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Research article

Reproductive Performance of Adult Male Rats Previously Exposed to Aflatoxicosis at Pre-puberty

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Abstract

Aflatoxin-induced reproductive toxicity was investigated in adult male rats. To investigate the effect of pre-pubertal exposure to aflatoxin B1 (AFB1) on semen quality in male rats after puberty. Eighty premature male rats (35 days old) were assigned equally to control and treatment groups. The males were daily administered with distilled water and AFB1 (0.3mg/kg/day) per os, respectively. After 15, 25, and 35 days of treatment (pre-puberty, puberty, and post-puberty stags, respectively), ten males from each group were weighed, anesthetized, and sacrificed. Testes, epididymis, seminal vesicles, and prostates were dissected and weighed. At puberty (25 days of treatment) and post-puberty (35 days of treatment), the tail of the epididymis was dissected for semen analysis, including sperm motility, sperm count, sperm viability, and sperm abnormality. Following 15, 25, and 35 days of exposure, the AFB1-treated group revealed a decline in the relative weight of testes, epididymis, seminal vesicle, and prostate than the control group, early at the pre-pubertal stage, which continued at pubertal and post-pubertal-stages. Sperm motility, sperm count, and sperm viability were significantly decreased, while sperm abnormality was significantly increased in the AFB1 group, at puberty and post-puberty. Females matted with AFB1 treated males revealed significant decrease of pregnancy rate, number of offspring, and litter weight at birth in comparison with those matted with control males. Altogether, these results showed an adverse effect of pre-pubertal exposure to AFB1 on male reproductive performance with impaired spermatogenesis after puberty.

Keywords: Aflatoxin B1, Fertility index, Reproductive performance, Semen, testes.

Introduction

Aflatoxins are toxic substances made by the *Aspergillus* species of mold that primarily contaminate food in tropical regions. Aflatoxin B1, the most dangerous aflatoxin, is a substantial contributor to hepatocellular carcinoma (HCC) in these nations (1). Mycotoxins are secondary metabolites created by mycotoxin fungi such as *Penicillium*, *Aspergillus*, and *Fusarium* (2). It has economic significance for its impact on human and animal health and domestic and international trade (3,4). In tropical nations, consumption of infected foods such as grains, nuts, eggs, milk, and meat exposes people to aflatoxins, which are secondary metabolites produced by *Aspergillus* species molds (5). Hepatotoxicity and carcinogenesis have been associated with AFB1-exo-8,9-epoxide, the highly reactive metabolite of AFB1 that binds to DNA and produces adducts (6,7). Aflatoxins are known to cause a variety of health problems, as the toxicity of AFB1 is quite complicated and is highly connected to the dosage, solvent, route of administration, duration of exposure, age, gender, species, target organs, and other factors (8). In animals, aflatoxicosis damages the liver, reduces milk and egg production, and makes them more susceptible to infections from hazardous

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microbes (such salmonellosis) since it suppresses their immune systems (8). Aflatoxisis affects human fertility, anemia, and gastrointestinal function (9,10). The mycotoxins known as aflatoxin come in various forms. *A. parasiticus* produces aflatoxins B1, B2, G1, and G2 while *A. flavus* only produces aflatoxins B1 and B2 (11,12). The most prevalent aflatoxin, as well as the one with the greatest harmful effects on people and carcinogenic effects on sensitive lab animals, is aflatoxin B1 (13).

In humans and experimental animals, it has been shown that aflatoxins, notably AFB1, may affect the endocrine glands and reproductive system to varied degrees (14). Aflatoxin treatment of adult male rats produces pathological alterations in the testicles and epididymis (15), decreased spermatogenesis (16), decreased weights of epididymis, prostate, and seminal vesicles (17), reduced spermatid number and testosterone levels, while causing sperm tail and nuclear structural defects (16), degeneration of the Sertoli cells and increased apoptosis (18), reduced number of Leydig cells, production of immature, giant, and multinucleated sperm in males (19).

The current study hypothesized that pre-pubertal aflatoxin B1 exposure may have an impact on adult spermatogenesis, therefore, the study aims to investigate the impact of prepubertal aflatoxicosis on the semen quality after puberty.

