Original paper

DURATION-DEPENDENT HISTOPATHOLOGICAL AND HISTOMETRIC CHANGES IN THE TESTIS OF AFLATOXIN B1-TREATED MICE

Faridha A, Faisal K and Akbarsha MA

Department of Animal Science, Bharathidasan University, Tiruchirappalli - 620 024, India.

SUMMARY

In the background of the decreasing trend of male reproductive health in men and animals, the present study was aimed at finding gravimetric, histopathologoical and histometric changes in the testis of Swiss mouse in response to treatment of aflatoxin B_1 (AFB_1) in a chronic toxicity testing over different periods of time and at finding recovery, if any, after cessation of the treatment. AFB_1 suspended in corn oil and ethanol, was administered through intraperitoneal route to 90 day old Swiss mouse at a daily dose of 50mg/kg body weight for 7, 15, 35, 45 days. In the recovery group the mice were kept, after cessation of the treatment, under observation for 35 or 70 days. The testicles and seminal vesicles of the animals were subjected to histopathological analysis adopting paraffin/resin embedding and light microscopy. Computer assisted histometric analysis of several parameters was also made. In general there was little impact of the treatment in the animals treated for 7 days. In the animals treated for 15, 35 and 45 days, there was duration-dependent regression of the testis and seminal vesicles. Histopathological changes were observed in both the spermatogenic and androgenic compartments of the testis. Fragmentation of chromatin of pachytene spermatocytes, generation of uni- and multinucleate gaint cells, and premature loss of spermatids and spermatocytes were observed. Whereas the seminiferous tubules regressed, the Leydig cells underwent hypertrophy and distortion of shape of the nucleus. Generation of multiple micronucleate gaint cells and extensive loss of germ cells from the seminiferous epithelium were observed. Fertility of the mice was severely hampered. The various changes are discussed in relation to the possible effects of AFB_1 on the microtubules of the spindle fibers and the DNA of chromatin. Since spermatogonia and Sertoli cells are not vulnerable targets to AF toxicity, the treated animals recovered over a period of time.

Key words: Aflatoxin B1, male reproductive toxicity, testis, seminal vesicles

INTRODUCTION

In the recent times, there has been a great concern about the increasing trend of male infertility in men (1). There is also equal concern about the declining semen quality and decrease in sperm counts in otherwise normal men (2, 3), domesticated (4) and wild animals (5). The causative factors are essentially environmental, occupational and/or dietary. Dietary toxins such as mycotoxins are among the major contributors to deterioration of male reproductive health (6). These toxic compounds, aflatoxins (AFs), ochratoxins, zearelenone, fumonisins, tremorgenic toxins, etc., are produced by certain strains of fungi which grow on moist cereals, nuts, seeds, herbs, medicinal plants, dried vegetables and food preparations (6-10). AFs are mycotoxins produced by *Aspergillus flavus* and *A. parasiticus*. They occur in a wide range of food and feed commodities (10, 11). In countries with high dietary AF intake, a daily exposure of 1.7 µg/kg body weight has been estimated but it could exceed 1 mg/ day at certain times of the year (7). Epidemiological studies in men and experimental studies in laboratory animals have shown AFs at acute or chronic concentrations to be among the most potent mutagens and hepatocarcinogens (12-14). At smaller doses during subchronic toxicity, as would usually happen during dietary exposure, AFs produce a milder effect known as aflatoxicosis, which is reflected as feed refusal, decreased feed efficiency, stunted growth, decreased milk production and impaired reproductive efficiency (15, 16). Four major aflatoxins *viz.*, B_1, B_2, G_1, and G_2, are direct contaminants of cereals, grains and fruits and thereby the feeds produced out of them. AFB_1 is the most potent of AFs. In the rat AFB_1 is rapidly absorbed *via* the small intestinal tract, following the first-order kinetics (17). Oral AFB_1 is metabolized in the intestinal tissue and liver by the various microsomal cytochrome P450s (primarily cytochrome P450 $3A_4$ and IA_2), resulting in AFB_1-8, 9-epoxide, which binds to DNA forming AFB_1–guanine adducts (18-20).

Oral feeding or intraperitoneal administration of AFB_1 to experimental animals produced adverse effects

Correspondence to be addressed to: Prof. M.A. Akbarsha E.mail: akbarbdu@yahoo.com

on aspects of male reproduction such as delayed testicular development, morphological, regressive and degenerative changes in the testis, hypertrophy of Leydig cells and drop in testosterone, decrease in the percentage of live sperm and increase of sperm abnormalities, *etc*. (21-29). Formation of testicular AFB_1-DNA adducts in rats has been shown (30). AFs have been detected in the human semen (31) and boar sperm (32). In the latter case, the highest AF residues were recorded in March to May and were related with AF concentration in the feedration.

The various experimental studies, which indicate deterioration of male reproductive health, do not provide insight into the impact of AFB_1 on the spermatogenic and androgenic compartments of the testis when the toxin is administered over different periods of time. Further, the various studies indicate only qualitative histological changes. The present study reports the gravimetric, histological and histometric changes in both the spermatogenic and androgenic compartments of the testis of mice administered AFB_1 over different periods of time in a chronic toxicity testing. Fertility testing and recovery study were also conducted.

MATERIALS AND METHODS

The methodology of chronic male reproductive toxicity testing was adopted from Linder *et al*. (33). Swiss strain male albino mice (90 day old), weighing approximately 30g, were used in the study. Female mice used in the fertility tests also belonged to the same strain and age. Mice were developed from a stock procured from International Institute of Biotechnology and Toxicology (IIBAT), Padappai, India. Mice were housed in polypropylene cages with metal grill tops, and fed with standard pellet feed (Gold Mohur Laboratory Animal Feeds, Lipton India Ltd., Bangalore) and water *ad libitum*. The toxin, aflatoxin B_1 (AFB_1), was obtained from SIGMA Chemical Co (St. Louis, MO, USA).

The AFB_1 was quantitatively prepared in corn oil and ethanol (95:5) according to Egbunike *et al*. (34). AFB_1 at a dose of 50µg/kg/day was administered to the experimental animals through *intra-peritoneal* route for 7, 15, 35 and 45 days. Each experimental group consisted of 45 animals. Control mice were maintained parallel to each experimental group, and consisted of 45 animals each. At the end of each experimental period, 5 animals from each of the experimental and control groups were weighed and killed through cervical dislocation under mild sodium pentabarbital anasthesia. The animals were dissected and the testes and seminal vesicles were used for gravimetric as well as histological analysis adopting paraffin-embedding and PAS and hematoxylin staining. In another 5 mice from each of the experimental and control groups the reproductive system was perfused with Karnovsky's (35) fluid (glutaraldehyde 1%, paraformaldehyde 1%, in phosphate buffer) and the animals were sacrificed under anasthesia. The right testis was removed and thin slices of the tissue were fixed in 2.5% glutaraldehyde in cacodylate buffer and post-fixed in 1% osmium tetroxide for obtaining semithin sections (1µm thickness). The sections were stained in toluidine blue O (TBO). 5 mice in each of the experimental and control groups were tested for fertility. Fertility test was carried out after withdrawal of the toxin treatment or after the period of recovery, by allowing a male mouse to mate with two female mice at estrus. Mounting, if any, was observed overnight. The following morning, vaginal smears were examined in a microscope for spermatozoa. Subsequently, the female mice were separated and allowed to go to term and the litter size was recorded. Thus, male fertility was evaluated indirectly by registering female fertility. The remaining 30 animals in each of the experimental and control groups were left for recovery. For these mice, treatment of AFB_1 was discontinued after the defined period. 15 animals were sacrificed and subjected to analysis as above after 35 days of recovery, the duration of one spermatogenic cycle (36) and the remaining 15 animals sacrificed and subjected to analysis after 70 days. Fertility testing of animals in the recovery groups was the same as for AFB_1-treatment.

