sperm cell morphology and count

Isolation of sperm cells

Sperm cells were collected from the vas deferens of the sacrificed rats; the rats were sacrificed and the vas deferens located and ligated with a minimum of 36mm length, both extremities of the vas deferens were ligated, cut and placed in a sterile petri dish. To the petri dish, 6 μl of normal saline already adjusted to $37 \pm 2^\circ\text{C}$ was added. The Vas deferens was teased to allow the sperm cell diffuse out of it. A drop of the sperm cell from the petri dish was placed on a grease free clean slide and covered with a transparent cover slip.

Motility of spermatozoa

The sperm cell motility were determined with the correlation between progressively motile sperm cells after ejaculation and the fertility. The motility were evaluated with regards to three variables: Progressively motile, Non-progressive motility and Immotile spermatozoa and it is usually expressed in percentile. Spermatozoa can show good motility and viability in the seminal plasma 24 hours after ejaculation but in some semen samples the motility may decline much faster (Brazil, *et al.* 2004; WHO, 2010, and Ibeh, *et al.* 2018).

A drop of the sperm cell was taken from the petri dish and dispensed on a clean grease free slide and further covered with a transparent cover slip. The slide was placed on the microscope and viewed with the x20 and x40 objective magnification lens. The motility was scored in percentage according to their nature of motility as, Progressive, Non progressive and Immotile sperm cells.

Vitality testing

One volume of semen (a drop) was mixed into two volumes of eosin solution (1% diluted water). After 30 seconds three volumes

of nigrosine solution (10% nigrosine) was added and the sample homogenized. A thin smear was then made immediately and air dried. The Stained slide was examined under the oil immersion objective lens (x100). Live spermatozoa were unstained (white) and the dead ones were red (WHO, 2010).

Morphology

The sperm cell morphology was assessed by staining the slide with the Improved Eosin and Leishman stain (Ibeh., *et al.* 2018).

A drop of the sperm cells was dispensed on a grease free clean slides and a smear was made, the slide was left to air dry. The slide was flooded with the Improved Eosin and Leishman stain for 15 mins. The stain was rinsed and the back was blotted dry with cotton wool and left to air dry. The slide was placed in a microscope with the magnification lens at x100. The slide was viewed with at least 30 magnification field, the normal and abnormal sperm cells were spotted and scored in percentage.

Statistical analysis

The SPSS software for the analysis of variance was used to determine the significant difference at ($p < 0.05$) for the effect of Dietary Aflatoxin M1 on sperm cell quality in adult male Wister rats.

Results

The results of the effect of Aflatoxin M1 on Adult male Wister rats revealed the negative effect on sperm cell quality as compared with the control group. The descriptive statistics of the sperm cell motility and count between and across the study groups; revealed that the highest and lowest progressively motile cells and the highest and lowest total sperm cell counts were 71.67 ± 3.07 , 37.50 ± 9.97 and 280 ± 24.358 , 140 ± 25.690 as compared with the control group of 85.83 ± 2.73 and 378 ± 15.147 respectively.

The results of sperm cell morphology and count between and across the study groups with the highest and lowest normal morp-

hology with mean of 75.00 ± 2.236 and 64.17 ± 3.745 as compared with the control group 90.83 ± 0.833 .

For the progressively motile sperm cells, the number of motile cells dropped from 71.6% in rats after 2 weeks of being fed Aflatoxin diet to 55% in 4 weeks and then 37.5% in 6 weeks, compared to values of the control group not fed Aflatoxin diet with 85.8% progressively motile sperm cells.

The Non progressively motile sperm cells did not drop in rats after 2 weeks and 4 weeks of being fed Aflatoxin M1 diet with 11.6% respectively, but dropped to 10.8% in rats after 6 weeks on dietary Aflatoxin M, compared to the control group with 5.8%. The Immobile sperm cells increased from 16.6% in rats after 2 weeks of Aflatoxin M1 diet to 35.0% in 4 weeks and then 53.3% in 6 weeks, compared to the control group with 8.3%. The Total sperm cell count dropped from 280×10^6 cells/mm³ in rats after 2 weeks of Aflatoxin M1 diet to 206×10^6 cells/mm³ in 4 weeks and then 140×10^6 cells/mm³ in 6 weeks of being on Aflatoxin M1 diet, compared to the control group with 378×10^6 cells/mm³ respectively.

There was an increased levels of abnormal sperm cell from 25% in rats after 2 weeks of being fed Aflatoxin M1 diet to 30% in 4 weeks and 35.8% in 6 weeks, compared to values of the control group with 9.1% abnormality.

The presence of abnormal sperm cell formation as seen in rats 2 weeks of being fed Aflatoxin diet presenting with headless sperm cells, multiple tailed sperm cells, short tailed sperm cells respectively (Plate 1a). In week 4 there was more abnormal sperm cells presenting with headless sperm cells, bent headed sperm cells, and tailless sperm cells respectively (Plate 1b). In week 6 six of exposure to dietary Aflatoxin M1, the marked abnormality presented were more of degenerated forms, co joined headed sperm cells and premature sperm cells (Plate 1c), compared to the control group with no abnormal sperm cells as shown in (Plate 1d).

Plate A-D: Sperm Cell Morphology Stained with the Improved Eosin and Leishman Stain (Ibeh et al., 2018).

