The Effect of aflatoxin b1 on the sperm characteristics, sperm DNA damage, fertility potential and early embryonic development in NMRI mice

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ABSTRACT

The increasing prevalence of infertility cases is becoming a major public health problem in developing countries due to changes in diet and lifestyle. aflatoxins are among the major dietary toxins which contribute to deterioration of male reproductive structure and function. More than 5 billion people in developing countries worldwide are at risk of chronic exposure to naturally occurring aflatoxins and more so in the tropical regions. the main aim of the present study was to identify the effects of aflatoxin b1(AFB1) on sperm characteristics, sperm DNA damage and sperm chromatin integrity. The fertility potential in male mice was also evaluated. NMRI male mice of 8-week old were included in this study and they were randomly divided into three groups. The first group was received low dose (LD) of aflatoxin b1 (100 μg kg-1, PO) and the second was treated with high dose (HD) of aflatoxin B1 (700 μg kg-1, PO) for 35 consecutive days. The control group were only treated with oral corn oil for 35 consecutive days. Sperm cells were removed from cauda epididymis and analyzed for sperm characteristics (count, viability, motility, morphology), chromatin integrity and DNA damage. In addition, the rate of fertilization, two cell embryos, blastocysts, arrested embryos and their types was examined using zygotes cultured in human tubal fluid - bovine serum albumin (HTF-BSA) medium. The data were later compared and statistically analyzed by one way ANOVA and Tukey multiple comparison posthoc tests. (p<0.05). Concomitant significant increase in DNA damage and protamine deficiency of the sperm cells aflatoxin treated mice were observed (P < 0.05). In addition, the sperm motility and viability and Sperm count was significantly lower than that of control mice, but the embryo arrest rate in treated mice was significantly higher than that of control group (P < 0.05). In conclusion AFB 1 was able to induce DNA damage and chromatin abnormalities of sperm cells which could be contributed in the observed low fertilization rate and retarded embryonic development.

INTRODUCTION

Approximately 15-20% couples in the reproductive age are suffering from infertility in which male infertility is a contributory factor in half of all these couples [17,26]. Among the variety of causes, environmental factors such as dietary toxins and lifestyle factors seem to be among the most important factors of infertility [30,14,6,31,35]. one of the well-known and widely prevalent groups of food-borne toxins is the aflatoxins. Aflatoxins, are secondary toxic fungal metabolites produced by Aspergillus flavus and Aspergillus parasiticus. the toxin are usually found as a mixture AFB1, AFB2and AFG1and AFG , of which AFB1is the most potent toxic, carcinogen and mutagen. Toxin is activated to AFB1-8,9 epoxide and forms adduct primarily at N7 position of guanine and is responsible for its mutagenic and carcinogenic effects. In addition, lipid peroxidation and oxidative DNA damage are also the manifestations of aflatoxin B1- induced toxicity [27,32,20,33,22,1], at the high level, aflatoxin cause clinical illnesses and death but the chronic exposure of low level of toxin causes a disease state known as aflatoxicosis [21,34]. Aflatoxicosis can affect aspects of male reproduction, namely spermatogenesis [11], Leydig cell function [10], sperm production [3,4], testicular structure and secretion of androgens [9]. It was reported that the semen quality and embryonic development may be interrelated [18]. Janny and Menezo [16] have found that low cleavage rate and reduction in blastocyst percentage was correlated with sperm abnormality. Chromatin abnormalities and DNA damage can be
considered as male subfertility indicator regardless of the routine indications of male infertility such as sperm concentration, motility and morphology. Given the paternal gametes affect embryonic development up to the blastocyst stage, any complement sperm parameter evaluation is of great importance [28]. In the present study, sperm characteristics, DNA damage and chromatin integrity and their influences on infertility in an animal model have been investigated.

MATERIALS AND METHODS

Animals and treatment groups:

8-week old male NMRI mice were used in this study. The mice were obtained from the animal resources center of the Faculty of Veterinary Medicine, Urmia University, Iran and kept under controlled environmental conditions (22 ± 2 °C, 30-60% relative humidity, 12/12h dark-light cycle). Following one week acclimatization, animals were randomly assigned to three groups each of four male mice as control-sham and test groups. All mice were fed ad libitum with a commercial mouse chow diet.