In the case of gravimetric analysis the paired testicles and seminal vesicles from each animal were weighed separately. Data for each organ from each group were used to calculate the respective means and the standard deviations. Light microscopic observation was made in a Carl Zeiss Axioskop 2 plus (Carl Zeiss, Gena, Germany) research microscope connected to a computer through Sony DxC-151a/151HP 2/3 CCD camera (Sony, Tokyo, Japan). Photomicrographs were obtained and processed using Axiovision software. Histometry/cytometry was conducted using the same software. The software is a modular image-processing and analysis system for use in modern microscopy. Sections stained in PAS and hematoxylin or TBO were viewed in the microscope and fields with sections of seminiferous tubules (STs) in circular transverse section were selected at x100 magnification. Using the software,

the boundary of the each of the ST was demarcated, and the total area of the STs in the field was deducted from the area of the field. Thus, the area of the STs and the area of the interstitium were duduced. This was done for five different fields from the tissue of each mouse, thus making 25 measurements for each sub-group. The perimeter and the diameter of five different circular transverse sections of STs from each mouse were also measured at x400 magnification making 25 measurements of each parameter for five animals in each sub-group. At x400 magnification the epithelium of STs was analyzed for number of uninucleate giant cells (UNGCs) and multinucleate giant cells (MNGCs). This was done in STs in semithin sections and a total of 25 tubules were assessed in each group. The data were used to find the total number of cells in 10^4 mm^2 area per tubule, and among them the number of UNGCs and MNGCs.

The number of Leydig cells per 10^3 mm^3 of the interstitial area was counted in five fields from each animal, at x1000 magnification, making it 25 counts for each sub-group. The perimeter of Leydig cells and the diameter of the nuclei of Leydig cells were also measured for 100 Leydig cells in the testis of each group. The data were subjected to analysis using the Carl Zeiss software as above. The mean and the standard deviation were calculated for data in respect of each parameter in each group. Data for each parameter for the respective control and treatment groups were used for application of Students' *'t'* test to find the level of significance of the difference.

RESULTS

Decrease in the weight of the testicles and seminal vesicles

Gravimetric analysis of testicles and seminal vesicles revealed duration-dependent decrease in their respective weights (Table 1). As calculated from the mean values the decrease in weight of these organs was not significant in the mice treated AFB_1 for 7 days whereas in the mice treated for 15 days, the weight of testicles decreased significantly to 73%, in those treated for 35 days to 68% and in those treated for 45 days to 51%. Weight of the seminal vesicles also decreased to 76% in mice treated for 15 days, to 69% in those treated for 35 days and to 59% in those treated for 45 days.

Table 1. Weight of the paired testicles and seminal vesicles of control and AFB1 - treated mice (Mean ± SD)

Duration of Treatment	Weight of testicles (mg)		Weight of the seminal vesicles (mg)	
	Control	Experimental	Control	Experimental
7 days	213±16	203±13 (95)	85±8	79±7 (93)
15 days	217±14	158±12*(73)	84±9	64±6*(76)
35 days	219±21	148±08*(68)	86±8	59±6*(69)
45 days	220±19	112±06*(51)	88±7	52±6*(59)

* $p<0.01$

Number in parenthesis, percentage of the control value

Recovery group I - 35 days

Duration of Treatment	Weight of testicles (mg)		Weight of the seminal vesicles (mg)	
	Control	Experimental	Control	Experimental
7 days	218±16	212±12	84±6	82±6
15 days	221±18	189±14	86±7	72±5
35 days	222±15	172±16	85±4	69±4
45 days	221±17	163±17	87±6	68±6

Recovery group II - 70 days

Duration of Treatment	Weight of testicles (mg)		Weight of the seminal vesicles (mg)	
	Control	Experimental	Control	Experimental
7 days	221±17	218±16	86±7	85±5
15 days	222±18	218±17	82±6	81±6
35 days	221±16	220±18	85±8	78±5
45 days	223±19	221±19	85±6	77±6

Gross histological and histometric changes in the testis

In the control mice the STs were compactly arranged, possessing a spacious lumen, containing sperm or empty, and the seminiferous epithelium (SE) was highly intact. The architecture of the STs differed between different tubules in relation to the stages in the cycle of the SE. The interstitium was confined to the angular spaces between the tubules and contained densely packed Leydig cells (Fig. 1A). Histopathological changes were observed in the testis of mice belonging to all the four experimental groups and the impact clearly reflected dependence on the duration of treatment. In general the trends were decrease in size of the STs, appearance of small to large vacuoles in the epithelium, decrease or absence of elongating spermatids,

