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THE IMPACT OF AFLATOXIN B1 ON SPERM QUALITY AND SPERM DNA DAMAGE IN NMRI MICE

Tohid Mohammadi[1], Iraj Pousty[2], Hassan Gilanpour[3]

1,2,3. Department of Anatomical Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran.

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ABSTRACT

Background and objective: A number of papers have reported the deficiency of reproductive health in human, as animals, over the past years. The factors causing these deficiencies, including environmental and industrial factors, treatment, food toxins, and lifestyle, have been investigated. Among the food toxins, aflatoxins are one of the factors causing deficiency in reproductive health of men. In the current research, we aim to investigate the impact of aflatoxin B1 on sperm characteristics and chromatin quality and sperm DNA damage using aniline blue and acridine orange technique.
Methodology: In the current research, 40 adult male NMRI mice were used in four groups (each group containing 10 mice). Experimental groups received aflatoxin B1 per day with doses of 100-350 and 700 mg / kg for 35 days in the form of gavage at the same maintaining conditions. However, the control group received only the aflatoxin solvent in the same way. Spermatozoids were isolated from tail of epididymis. The number of sperm and survival rate, mobility, morphology, DNA damage, and quality of sperm chromatin were examined.
Findings: The data obtained in the current research revealed that sperm motility and survival rate and the number of them decreased significantly in groups treated with aflatoxin compared to control group. Moreover, the percentage of DNA-damaged sperms and abnormal chromatin and non-adult forms increased significantly in mice treated with aflatoxin.
Discussion: Reproductive dysfunction is one of the secondary complications of sub-lethal dose of aflatoxin, associated with disability in sexual power and reduced sperm quality caused by testicular dysfunction. The obtained findings confirm the hypothesis that aflatoxin has direct impact on sperm production and parameters and DNA and cramatine quality of sperm by inducing damage to testicle.

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Introduction

Nowadays, concern on side effects of various drugs and toxic substances on the reproductive system of the sexual cells is increasing in different communities. During the spermatogenesis process, male sexual cells pass through different evolutionary stages, in which they are invaded by a toxic substance .Production of sexual cells damaged in terms of function and quality can result in congenital anomalies, fetal death, or provide the conditions for cancer (1). Vulnerability in sperm function and natural morphology of sperm and damage in the spermatogenesis process is one of the most common causes of infertility in men. One of the influential factors in this regard is oxidative stress caused by reactive oxygen species (ROS) radicals, known as cause of spermatozoid vulnerability .Aflatoxin B1 is a very strong mycotoxin, which is produced by Aspergillus group fungi.

This toxin in high dose causes a gene mutation and cancer, and eventually death. However, in low doses received daily in diet of humans and animals, it causes a disease called as aflatoxicosis (2). Aflatoxicosis decreases the level of growth (3) and causes defect in immune system (4). It also flows towards the genital tract and influences different aspects of pregnancy,

Corresponding Author: Iraj Pousty, Department of Anatomical Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran, Email: Pousty@srbiau.ac.ir

including spermatogenesis in the male gender (5). As the impact of aflatoxin on spermatozoids has not be investigated adequately, the current research was conducted to investigate the impacts of oral exposure to aflatoxin B1 toxin on the quality and quantity of sperm and the impacts of toxin on DNA breakdown and chromatin maturity of sperm.