Materials and methods

Chemicals

Aflatoxin-B1 with a purity of 98%, extracted from *Aspergillus flavus* (Formula: C17H12O6, molecular Weight: 312.3 AMU, white to faint yellow powder, and soluble in chloroform) was purchased from Sigma Company, USA. All the additional compounds utilized in the current study were of high analytical quality and were obtained from commercial establishments.

Animal ethics and care

The current research was carried out according to the guidelines of the National Research Council for the Care and Use of Laboratory Animals. The Ethical Council in the College of Veterinary Medicine, University of Al-Qadisiyah agreed to conduct this experiment.

Experimental animals

The study was conducted on 80 premature male rats, 35 days old, and 75-88 g. The animals were kept at 22 to 25 °C, 75-76% relative humidity, and 12:12 hours of dark and light cycle. The animals had free access to water and laboratory food at all times. After acclimatization, the animals were weighed before treatment and after each period of the experiment.

Experimental design

The eighty premature male rats were allocated to control and treatment groups (40 males each). Control male rats were daily administered with distilled water per os. Treatment male rats were daily administered with Aflatoxin-B1 (0.3mg/kg/day) (9,20). After 15, 25, and 35 days of treatment (pre-puberty, puberty, and post-puberty periods, respectively), ten males from each group were weighed, anesthetized by intra-peritoneal injections of ketamine (90 mg/kg BW) and xylazine (40 mg/kg BW), and sacrificed. Testes, epididymis, seminal vesicles, and prostates were dissected and weighed. The relative weight of each organ (g/100g BW) was calculated.

At puberty (25 days of treatment) and post-puberty (35 days of treatment), the tail of epididymis was dissected for semen analysis, including sperm motility (21), sperm count (22), sperm viability (23),

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and sperm abnormality (24). In brief, sperm number was calculated by cutting the epididymis tail into small pieces using a microsurgical blade, and it was stored at 37 °C for examination after being completely mixed in 1 ml of physiological saline. One drop of the diluted semen was put on a slide using a micropipette, covered by a cover slide. Using a microscope's objective lens at a magnification of 40X, sperm counts were counted in 10 tiny areas. The mean of computed sperms in ten fields was used to calculate the concentration of sperms (sperm/milliliter), and the mean was then multiplied by 1 million (according to the following equation).

Sperm concentration (sperm/ml) =Mean of calculated sperms in ten field x 10^6

Abnormal sperm percentage was calculated by adding a drop of the epididymal tail mixture to a slide, mixing for 30 seconds, and then spreading with another slide to generate a smear. The smear was observed through an oil lens after drying (X100). 200 sperm were thought to be present, and the equation below was used to identify the abnormal sperm

Abnormal sperms (%) = No. of abnormal sperms / Total No. of sperms x100

Total sperm motility was calculated by placing the epididymal tail mixture on a clean, warm, and dry slide, and mixed thoroughly. Then carefully inspected using a light microscope with a 10X objective lens using the following equation:

Sperm motility (%) = No. of motile sperms / Total No. of sperms x 100

Sperm viability (%) was calculated by mixing a drop of eosin-nigrosine stain with a drop of the epididymal tail mixture and examined under the oil lenses (X100). Since the heads of dead sperm will be stained while those of live sperm will not, 200 sperms were calculated. The following equation was used to compute the proportion of living sperm:

Live sperm (%) = No. of live sperms/Total No. of sperms x 100

The remaining 10 males from each group were matted with experienced females (1 male: 2 females) to find out the fertility index, including pregnancy rate (%), offspring number/dam, duration of pregnancy (day), and weight at birth (g).

Statistical analysis:

The statistical analysis was conducted using GraphPad Prism version 5. The mean ± standard deviation (M±SD) was used to present the data. One-way ANOVA with Newman-Keuls posthoc analysis determined the significant differences between the periods within each group. Additionally, the Student's t-test was used to determine the significant differences between the groups at each period. The level of ($p<0.05$) was considered significant (25).