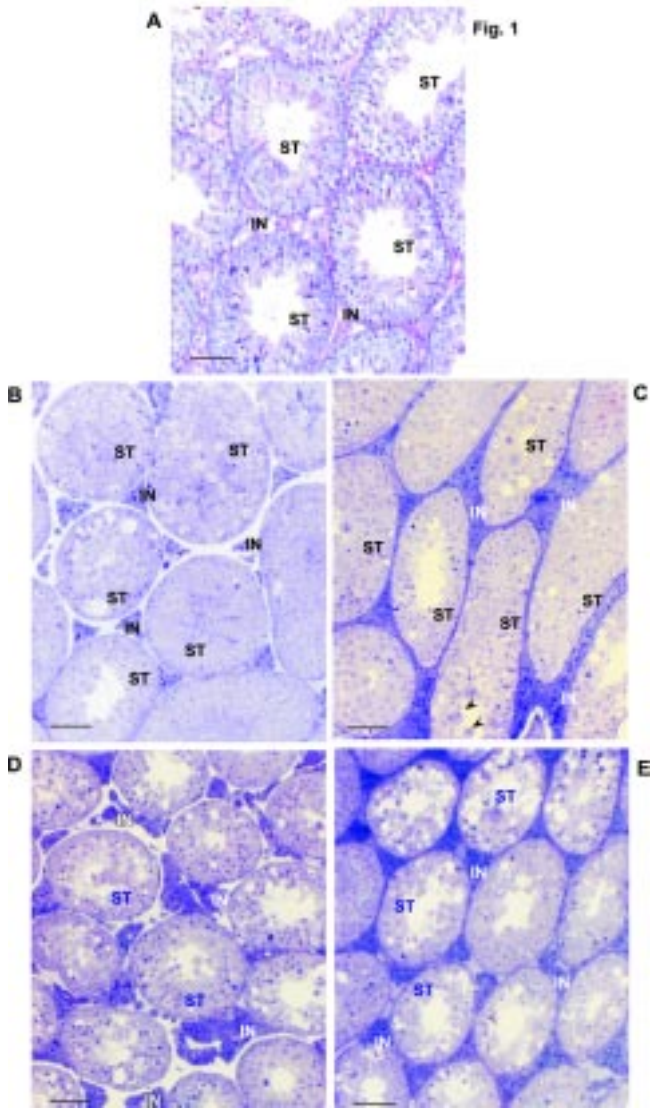

Fig. 1A-E. Section of the testis of control and treated mice. **A**: Control mouse; paraffin section, PAS & H staining. **B-E**: AFB_1-treated mice; semithin sections, TBO staining. Note depletion of lumen. B: 7 day treatment. **C**: 15 day treatment. Note occurrence of giant cells (arrowheads). Interstitium is hypertrophied. **D**: 35 day treatment. Note hypertrophy of interstitium. **E**: 45 day treatment. Note hypertrophy of interstitium. ST, seminiferous tubules; IN, interstitium. Scale bar 35µm.

appearance of uni- and multinucleate giant cells, occurrence of double the normal-sized spermatids with several small nuclei, partial to complete obliteration of lumen or increase of lumen and/or increase in the interstitial area and the density of interstitial cells (Fig. 1B-E). Histometric analysis revealed duration-dependent decrease in the percent area of STs and increase of interstitial area. As calculated from the mean values the difference in respect of ST area, when compared to the corresponding control, was not significant in mice treated for 7 days; in those

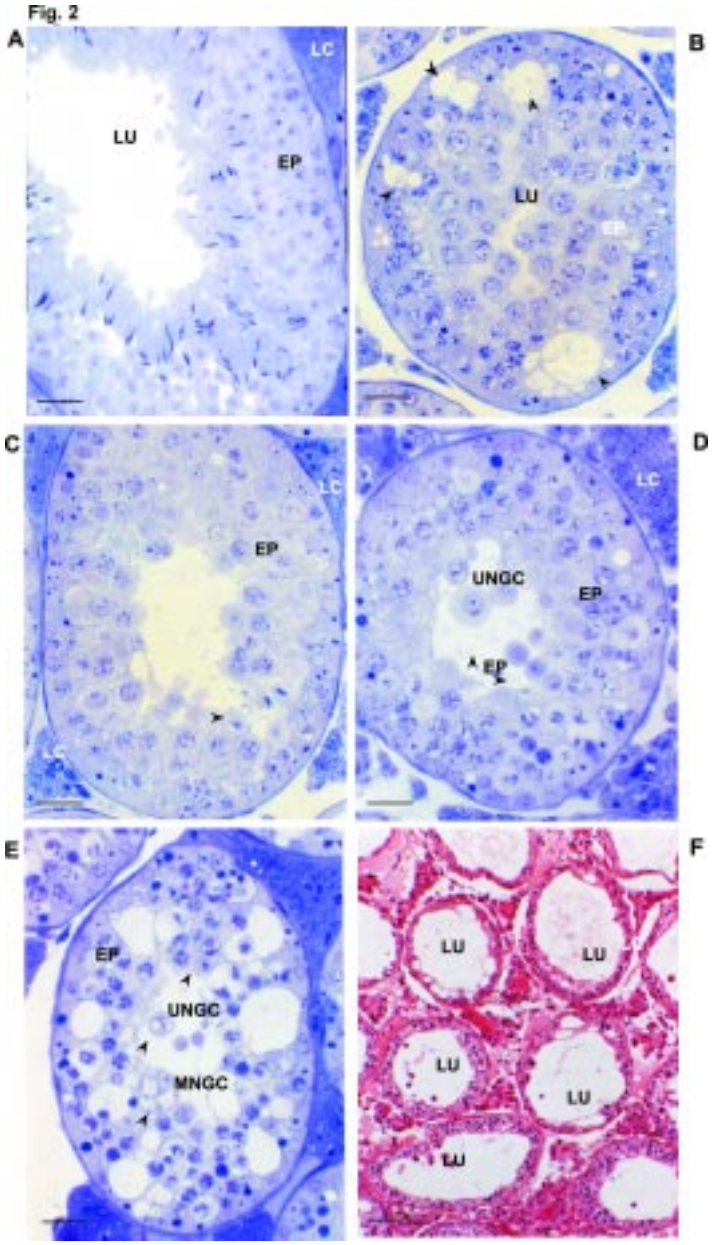

Fig. 2A-F. Seminiferous tubules of control and treated mice. A-E, semithin sections, TBO staining; F, paraffin section, PAS & H staining. **A:** Control. **B:** Treated (7days). Note loss of intercalary germ cells (arrow-heads). **C:** Treated (15 days). Note loss of germ cells (arrowheads) from the epithelium. The Leydig cells are densely granulated and/or vacuolated. **D:** Same as 3C, a different tubule. Note absence of elongating spermatids and presence of uninucleate giant cells towards the lumen (arrowhead). **E:** Treated (45days). The trend is same as in D, but more intensified. **F:** Treated (45 days). Note decreased epithelial height due to loss of germ cells and an increase of the lumen with immature germ cells in it. EP, epithelium; LC, Leydig cells; LU, lumen; MNGC, multinucleate giant cells; UNGC, uninucleate giant cells. Scale bar 18µm.

treated for 15 days, the area of STs decreased to 77%, in those treated for 35 days to 49% and in those treated for 45 days to 33% (Table 2).

Table 2. Percent area in transection occupied by the seminiferous tubules and the inter sritium in the testis of mice treated with AFB1. Each value is mean ± SD of 25 determinations atx100 magnifications from sections obtained from the right restis of 5 animals.