Methodology

To carry out this experimental study, 40 male NMRI mice (8-10 weeks old) with an average weight of 20 grams, whose fertility has been already tested through mating with female mice, were used. After two weeks of adaptation to environmental conditions, they were divided into three treatment groups and one control group, and kept in standard and same conditions, and water and food were provided freely for them. In the control group, mice received aflatoxin solvent, which were DMSO and corn oil. Three experimental groups were as follows .The first group received aflatoxin B1 (made by Sigma Company of USA) every day at amount of 100 mg / kg. The second group received the toxin at amount of 350 mg/ kg, and the third group received aflatoxin B1 at amount of 700 mg/kg. All toxins were prescribed to mice orally through gavage catheter for 35 days.
After completing the treatment period, mice were sampled. For this purpose, after anesthesia and displacement of the neck, the tail of the epididymis was isolated after isolating the surrounding connective tissues. Then, they were placed within the HTF-containing culture, which contained 4 mg/ml of bovine serum albumin (BSA) (Sigma Company of USA), reached to balance before in 5% carbon dioxide and 37 ° C conditions .The tail of epididymis was divided into different parts and remained in the culture for 20 minutes. It should be noted that sterile plates containing the sperm samples were placed inside the CO2 incubator during this period. After 30 minutes, tissue parts were removed from the culture (6). Sperm samples were extracted using the above method to evaluate the sperm motility. After diluting the sperms for each mouse in each group, 10 µl of the sperm suspension was used to evaluate the sperm motility percentage through Neubauer slide. To evaluate the number of sperms per epididymis tail, Neubauer slide was used, which 20 µl of sperm suspension was used for each mouse in each group .For this purpose, 1 to 50 dilution of the mentioned suspension was prepared, so that 980 µl of distilled water was poured into a 1 ml microtube, and then, 20 µl of sperm suspension was added. Then, 10 µl of the solution was removed and pouted on Neubauer slide, which stone slide has been already placed on it; the sperms were count in this way (7). To evaluate the survivability and determining the percentage of living sperms, Eosin Nigrosin staining was used. The procedure is based on the point that sperms become permeable against the mentioned color as a result of damage to the plasma membrane. Thus, those sperms that their heads were colored were known as dead sperm, and the results were stated as percentage. For morphological evaluation accurately and cytoplasmic residues detection, indicating the morphologic immaturity of sperms while passing through the epididymis, Eosin Nigrosin staining was used. Those sperms contained cytoplasmic residuals were considered as abnormal sperms (morphologically). Abnormal sperms were evaluated in form of 3 groups of sperms, which showed dysfunction in the head, neck and tail, or simultaneous dysfunction in the head and tail or contained cytoplasmic residuals (8). On the other hand, aniline blue staining was used to evaluate the sperm maturity. This analysis is based on the point that protamine is replaced by a histone in the nucleus chromatin in the spermiogenesis stage. This replacement is very important in the density and stability of sperm. Immature sperms turn to dark blue due to high histone in this staining. To evaluate any breakdown in two sperm DNA sequences, acridine orange staining was used. This type of staining is used to detect those sperms whose DNA has been broken down, so that healthy sperms are seen in green color and in sperms whose DNA has been broken down after staining, DNA is seen from yellow to fluorescent red .As a result, those sperms that showed this state were considered as DNA-damaged sperms and the findings of this study were stated as percentage (9). All data obtained in this research and comparing the sperm characteristics among experimental groups were analyzed using SPSS 19 software and ANOVA method and post-hoc Tukey test with a significance level of less than 0.05, and findings were stated as table shows.

Findings

Comparing the findings obtained from sperm density (number) per epidermis in Table 1 revealed that prescribing the aflatoxin B1 led to significant reduction in number of sperms compared to control group, and this significant reduction was seen in groups treated with dose of 350 and 100 ($P<0.05$). The percentage of living sperms specified in Eosin and Nigrosin staining based on Figure 1 was obtained in experimental groups (Table 1). In the groups treated with aflatoxin B1, significant reduction in the number of living sperms was seen, compared to control group ($P <0.05$). However, among the groups treated with toxin, significant reduction was not seen. Based on Table 1, the percentage of mobile sperms was investigated in four experimental groups, which it was reduced significantly in groups treated with aflatoxin, compared to control group, while this reduction was not significant among the groups treated aflatoxin ($P>0.05$). Sperms with abnormal morphology were counted in in experimental groups in Table 1 and they were stated as percentage, and significant difference was found between treatment groups and control group ($P<0.05$). Based on the acridine orange evaluation method, the number of damaged chromatin-containing sperms damaged was calculated using fluorescence microscope in six groups and their percentage was obtained. In Table 1, it has been stated that sperms with natural green nucleus and yellow to red nucleus are considered as DNA-damaged sperms, depending on level of damage to chromatin. The difference between the mean percentage of DNE-damaged sperms in groups treated with aflatoxin B1 and the control group was significant, while this difference was not significant between e different doses of toxin ($p<0.05$). The number of sperms with immature chromatin was calculated in 4 experimental groups in

Table 1 and their percentage was significant. The significant difference between the control group and the groups treated with aflatoxin B1 was evident.