- (A) (i) Abnormal sperm cell, deformed head (ii) Headless spermatozoa
- (B) (i) Tailless sperm cell (ii) bent sperm cell
- (C) (i) Headless sperm cell (ii) Tailless sperm cell
- (D) (i) Bent head (deformed) (ii) Headless sperm cell (iii) Tailless sperm cell

Groups of Rats Fed on Aflatoxin M1 Diet (Weeks)	Total Sperm Cell Counts (Mean ± S.E) N=6 (x10 ⁶ cells/mm ³)	Progressive Motility (Mean ± S.E) N=6 (%)	Non Progressive Motility (Mean ± S.E) N=6 (%)	Immotile (Mean ± S.E) N=6 (%)
2	280.00 ± 24.35	71.67 ± 3.07	11.67 ± 1.66	16.67 ± 2.10
4	206,67 ± 14,53	55.00 ± 9.91	11.67 ± 1.66	35.00 ± 11.4
6	140.00 ± 25.92	37.50 ± 9.97	10.83 ± 2.00	53.33 ± 12.2
Control	378.33 ± 15.14	85.83 ± 2.71	5.83 ± 2.00	8.33 ± 1.05

Table 1: Effect of Aflatoxin M1 on the sperm cell quality, comparing the control group and the treated group.

Groups of Rats Fed Aflatoxin Diet (Weeks)	Normal Morphology (Mean ± S.E) N=6 (%)	Abnormal Morphology (Mean ± S.E) N=6 (%)
2	75.00 ± 2.23	25.00 ± 2.23
4	70.00 ± 0.00	30.00 ± 0.00
6	64.17 ± 3.74	35.83 ± 3.74
Control	90.83 ± 2.04	9.17 ± 0.83

Table 2: Sperm Cell Morphology of Adult Male Wister Rats Fed with Aflatoxin M1 Diet.

Discussion

The spermatozoa harvested from rats dietary fed aflatoxin M1 showed rapid decrease in their motility with the increase in time of treatment with 71.67 ± 3.07 in 2 weeks to 55.00 ± 9.91 in 4 weeks and 37.5 ± 9.97 in 6 weeks, compared to the control of 85.83 ± 2.17 respectively. The differences in the average spermatozoa motility of the test Wister rats and the control were statistically significant (P<0.05). The depression of spermatozoa motility in rats exposed to aflatoxin M1 suggest that aflatoxin M1 impaired the metabolic activities of the sperm cell which is also reported in previous studies [21,22]. The adhesion of aflatoxin M1 to the nucleic acid of a sperm cell may inhibit the cells response to chemotactic agents thereby decreasing its motility (Patten, 1981). The loss of nucleic acids and certain enzymes by sperm cells may contribute to their low motility and viability [23,24].

The immotile sperm cells also signifies dead cells and dysgenesis was highest in the 6 weeks of exposure with 53.33 ± 12.2 and the lowest immotile sperm cells was seen with 16.67 ± 2.10 in 2 weeks of rats dietary exposed to Aflatoxin M1 as compared with the control with 8.33 ± 1.054. Due to the active spermatotoxic effect of high accumulation of aflatoxin M1 in semen, this sets up an oxidative stress in the testicular region thus leading to the depletion of preformed antioxidants [25,26].

In this study the Spermatozoa harvested from the rats decreased in sperm cell count over the period of exposure to dietary aflatoxin M1. This difference is expressed in the average spermatozoa count of test rats and the control which were significantly significant (P<0.05). The reduction in spermatozoa count in rats exposed to dietary aflatoxin M1 from 280 ± 24.35 in 2 weeks of rats dietary exposed to aflatoxin M1 to 206 ± 14.5 in 4 weeks and 140.0 ± 25.69 in 6 weeks as compared with the control group with 378.3 ± 15.4 (x10⁶ cells/mm³), thus suggest that aflatoxin M1 caused the destruction of sperm cells. This effect must have been achieved through several mechanism: Changes in the tonicity (osmolality) of the extracellular fluids which is as a result of acid-base imbalance; binding of aflatoxin M1 to sperm cells leading to active membrane damage and subsequent lysis of the sperm cell. All these factors may act independently or in concert to bring about a decrement in spermatozoa count. The observed decrease in spermatozoa count are in tandem with works on rats exposed to aflatoxin M1 [27-30].

Abnormalities were observed when rats were exposed to aflatoxin M1 as reported in this study compared with the control group that had as high as 90.83 ± 0.833 (%) normal sperm cell and only 9.71 ± 0.833 (%) abnormal sperm cell. Some of the abnormalities as shown in the micrograph (Plate 1, 2, and 3) where, Headless spermatozoa, disjoined body, bent head, tailless sperm cell, double tailed sperm cells and short tailed sperm cells. There was a significant increase in abnormal sperm cell in week six with 35.83 ± 3.745(%), 30.0 ± 0.00 in week four and the lowest abnormalities in week two one (25.00 ± 2.236) (%), compared to the control group. As documented in previous studies which showed a 95 ± 5 (%) normal sperm cells in the control groups (Akbarsha, *et al.* 2007), which is also in tandem with this study. Abnormal sperm cells were significantly lower as observed in this study, compared to previous work that showed a higher percentage of abnormal sperm cells. This may be due to our short time of exposure of the rats to dietary aflatoxin as compared to other studies with a minimum of fifty six days (56) exposure to aflatoxin M1 [31].

The increase in spermatozoa abnormalities may be directly related to the toxicity of aflatoxin M1 on the spermatocytes, which progresses to abnormal mitotic and meiotic division of the primary and secondary spermatocytes [32]. The basis of induced abnormalities can also be caused due to the phenolic ring structure of aflatoxin which induces mitochondria uncoupling and oxidative phosphorylation which eventually results in the swelling of the spermatid mitochondria [33,34]. Leakages of cellular materials following the alterations of sperm membrane permeability may also contribute to spermatozoa abnormalities [35-61].

Conclusion

The results of this study has revealed that aflatoxin M1 ingestion negatively affects sperm cell quality in male Wister rats. Sperm cell motility, count decreased and abnormalities increased with increased time of dietary exposure, from 2 to 6 weeks.

Conflict of Interest

There were no conflict of interest associated with this study.

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