Treatment group

Duration of Treatment	Seminiferous tribule area		Interstitial area	
	Control	AFB1-treated	Control	AFB1-treated
7 days	86.88±1.93	82.12±1.63*	13.12±1.93	17.88±1.64
15 days	86.94±2.08	76.88±3.20*	13.01±2.04	23.12±2.36*
35 days	86.10±2.04	48.88±6.22*	13.90±2.04	51.12±6.22*
45 days	85.34±2.67	33.38±5.96*	14.66±2.68	66.62±5.96*

*p < 0.001

Recovery group II - 35 days

Duration of Treatment	Seminiferous tribule area		Interstitial area	
	Control	AFB1-treated	Control	AFB1-treated
7 days	88.23±2.15	82.12±1.63	12.15±1.25	22.43±6.72
15 days	87.75±1.32	79.46±2.32	12.32±1.75	28.16±7.09
35 days	87.62±2.55	65.72±2.26	13.05±1.92	32.24±6.24
45 days	87.98±2.62	56.59±1.75	12.75±1.78	45.72±6.35

Recovery group II -70 days

Duration of Treatment	Seminiferous tribule area		Interstitial area	
	Control	AFB1-treated	Control	AFB1-treated
7 days	86.24±2.05	84.69±1.75	13.17±1.89	14.65±1.72
15 days	86.92±2.21	84.23±2.65	13.21±2.65	17.11±1.85
35 days	87.02±1.92	80.72±2.32	13.56±2.42	24.32±1.82
45 days	86.56±1.89	75.02±2.05	13.95±2.35	28.75±2.03

The trend in respect of the interstitial area was opposite to this. Critical observation of the individual STs revealed decrease to almost total absence of elongating spermatids. Spermiated spermatozoa were invariably absent in the lumen (Fig. 2A-F). The height of the SE either increased (Fig. 2A-E) or decreased (Fig. 2F) and, correspondingly, the lumen was either almost obliterated or increased. A duration-dependent appearance of uni- (Fig. 2D) and multinucleate (Fig. 2E) giant cells was noticed. The SE of the mice treated for 15, 35 and 45 days possessed small to large vacuoles or empty spaces increasing in magnitude in relation to the duration of treatment. The vacuoles were empty or contained cell debris. Cell shrinkage and necrosis or pycnosis of the nuclei were also noticed. A few of the giant cells in the epithelium as well as in the lumen possessed vacuolated cytoplasm and pycnotic nuclei or nuclei with marginalized chromatin. Data on the perimeter and diameter of the STs are presented in Table 3. Both the parameters decreased. The SE of the control mice did not indicate any degeneration, whereas in the AFB_1-treated mice the tubules with indication of epithelial degeneration increased in the order of the duration of treatment, and in the mice treated for 45 days no tubule was spared (Figs. 1D, E, 2B, E,).

Table 3. Perimeter and diameter of the seminiferous tubules of mice treated AFB1. Each value is mean ± SD of 25 measurements made at x400 with sections from the right testis of 5 animals

Treatment group

Duration of treatment	Perimeter (mm)		Diameter (mm)	
	Control	AFB1-treated	Control	AFB1-treated
7 days	444.69±10.64	389.92±16.34*	165.65±3.70	120.56±4.71*
15 days	453.00±8.12	332.11±15.09*	164.76±3.19	103.98±8.45*
35 days	462.39±5.34	309.10±19.92*	163.84±2.09	94.01±3.25*
45 days	464.03±2.91	232.53±11.62*	164.56±2.32	81.44±5.71*

* p<0.001

Recovery group I -35 days

Duration of treatment	Perimeter (mm)		Diameter (mm)	
	Control	AFB1-treated	Control	AFB1-treated
7 days	444.69±8.72	412.73±16.34	164.96±2.86	145.24±4.21
15 days	462.14±9.64	364.72±12.36	164.14±2.36	136.72±6.42
35 days	364.24±4.68	349.52±12.73	165.12±2.39	129.92±4.16
45 days	465.86±4.32	303.16±9.59	164.87±2.86	115.33±4.18

Recovery group II-70 days

Duration of treatment	Perimeter (mm)		Diameter (mm)	
	Control	AFB1-treated	Control	AFB1-treated
7 days	451.36±8.23	438.17±13.16	163.74±4.21	156.26±3.14
15 days	456.17±12.45	438.76±8.92	163.17±2.85	152.17±6.35
35 days	464.68±8.93	448.86±12.83	165.19±3.28	149.86±4.82
45 days	468.14±6.31	436.73±9.76	166.45±3.05	139.32±4.48

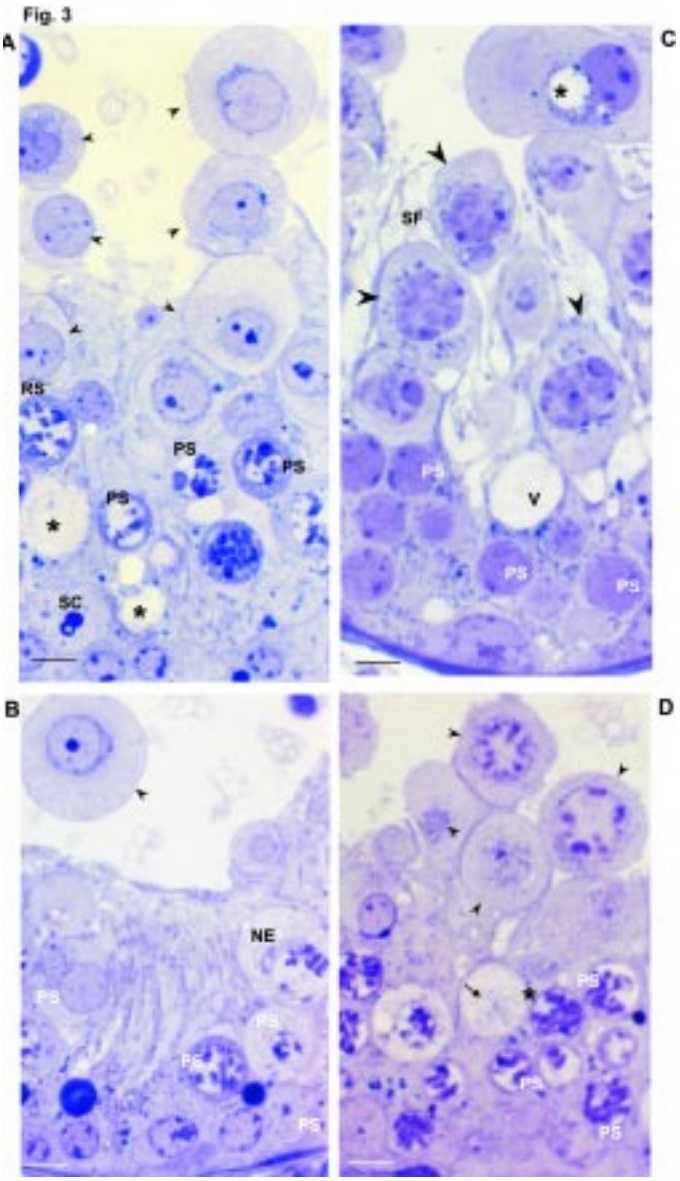

Fig. 3A-D. Seminiferous epithelium of treated mice. Semithin sections, TBO staining. **A:** Shows uninucleate giant cells (arrowheads) which are spermatids in the epithelium, damage to chromatin of pachytene spermatocytes, and loss of intercalary germ cells (asterisks). **B:** The uninucleate giant spermatid (arrowhead) is seen in the lumen of the seminiferous tubule. Necrosis of pachytene spermatocytes is also evident (asterisks). **C:** The UNGCs (arrowheads) are pachytene spermatocytes. Note doubling of size of the nucleus, compared to those which underlie them. The giant cells are in the process of being released and one of them is vacuolated (asterisk). **D:** The giant cells (arrowheads) are in the process of being released into the lumen. In the area marked with asterisks, germ cells are totally lost. NE, necrosis; PS, pachytene spermatocytes; SC, Sertoli cell; SF, Sertoli cell fibrosis. Scale bar, 4µm.