Results

Relative genital organ's weight

Table (1) indicates a significant increase ($p<0.05$) of testes, epididymis, seminal vesicles, and prostates in both study groups with age from pre-puberty, puberty, and post-puberty. As illustrated in the same table, the relative weight of aflatoxic male's testes, epididymis, seminal vesicle, and prostate decreased significantly ($p<0.05$) than control, early after 15 days of treatment (pre-pubertal stage). These significant differences continued after 25 days and 35 days of treatment (pubertal and post-pubertal stages, respectively).

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Table 1: Relative genital organ's weight in aflatoxin-B1 treated male rats.

Relative organ wt (g/ 100 g bw)	Period	Groups	
		C	Af
Testes	**15 day**	0.522±0.049 Ac	0.401±0.066 Bc
	25 day	0.774±0.063 Ab	0.617±0.052 Bb
	35 day	0.942±0.077 Aa	0.728±0.048 Ba
Epididymis	**15 day**	0.332±0.036 Ac	0.227±0.042 Bb
	25 day	0.518±0.057 Ab	0.384±0.034 Ba
	35 day	0.571±0.063 Aa	0.402±0.062 Ba
Seminal vesicle	**15 day**	0.244±0.062 Ac	0.214±0.054 Ab
	25 day	0.378±0.054 Ab	0.257±0.033 Bab
	35 day	0.437±0.048 Aa	0.277±0.037 Ba
Prostate	**15 day**	0.435±0.028 Aa	0.243±0.036 Bb
	25 day	0.731±0.046 Aa	0.458±0.041 Ba
	35 day	0.772±0.036 Aa	0.434±0.038 Ba

The values are depicted as Mean ± SD. The different uppercase letters indicate significant differences ($p<0.05$) between groups during each period. Lowercase letters indicate significant differences ($p<0.05$) between the periods for each group.

Semen quality

In comparison with control male rats, aflatoxin-treated male rats revealed a significant decrease ($p<0.05$) of sperm motility, sperm count, and sperm viability and a significant increase ($p<0.05$) of sperm abnormality, after 25 and 35 days of treatment (puberty and post-puberty, respectively). When comparing the treatment periods, sperm count increased significantly ($p<0.05$) in control males and decreased significantly ($p<0.05$) in treated males at 35 day period compared with 25 day period, whereas sperm motility, viability, and abnormality showed convergence between the two periods in both groups (Table 2).

Table 2: Semen quality in aflatoxin-B1 treated male rats.

	Period	Groups	
Semen profile		**C**	**Af**
Sperm motility (%)	**25 day**	78.234±8.327 Aa	43.272±6.133 Ba
	35 day	81.742±8.166 Aa	47.748±4.279 Ba
Sperm count (million/mL)	**25 day**	58.744±7.221 Ab	41.668±5.115 Ba
	35 day	72.883±6.892 Aa	34.952±4.056 Bb
Sperm viability (%)	**25 day**	70.833±4.325 Ab	34.977±4.118 Ba
	35 day	78.178±4.422 Aa	33.813±5.362 Ba
Sperm abnormality (%)	**25 day**	18.934±1.564 Aa	75.387±5.819 Ba
	35 day	19.286±1.835 Aa	73.036±7.854 Ba

The values are depicted as Mean ± SD. The different uppercase letters indicate significant differences ($p<0.05$) between groups during each period. Lowercase letters indicate significant differences ($p<0.05$) between the periods for each group.

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Fertility index

Females matted with aflatoxin-treated male rats revealed a significant decrease ($p<0.05$) in pregnancy rate, number of offspring, and litter weight at birth in comparison with those matted with control male rats, whereas duration of pregnancy showed no significant differences ($p>0.05$) between the study groups (Table 3).

Table 3: Fertility profile of females mated with aflatoxin-B1 treated male rats.

	Groups	
Fertility profile	**C**	**Af**
Pregnancy rate (%)	100	32.33
Litter No./dam	8.238±0.787 A	3.344±0.382 B
Duration of pregnancy	21.120±0.602 A	21.383±0.864 B
Litter wt (g) on the 1st day	5.782±0.109 A	4.226±0.112 B

The values are depicted as Mean ± SD. The different uppercase letters indicate significant differences ($p<0.05$) between groups.