Aflatoxin B1 (AFB1) was obtained from Sigma Chemical Co was dissolved in corn oil and Dimethyl sulfoxide (DMSO) (95:5, v/v) and administered by gavage once daily for 35 consecutive days in doses of of 100 μg kg-1 (low dose) and 700 μg kg-1 (high dose). Solution of Aflatoxin was given concurrently to control animals. At the end of the study period, the animals were euthanized by decapitation according to recommendation of the institutional ethical committee. Both epididymides (cauda and vas) of each mouse were transferred to a 60 mm Petri dish containing 1 mL of HTF-BSA (Sigma, USA) medium pre-warmed to 37 °C. The cauda was minced making 5-7 slices with a 30 gauge needle of an insulin syringe. Then using forceps, the vas “walk down” to push out any remaining sperm. After 30 min incubation at 37 °C in an atmosphere of 5% CO2, the epididymal tissue was separated from the released spermatozoa [13]. Then sperms were analyzed for count, motility, viability, abnormality, chromatin integrity and DNA damage.

Assessment of sperm motility:

One drop of sperm suspension was placed on a microscope slide, and a cover slip was placed over the droplet. At least 10 microscopic fields were observed at 400× magnification using a phase contrast microscope, and the percentage of motile sperm was evaluated microscopically within 2–4 min of their isolation from the epididymides and was expressed as a percentage of motile sperm of the total sperm counted.

Assessment of Epididymal sperm count and viability:

Epididymal sperm counts were obtained by the method described in the WHO Manual (1999). Briefly, a 5 μl aliquot of epididymal sperm was diluted with 95 μl of diluent (0.35% formalin containing 5% NaHCO3 and 0.25% trypan blue) and approximately 10 μl of this diluted specimen was transferred to each of the counting chambers of the hemocytometer, which was allowed to stand for 5 min in a humid chamber to prevent drying. The cells sediment during this time and were counted with a light microscope at 400×.

A 20 μl of sperm suspension was mixed with an equal volume of 0.05% eosin-Y. After 2 min incubation at room temperature, slides were viewed by bright-field microscope with magnification of 400×. Dead sperms appear pink and live sperms are not stained. Two hundred sperms were counted for each sample and viability percentages were calculated.

Assessment of sperm morphology:

For the analysis of morphological abnormalities, sperm smears were drawn on clean and grease-free slides, and allowed to air dry overnight. The slides were stained with 1% eosin-Y/5% nigrosin and examined at 400× for morphological abnormalities such as amorphous, hook less, bicephalic, coiled, or abnormal tails.

Assessment of sperm single-stranded DNA:

Semen samples were washed three times in phosphate buffered saline (PBS). Thick smears of washed spermatozoa were prepared on pre-cleaned degreased slides and allowed to air-dry for 10 min. The smears were fixed for 1 h in ethanol-acetone (1:1) at 4 °C and allowed to dry for a few minutes before staining with Acridine Orange (AO) (0.19 mg mL-1) for 7 min at room temperature. After staining, the slides were gently rinsed in a stream of distilled water and air dried. This was followed by evaluation under fluorescence microscope (Model G57, Nikon, Japan) with a 100 oil immersion objective. The percentage of spermatozoa stained with Acridine Orange was determined by counting 200 spermatozoa per slide. The monomeric AO, bound to normal double-stranded DNA, produces a green fluorescence, whereas the aggregated AO on single-stranded DNA yields a yellow to red fluorescence.
Assessment of sperm chromatin integrity:
The Acidic Aniline Blue (AAB) stain specifically reacts with lysine residues in nuclear histones and reveals differences in the basic nuclear protein composition of the sperm. Histone-rich nuclei of immature sperms are rich in lysine and will consequently take up the blue stain. On the other hand, protamine rich nuclei of matured spermatozoa are rich in arginine and cysteine and contain relatively low levels of lysine, which means they will not be stained by AAB. The air-dried fixed smears were stained for 7 min with 0.5 % Aniline Blue in PBS buffer. The pH was adjusted to 3.5 using acetic acid. Slides were gently rinsed in distilled water and air dried. Sperm heads containing immature nuclear chromatin stain blue and those with mature nuclei do not take up the stain. The percentage of spermatozoa stained with aniline blue was determined by counting 200 spermatozoa per slide under bright field microscope.

Oocyte collection:
Female mice of 6-8 weeks old were superovulated with pregnant mare’s serum gonadotrophin (PMSG: Folligon, Holland) (7.5 IU, IP) and human chorionic gonadotrophin (hCG: Folligon, Holland) (7.5 IU, IP) 48 h apart. At 13 hours post-hCG administration, female mice were sacrificed by cervical dislocation. Both oviducts of each female were transferred to a Petri dish containing 2.0 mL, HTF-BSA medium. Using a stereomicroscope, the swollen ampulla was found and oocytes dissected out for in vitro fertilization (IVF) [13].