Fig.4

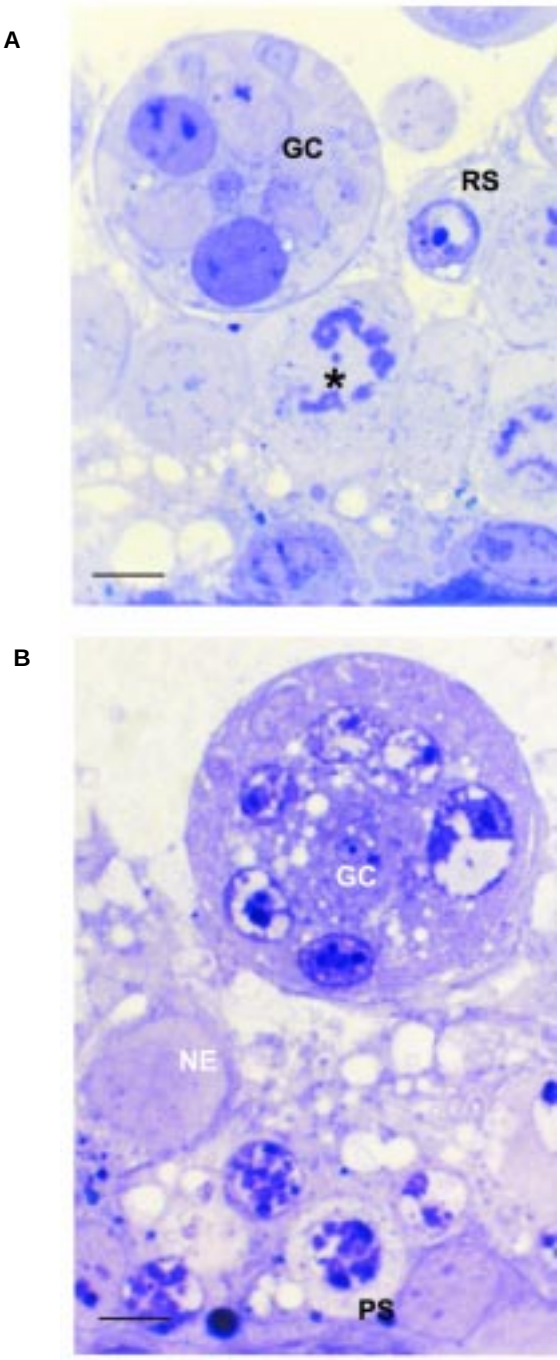

Fig. 4A, B. Seminiferous epithelium of treated mice showing multinucleate giant cells (GC). Semithin, TBO. **A:** The MNGC, with intact nuclei, lies towards the lumen. Note spermatocytes arrested in M2 (asterisk). A round spermatid reflects apoptotic morphology. Note abnormality in the entire adluminal compartment. The basal compartment is intact. **B:** The nuclei of the multinucleate GC reflect apoptotic morphology and the cytoplasm is also vacuolated. Necrosis of round spermatids and chromatin damage in pachytene spermatocytes are also evident. NE, necrosis; PS, pachytene spermatocyte; RS, round spermatid. Scale bar 4µm

Critical observation of the STs of AFB_1-treated mice, particularly those in the 35 and 45 day treatment groups, revealed occurrence of pachytene spermatocytes or spermatids of size double that of the respective normal cells (Fig. 2D, E). Such cells are designated as UNGCs. They were present in the epithelium along the luminal profile (Fig. 3A), some projecting into the lumen but still adherent to the Sertoli cells (Fig. 3B) or lying loose in the lumen (Fig.3C). In several cases the UNGC possessed highly vacuolated cytoplasm, and the nucleus was altered in morphology (Fig. 3D).

Another observation made in several of the STs of the AFB_1-treated mice belonging to 15, 35 and 45 day treatment groups was occurrence of MNGCs (diameter, 40-52 mm dia) (Fig. 2E). Such cells possessed two to 16 nuclei. The nuclei were either intact (Fig. 4A) or had marginalized chromatin (Fig. 4B). The cytoplasm indicated little (Fig. 4A) to extensive (Fig. 4B) vacuolation. One of the observations was appearance of large cells (diameter 20-30 mm) containing several micronuclei (Fig. 5A). Such cells are designated as multiple micronucleate giant cells (MMGC). They were present in the epithelium as well as the lumen; when present in the epithelium, they were separated from the Sertoli cells to a great extent, indicating that they were being released into the lumen and would result in the appearance of vacuoles in the epithelium. The micronuclei had the appearance of dot-like dense chromatoid bodies and the cytoplasm formed into two distinct zones, a thin peripheral zone and a large central zone containing the micronuclei. In a few tubules UNGC, MNGC and MMGC coexisted (Fig. 5B).

Data on the counts of cells per unit area, and among them those that were UNGC, MNGC and MMGC are presented in Table 4. The data reveal that in the mice treated AFB_1 for 7 days UNGC, MNGC or MMGC was not generated, whereas in those treated for 15, 35 and 45 days all the three versions of giant cells were generated. Loss of germ cells in a few tubules was so acute that hardly any germ cell was present in the adluminal compartment, with the epithelium manifesting small to large vacuoles (Figs. 2E, F, 6A). In some of the tubules the Sertoli cells themselves, from above the level of the ectoplasmic specialization, *i.e.*, the tight junction of blood-testis barrier, had broken away and such broken portions were carrying with them the pachytene spermatocytes, rendering the epithelium comparable to Sertoli cell-only syndrome, though careful observation revealed the presence of spermatogonia (Fig. 6A, B). The immature germ cells thus lost from the STs could be traced to the rete testis (Fig. 6C). The lumen of the epididymal duct, particularly in the mice treated AFB_1 for 35 and 45 days, contained such immature germ cells instead of sperm (Fig. 6D). Several of the immature germ cells in the lumen of the

Table 4. Number of germ cells in 10^4 mm^2 area of the STs, number of those which were uninucleate giant cells and number which were multinucleate giant cells. Data are Mean ± SD of values from 25 STs, each in semithin sections at x400, from the right testis of 5 animals in each duration of treatment (Data from control animals not included in view of absence of giant cells).