Discussion

The results of the study showed a decrease in the relative weights of reproductive organs, sperm parameters, and fertility index with increased exposure to AFB1 before puberty for a period of 15, 25, and 35 days. These alterations could be a result of the hypothalamic-pituitary-gonadal axis indirectly suppressing reproduction or the direct harmful action of AFs on the reproductive organs. Aflatoxin toxicity is caused by extensive and non-specific interactions between various cell proteins and aflatoxins or their activated metabolites, which can impair essential metabolic processes and protein production and result in cell death (7).

In aflatoxicosis, the intermediate metabolite AFB1-exo 8,9-epoxide (FBO) is produced as the first phase of AFB1 metabolism by microsomal cytochrome enzyme (CYP450), which is thought to be ultimately accountable for genotoxicity. AFBO is an unstable chemical that interacts with proteins, phospholipids, nucleic acids, and other cellular macromolecules to cause a variety of genetic, metabolic, signaling, and cell structural disturbances (26,27). However, additional data showed that AFB1's induction of oxidative stress (OS) had equally dramatic or greater impacts on cell function and integrity (28-30). Genotoxic stimuli, such as exposure to AFB1, which causes severe DNA damage, primarily trigger p53-dependent apoptosis, involving two main regulatory proteins of the family of the Bcl-2 homology domain 3 (BH3)-only, PUMA and NOXA, with PUMA being involved in virtually all p53-dependent apoptotic activities (31).

Furthermore, the development of oxidative stress (OS) caused by AFB1 metabolism has also been linked to the mutagenicity of aflatoxins. Oxidative DNA damage (ODD) is caused by the OS acting directly on the DNA or indirectly by the production of byproducts from the lipid peroxidation (LPO) of membrane phospholipids (29,32). Reactive oxygen species (ROS) are produced in excess as a result of the processing of AFB1 by CYP450 enzymes in the liver. ROS can attack DNA's nitrogen bases and deoxyribose moieties and produce more than 100 distinct DNA adducts, such as 7,8-dihydro-

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8-oxo-2′-deoxyguanosine which has been utilized as a biomarker for oxidative DNA damage (29,33,34).

Moreover, physiological strategies involving antioxidant systems (enzymatic and non-enzymatic antioxidants) or antioxidant metabolites are typically used to combat OS, regardless of its source (29,35). According to the current study, the high levels of OS caused by aflatoxicosis create an imbalance between oxidants and the body's antioxidant defense mechanisms, making modulatory means (antioxidants) ineffective at preventing DNA damage that must be repaired before replication to maintain genomic stability and prevent cumulative mutations and genotoxicity. AFB1 can, however, indirectly cause DNA damage via OS by generating ROS, which then attack oxidatively degraded membrane phospholipids and generate a variety of mutagenic aldehydes, including malondialdehyde (MDA) (29).

The indirect reproductive suppresses of the HPG is the pituitary gland's diminished ability to express FSHβ and LHβ genes leads to lower serum levels of gonadotropins from pituitary gland, and subsequently testosterone concentrations (17). The primary regulator of normal spermatogenesis and male reproductive processes is the HPG axis (36). LH encourages testosterone secretion from the Leydig cells, which further increases sperm production, while FSH stimulates Sertoli cells and aids in the process of spermatogenesis (37).

Direct cytotoxic effects of AFB1 and/or decreased testosterone could be the reasons of decreased semen parameters and increased abnormal or dead sperms in the current study. According to previous studies, androgens are crucial in promoting spermatogenesis in adult rats (38). On the other hand, oxidative stress may have an impact on the reproductive system because both spermatogenesis (38) and Leydig cell steroidogenesis (40) are vulnerable to oxidative stress and lipid peroxidation and sperm fertility heavily depends on the existence of antioxidant mechanisms. In rodents, an increase in lipid peroxidation has been linked to flatoxicosis (39). Additionally, it has been demonstrated that aflatoxicosis raises oxidative stress indicators (41).

Conclusions

In conclusion, pre-pubertal exposure to AfB1 impairs semen quality in male rats after puberty. This could be related to impaired reproductive performance after puberty, either directly through oxidative stress-induced testicular cytotoxicity or indirectly through inhibition of the HPG axis and ensuing reduction in gonadotropin secretion, which inhibits both spermatogenesis and steroidogenesis.

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