Sperm preparation and IVF:
Approximately 12-13 h after hCG injection of the female mice, the male mice were euthanized. Samples of each group were prepared from the sperm suspension as described in animal and treatment groups section. Spermatozoa were obtained by swim-up and capacitated by incubating at 37 °C under 5% CO2 for at least 1 h. Then sperm with the concentration of 1×10^6 total sperm per mL was added to 500 μL fertilization drop of HTF-BSA medium containing oocytes from three females. After four to six hours of incubation at 37 °C under 5% CO2, the cumulus cell free fertilized oocytes were transferred to fresh drops of HTF-BSA medium for culture of embryos. All of the medium droplets were covered with mineral oil [19].

Assessment of fertilization:
Fertilized oocytes were evaluated by appearance of the pronuclei and polar bodies under the inverted microscope with magnification of 200×. Assessment of embryonic development. About 24 h after the zygotes culture, the two cell embryos rate was assessed and in vitro embryonic development was evaluated at 120 h under phase-contrast microscopy. The intact, fragmented and/or lysed embryos which did not develop were recorded as “arrested embryos”. In this experiment the rate of cell lyses was divided into three following categories:

- **Type I:** fully lysed, necrotic and/or fragmented embryos.
- **Type II:** embryos with partially lysed/fragmented blastomeres.
- **Type III:** embryos with some lysed/fragmented blastomeres and/or cytoplasmic vesicles [7].

Statistical Analysis
Statistical analyses of sperm parameters and assessment of the results of IVF were carried out using one-way ANOVA followed by bonferroni test using SPSS software Ver. 19 (SPSS, Inc., IL, USA)

Results
Sperm parameters
Sperm count and motility
As shown in Table 1, treatment of male mice with AFB1 resulted in reduced sperm count and motility (P < 0.05). There was no significant difference between LD and HD treated-mice (P > 0.05)

Sperm viability and motility
The percentage of viability and motility significantly decreased in treated groups in comparison to control group (P < 0.05). There was no significant difference between LD and HD treated-mice (P > 0.05) (Table1).

Sperm morphology:
The level of abnormal sperm in Afl B1-treated mice was significantly higher than that of control group (P < 0.05) but, there was no significant difference between LD and HD (P > 0.05) (Table1)

DNA damage and chromatin integrity of sperms.
The level of abnormal single-stranded sperm DNA in Afl B1-treated mice was significantly higher than that of control group (P < 0.05). There was no significant difference between LD and HD treated-mice (P > 0.05). The percentage of sperms with protamine deficiency in treated groups (LD and HD) was higher than those of control group (P < 0.05). (Table1).
Fertilization rate and embryonic development:
The results of fertilization rate and embryonic development in different groups are summarized in Table 2. The data show that fertilization rate and two cell embryos rate in the groups of mice treated with AFB1 were significantly lower than of the control group (P < 0.05) but the rate of two cell embryos in high dose treated-mice significantly lower than that of the low dose treated-mice. Percentage of embryos in blastocyst stage after culturing for 120 h in the control group was significantly higher than that of AFB1 treated groups (P < 0.05) (Table 2).

There was a marked increase in percentage of arrested embryos type I, II and III in AFB1 treated mice in comparison with the control group (P < 0.05) (Fig. 2C). Furthermore, embryonic arrest type I and II was more frequent in high dose treated-mice than in low dose treated-mice (P < 0.05).

Discussion:
The cauda epididymal sperm count was statistically significantly reduced in aflatoxin-treated mice (Table 1), which could be related to reduced spermatogenesis. Cauda epididymal sperm viability and motility were statistically significantly reduced in the aflatoxin-treated mice (see Table 1), which could be due to reduced mitochondrial function and/or decreased adenosine triphosphate (ATP) and ATPase activity. Roy (1968) reported mitochondrial swelling, and reduction in succinate dehydrogenase (SDH) activity also has been noted. Reduction in sperm motility could also be due to reduction in testicular and epididymal proteins. Androgen-binding proteins are important for several functions in the testis. The proteins in the epididymis are important for sperm maturation, motility, and fertility rate. Acidic epididymal glycoprotein in the epididymal luminal fluid of various species induces forward motility of caput epididymal spermatozoa (chinoy,1984). Therefore, a reduction in protein synthesis during aflatoxicosis may affect sperm function. The effect of aflatoxin on membrane permeability might be another major factor in the loss of sperm motility.