Treatment group

Duration of treatment	Cells /10^4 mm^2	Uninuclear Giant Cells		Multinucleare Giant Cells	
		Total	Percent	Total	Percent
7 days	191.81± 29.08	106.32± 11.02	56.00± 9.57	Nil	Nil
15 days	148.63± 6.31	83.41± 5.08	55.61± 3.05	10.21± 4.15	6.81± 2.86
35 days	128.36± 5.43	75.42± 10.14	58.62± 7.06	8.44± 4.56	6.22± 3.63
45 days	106.19± 13.85	57.81± 4.77	61.61± 4.56	8.21± 2.86	8.61± 2.88

Recovery group I - 35 days

Duration of treatment	Cells /10^4 mm^2	Uninuclear Giant Cells		Multinucleare Giant Cells	
		Total	Percent	Total	Percent
7 days	193.24± 24.02	52.42± 7.82	27.12± 6.52	Nil	Nil
15 days	180.69± 17.45	40.15± 6.55	22.22± 3.46	5.32± 3.75	2.75± 1.98
35 days	168.36± 15.25	33.49± 7.92	19.89± 5.93	4.56± 4.04	2.70± 2.01
45 days	150.42± 12.75	25.72± 9.01	17.09± 4.35	4.01± 3.98	2.66± 2.21

Recovery group II-70 days

Duration of treatment	Cells /10^4 mm^2	Uninuclear Giant Cells		Multinucleare Giant Cells	
		Total	Percent	Total	Percent
7 days	196.37± 8.92	34.21± 6.42	17.42± 5.35	Nil	Nil
15 days	190.43± 7.62	28.23± 6.42	14.82± 4.73	3.46± 0.82	1.76± 1.39
35 days	186.54± 9.73	26.14± 9.86	14.01± 4.22	4.91± 1.02	2.63± 1.84
45 days	171.14± 10.62	20.68± 5.37	12.08± 3.65	3.14± 0.81	1.83± 1.28

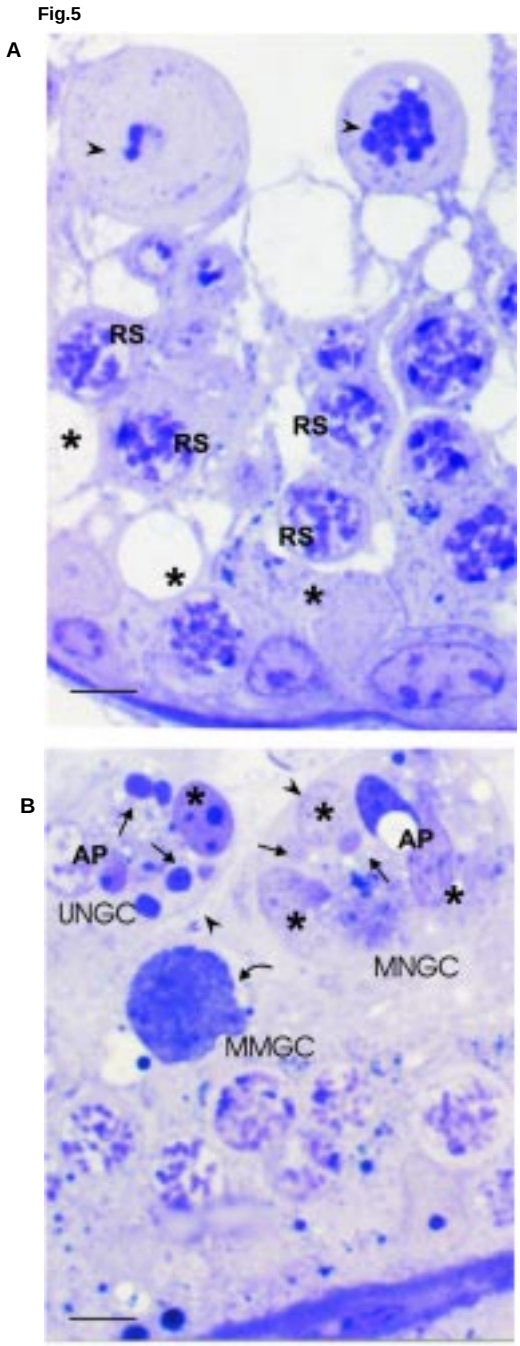

Fig. 5A: In this seminiferous tubule, chromatin of round spermatids (RS) is damaged and some RS are missing (asterisks). Two cells arrested in M2 with the chromosome pairs constituting the micronuclei are shown (arrowheads). **B:** The seminiferous epithelium in this tubule has two giant cells closer to the lumen (arrowheads), both possessing normal-sized nuclei (asterisks) and micronuclei (arrows). In each giant cell, there is one normal-sized nucleus in apoptosis (AP). An UNGC (curved arrow) is also present. Scale bar, 4µm.

epididymis invariably possessed marginalized chromatin. MMGCs were also present in the lumen of the epididymis in admixture with UNGCs and MNGCs, but a major difference between the nuclei of these two categories of

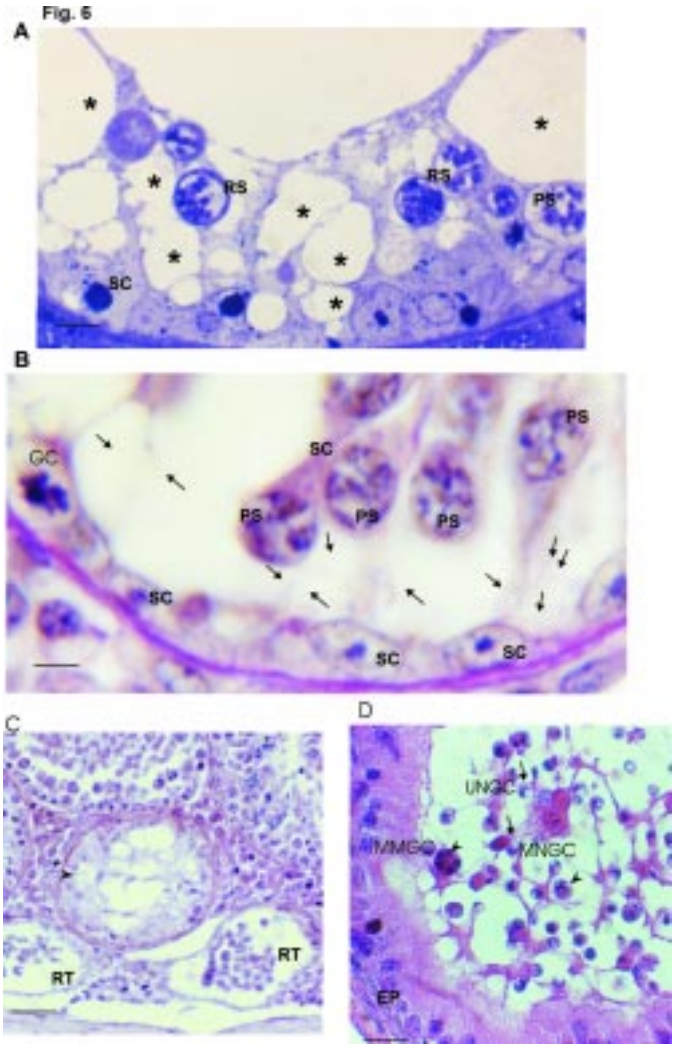

Fig. 6A-D: Seminiferous tubules (A-C) and epididymal duct (D) of treated mice. A: Damage to the chromatin of pachytene spermatocytes and round spermatids. Extensive loss of germ cells has resulted in thorough vacuolation of epithelium (asterisks). Basal portion of the Sertoli cell and the germ cells of the basal compartment are intact. Semithin. B: Pachytene spermatocytes including portion of Sertoli cell are being lost (arrows). But the body of the Sertoli cell and the few basal compartment germ cells are intact. Paraffin. C: Germ cell loss is evident (arrowhead), and such prematurely released germ cells are found in the rete testis. D: In this section of the cauda epididymidis the lumen has no sperm but is filled with prematurely released germ cells. MNGCs (arrowheads) and UNGCs with apoptotic morphology (arrows) are also seen. EP, epididymis; GC, basal compartment germ cells; PS, pachytene spermatocyte; RS, round spermatid; RT, rete testis; SC, Sertoli cell Scale bar, A, B 4 µm; C 20µm; D, 18 µm.

cells was marginalized chromatin of the UNGC and MNGC whereas in the MMGC the micronuclei were highly compact and dense and did not indicate any marginalization of chromatin.