Discussion

Aflatoxin B1 is a very strong mycotoxin, absorbed effectively and without spending energy in the duodenum (11, 10). Then, it is transformed to its active metabolite, AFB1-8-9 epoxid, by P 450 cytochrome enzymes available in cell (12, 13, and 14). This metabolite is able to peroxidase the lipids (LPO) and consequently create oxidative stress in the cell (15, 16, 17, and 18). In addition, epoxide created from aflatoxin is directly bound to DNA and RNA or proteins available in the cell, leading to DNA breakdown and lack of its transcription and change in structure of proteins (19, 20, and 21). According to the findings of current research and findings of several studies conducted on dysfunction in antioxidant system of cell caused by aflatoxin (22), it could be concluded that reduced number of sperms per epididymis in male mice treated with aflatoxin B1 can be due to depletion of semen-producing tubes of testicle from germ cells as a result of oxidative stress (23), which histologic and morphometric investigation of current research confirm it. Significant reduction in mobility can be due to toxic effects of oxidative stress on the energy production cycle in mitochondria and the disruption in midline part of sperm (24 and 25), and the toxin interference with sperm flagella, which causes change in structure of the flagella and disruption in movement of sperms (26). These results are in line with results of various studies. In addition, increase in sperms with abnormal morphology having cytoplasmic droplets is also due to spermicogenesis impairment as a result of changes in proteins present in the spermatogonal bridges (27) and inability in phagocytosing cytoplasmic droplets during permicogenesis process by the sertoli cells due to reduced number of these cells, which is a factor in increasing the spermatozoids with abnormal morphology. Histological studies confirm the dysfunction. Significant reduction in survivability and increase in DNA breakdown and sperm immature chromatin can be discussed by direct binding of toxin to protamine and other proteins involved in protamination, which causes chromatin maturity and increased sperm survivability. In addition, aflatoxin can disrupts the process of correct protamination of sperms' DNA and increases the probability of harmful external factors with DNA and its breakdown by directly binding to sperm DNA and transcriptional proteins on one hand, and by disrupting the function of sertoli cells on othe other hand (28). It finally leads toan increase in sperms whose DNA has been broken and immature chromatin, which this research indicates this change by aflatoxin.

Conclusion

Based on findings of current research, it can be concluded that aflatoxin causes oxidative stress by disturbing the oxidation-resuscitation balance, which this toxic biochemical stress causes toxicity of male reproductive system by causing disruption in stress to androgens and energy metabolism and apoptosis stimulation and inflammatory reactions and their development. This research for the first time shows that breakdown in sperm DNA and preventing sperm chromatin protamination cause disruption in permicogenesis and creation of sperms with lower survivability and movement, leading to increased number of dead sperms. This defects the male reproductive system, which leads to infertility.

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Paramete r Group	Number of sperms (ml/10^6)	Sperm survivabi lity percenta) ge of living (sperms	Percentage of mobile sperms (%motility)	Abnormal Sperm Morphology) (%	Broken strings of DNA %	immature chro-matin%
Control	65.25 ± .85	68.25 ±1.65	62.25 ±1.54	30.25 ± 2.46	.75 ± .47	1 ± .4

therapeutic 100	a 8 ±1.47	a 40.75 ± 2.86	a 37.5 ±3.79	a 73 ± 1.68	a 21.75 ± .85	a 15 ± 1.29
therapeutic 350	ab 17± 1.47	a 39.75 ± 2.32	a 35 ± 1.22	a 71.5±1.84	a 20±1.47	a 12.25±1.31
therapeutic 700	a 14.75±3.3	a 33.75 ± 2.28	a 28.25 ±1.54	a 62.25 ± 8.36	a 31 ± 5.4	a .5±.28

(a) represents significant difference in comparing the control group with other groups ($p<0.05$)
(ab) represents significant difference in comparing the therapeutic group 350 with other groups ($P<0.05$).

Figure 1- Sperm staining (Eosin Nigrosin, acridine orange, aniline blue)

Image A shows the living sperm with bright head on the right side and dead sperm with orange head on the left side in Eosin Nigrosin staining (Zoom × 1000). Image B shows the adult sperm with bright blue head and nucleus on the right side and immature sperm with a dark blue head and nucleus on the left side of the aniline blue staining (Zoom × 1000). Figure C shows sperm with healthy green DNA on the left side and DNA-damaged sperm with yellow nucleus on the right side of acridine orange staining.