Our findings showed that after AFB1 exposure, the percentage of immature sperms (sperms with protamine impairment) and sperms with single stranded DNA (DNA damaged sperms) were increased significantly. According to previous reports, sperm DNA damage and nuclear chromatin abnormalities occur during impaired spermiogenesis which in turn results into remarkable abnormality in DNA packing [25]. Consequently, free radicals especially reactive oxygen species (ROS) induce severe DNA damges [5] and/or lead to cellular apoptosis [12]. Our light microscopic analyses showed that the percentage of sperms with nuclear immaturity increased in AFB1-treated groups. Therefore, we came close to this fact that AFB1 induced detrimental effect on sperm chromatin packing and consequently elevated DNA damage. Abnormal endogenous nicks in DNA, malfunction, and/or any damages of nucleases are two different theories for abnormal chromatin packing. As AFB1 is known as mutagen and carcinogen due to binding proteins and DNA, thus we can conclude that AFB1 impairs creating and ligating nicks in DNA which in turn blocks protamination and consequently induces internal DNA damage by preventing its repair and increasing its susceptibility to damage. This theory has been proofed with elevated DNA damage in sperms after treatment with AFB1. Accordingly, higher percentage of the sperms underwent DNA damage in low and high dose CPFX-treated animals in comparison with sperms from control group (Table 1). The notable point was that, the DNA damage was not differed significantly in low and high dose AFB1-treated animals. Thus, it comes close that AFB1 is able to induce its pathological effect in low dose. damage was not differed significantly in low and high dose AFB1-treated animals. Thus, it comes close that AFB1 is able to induce its pathological effect in low dose. We also found that the fertilization rate and embryonic development in the AFB1-treated mice were lower than those of the control group. It is well known that, there is a positive correlation between the quality of the sperms with embryonic development in both in vivo and in vitro [29,23]. The ability of the embryo to survive appears to be negatively correlated with the level of DNA fragmentation in the germ line [15] Identified evidences of the present study suggested that disturbances in the organization of the AFB1-induced sperms genome, pathologically affected their fertilizing potential. Accordingly the percentage of arrested embryos was elevated significantly (P < 0.05) after AFB1 administration (Table 2). Similar to sperm DNA damage the results for IVF confirmed that the AFB1 exerted its adverse effect in low dose. The IVF outcomes for both low and high dose treated animals were not statistically significant. It has been shown that protamine deficiency and increased histone remnants in sperms as a result of any exposure with exogenous pathogens such as AFB1 have resulted in lower fertilizing ability and embryonic development. In the case of such impairment,

<table>
<thead>
<tr>
<th>Table 1: Effects of AFL B1 on sperm count, DNA integrity and chromatin quality. Data are presented as mean ± SEM.</th>
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<tbody>
<tr>
<td><strong>Sperm Count (x106/ml)</strong></td>
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<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>65.25 ± .85 *</td>
</tr>
<tr>
<td>Motility (%)</td>
</tr>
<tr>
<td>Viability (%)</td>
</tr>
<tr>
<td>Abnormal sperm (%)</td>
</tr>
<tr>
<td>Positive Acridine Orange staining (%)</td>
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<tr>
<td>Positive Aniline Blue staining (%)</td>
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<tr>
<td>* Different letters in each column indicate significant differences (P &lt; 0.05).</td>
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</tbody>
</table>
Table 2: The number and (percentage) of oocytes, fertilized oocytes, embryos (two cells and blastocysts) in groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number of oocytes</th>
<th>Fertilization rate N(%)</th>
<th>2 Cell Embryos rate N(%)</th>
<th>Blastocytes rate N(%)</th>
<th>Arrested embryos rate N(%)</th>
<th>Type of arrested embryos N(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>286</td>
<td>84.07a</td>
<td>98.31a</td>
<td>81.68a</td>
<td>18.31a</td>
<td>1.67a, 7.09b, 9.54c</td>
</tr>
<tr>
<td>Low dose</td>
<td>351</td>
<td>62.09b</td>
<td>96.82b</td>
<td>21.53b</td>
<td>78.46b</td>
<td>32.05b, 21.12b, 25.28b</td>
</tr>
<tr>
<td>high dose</td>
<td>348</td>
<td>60.86b</td>
<td>69.82c</td>
<td>20.31c</td>
<td>79.68b</td>
<td>34.39c, 23.59c, 21.68c</td>
</tr>
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</table>

Different letters in each column indicate significant differences (P < 0.05).

Fig. 1: In vitro development of embryos at 120 h of culture. A. Control group. Differentiation to expanded and hatched blastocysts B. Control group, blastocyst in hatching stage. C. Treatment groups. Embryos arrested and type I, II and III of embryo quality.

Conclusion:

Taking collectively, the present results highly support the idea that Aflatoxin induced testicular toxicity with adverse effect on sperm quality and fertilization rate in a dose-dependent manner. Although it is hypothesized that aflatoxins have adverse effects on birth outcomes, the replacement of histone proteins by protamines will be affected severely. Therefore, this impaired replacement will disable DNA to properly decondense after entering into oocyte (which is very essential in order to perform fertilization process).

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REFERENCES