Table 5. Certain parameters of Leydig cells of AFB1 - treated mice

Treatment group

Duration of treatment	Counts per 10^3 μm^2 area		Leydig cell perimeter (μm)		Leyding cell unclear diameter (μm)	
	Control	Treated	Control	Treated	Control	Treated
7 days	20.93± 1.32	14.15± 2.86*	123.32± 8.47	62.12± 6.43*	5.52± 0.63	4.68± 0.43
15 days	19.98± 1.36	20.93± 3.19	118.86± 9.66	54.41± 5.83*	5.36± 0.86	3.47± 0.64*
35 day	20.32± 1.86	28.86± 2.68*	121.92± 10.86	42.12± 4.94*	5.62± 0.81	3.16± 0.67*
45 days	20.18± 1.43	33.70± 3.92*	124.86± 10.32	31.68± 4.66*	5.43± 0.48	2.45± 0.52*

Recovery group I-35 days

Duration of treatment	Counts per 10^3 μm^2 area		Leydig cell perimeter (μm)		Leydig cell nuclear diameter (μm)	
	Control	Treated	Control	Treated	Control	Treated
7 days	19.75± 1.21	16.32± 2.45*	121.46± 8.47	94.96± 4.19*	5.21± 0.72	4.8± 0.42
15 days	20.89± 1.46	21.17± 2.38	122.11± 9.54	84.14± 4.61*	5.36± 0.70	4.19± 0.73*
35 day	19.25± 1.66	24.45± 1.89*	121.92± 9.11	78.32± 3.76*	5.19± 0.85	4.08± 0.62*
45 days	20.37± 1.22	26.86± 4.14*	120.90± 9.20	68.19± 5.49*	5.10± 0.59	3.92± 0.43*

Recovery group II-70 days

Duration of treatment	Counts per 10^3 μm^2 area		Leydig cell perimeter (μm)		Leydig cell nuclear diameter (μm)	
	Control	Treated	Control	Treated	Control	Treated
7 days	12.62± 1.32	19.86± 1.46	121.23± 6.72	120.86± 7.43	5.49± 0.48	5.46± 0.32
15 days	19.98± 1.36	18.92± 1.24	120.65± 8.94	111.34± 6.45	5.34± 0.62	5.12± 0.49
35 day	20.32± 1.86	22.42± 1.92	121.85± 9.64	114.39± 3.42	5.42± 0.71	5.23± 0.86
45 days	20.18± 1.43	23.11± 1.39	122.01± 9.22	108.63± 3.42	5.50± 0.48	5.31± 0.32

There was no indication of loss of germ cells in the control mice (Figs. 1A, 2A). In the AFB_1-treated mice, killing of germ cells could be located even in the mice treated for 7 days, and with increase in the duration of treatment the cells undergoing these pathological changes also increased. The loss of germ cells, particularly from the intercalary regions, resulted in vacuoles or empty spaces in the SE (Fig. 2E). The SE, in addition, reflected several other changes like fragmentation of chromatin of pachytene spermatocytes (Fig. 3A) and normal-sized round spermatids (Fig. 5A), necrosis of pachytene spermatocytes (Fig. 4B) and round spermatids, etc.

Effect of AFB1–treatment on the Leydig cells

In the mice treated AFB_1, two trends were noticed. In the mice treated AFB_1 for 7 days the Leydig cells underwent hypertrophy, and dark dense vesicles accumulated in the cytoplasm (Fig. 7A, B). In the mice treated for 15 and more days, there was a duration-dependent hyperplasia of the Leydig cells, distortion of shape of their nuclei and appearance in their cytoplasm of large vacuoles or dense granules (Fig. 7C, D). Histometric analysis of Leydig cells of AFB_1-treated mice showed increase in the counts of Leydig cells per unit area and decrease in the Leydig cell nuclear diameter; the changes were dependent on the duration of treatment (Table 5).

Fertility test

There was no change in the litter size of female mice mated with AFB_1-treated male mice for 7 days. In the 15 day treatment group there was a significant decrease in the litter size, whereas in 35 and 45 day treatment groups the females mated with the treated males did not deliver a litter (Table 6).

Recovery

The histological preparations and histometric data revealed that on cessation of treatment, followed by the period of recovery, there was partial restoration of the histoarchitecture of testis over 35 days, and almost complete recuperation over recovery for 70 days (Fig. 8A, B; Tables 1-6). Fertility test revealed that the animals in the recovery groups gradually regained fertility.

DISCUSSION

Results of the present study in the background of existing literature

The regression of testis, impairment of spermatogenesis, premature loss of germ cells and pathological changes in the Leydig cells, as observed in this study, have already been reported in a few studies. Continuous feeding of a diet containing 0.7ppm aflatoxin produced testicle degeneration in male goat (21). Testicular development in juvenile Japanese quail treated with AFB1 was delayed (22). Decreased reproductive potential caused due to AFB_1 treatment was noticed in male white Leghorn

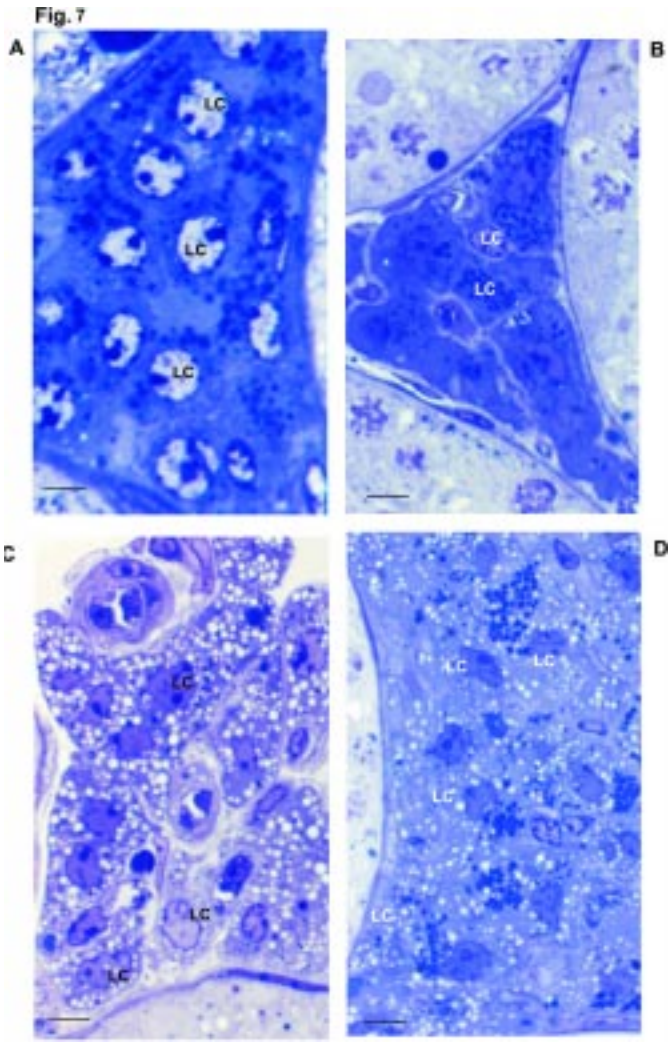

Fig. 7 A-D: Leydig cells of AFB1-treated mice. Semithin, TBO. **A:** Interstitial tissue of an AFB1-treated mouse (7days) showing hypertrophy of Leydig cells (LC), bloating of their nuclei and accumulation of dark dense granules in the cytoplasm. **B:** Same as A, but another mouse, showing regression of Leydig cells. **C:** Hyperplasia of Leydig cells and increased vacuolation in their cytoplasm. **D:** Shows intensification of the trend in **C** and distortion of shape of the nucleus. Scale bar, 4µm.

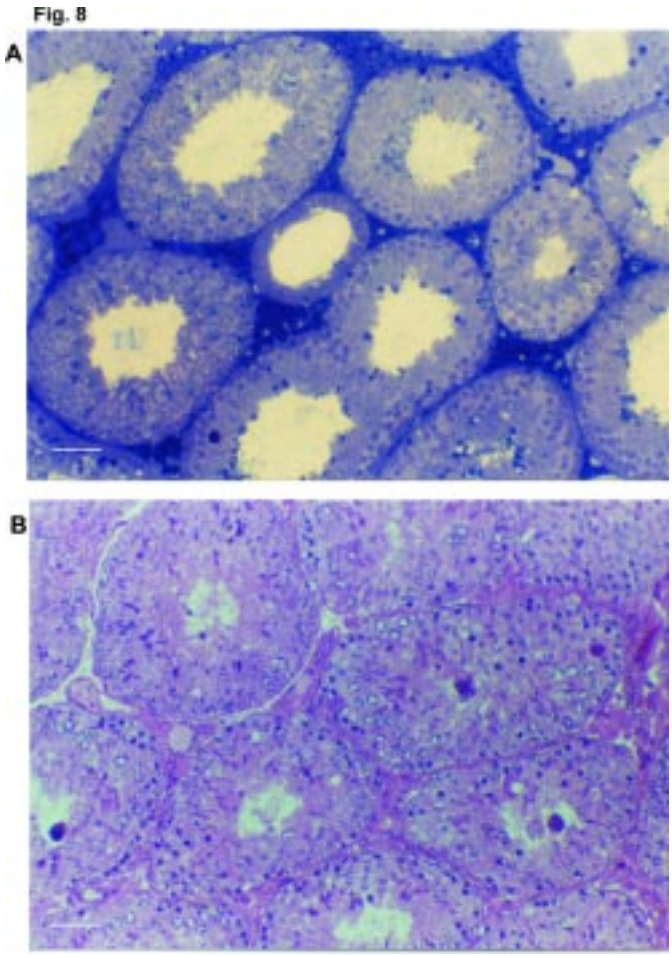

Fig. 8A, B: Sections of the testis of mice treated AFB1 for 45 days and left to recovery for 35 days showing partial recovery (Fig. 8A) and recovery for 70 days showing almost complete recovery (Fig. 8B). Scale bar A, 35 µm; B, 18 µm.

chicks (37). In an experiment treating roosters with AF at 5, 10 and 20 ppm concentrations in the diet for 8 weeks, it was found that the testis atrophied, no spermatogenesis occurred in several birds, incidence of abnormal spermatozoa increased, the epithelium was desquamated, the size and thickness of the generative layer decreased and the level of plasma testosterone also decreased (28). Morphological regressive changes were observed in the testis of aflatoxin-treated rat and pig (23). The percentage of live sperm markedly decreased and the sperm abnormalities increased in AFB_1-treated mice (27) and buffalo bulls (25). In a study on goslings and chicken it was found that, among all tissues, AFB_1 concentration was the highest in the gonads (38). After a single *ip* dose of 0.1 mg AFB_1/Kg body weight to the Chinese hamster, free AFB_1 was detected in the blood, liver, kidney and testis from minutes up to 8-10 hr after injection (39). Disruption of spermatogenesis, androgen synthesis and sperm function was observed in male mice treated AFB_1 at 50-60mg/kg body weight/day for 30 days (24, 34, 40-43). A gross reduction in serum testosterone level after AF-treatment was observed in male rats (44-46). Verma and Nair (29) found significant increase in cholesterol concentration of the testis of mice administered AF at doses 25 and 50mg/animal/day for 45 days and suggested that it may be due to either increase in biosynthesis of cholesterol or its decreased utilization during steroidogenesis. The gross changes noticed in the present study are in conformity with the above findings of male reproductive toxic effects of AFB_1. However, this study reveals the various cellular

targets in the testis to AFB_1 toxicity and throws light on the possible cellular mechanisms of action of the toxin in bringing about these effects. It could be stated at this point that AFB_1 brings about the toxic effects in the testis not through a unified mechanism of action, but through more than one mechanism.

Table 6. Results of fertility test

Treatment group

Duration of AFB1 treatement	Litter size	
	Control	**Experiment**
7 days	9.8±1.46	9.8±1.79
15 days	9.8±1.48	2.2±1.48
35 days	9.9±1.72	Nil *
45 days	9.2±2.28	Nil *

Recovery Group I-35 days

Duration of AFB1 treatement	Litter size	
	Control	**Experiment**
7 days	9.8±1.79	9.8±1.79
15 days	10±1.58	5.0±1.0
35 days	9.8±1.79	4.0±1.22
45 days	10±2.0	3.6±0.55

Recovery Group II-70 days

Duration of AFB1 treatement	Litter size	
	Control	**Experiment**
7 days	10±1.10	9.2±1.10
15 days	9.6±1.41	8.0±1.41
35 days	9.6±1.10	7.2±1.10
45 days	9.6±1.10	7.2±1.10

Justification of the dose of AFB_1

As has been stated elsewhere the toxicity testing in the present study has been chronic toxicity testing. According to Groopman *et al.* (14) and Krishnamachari *et al.* (12) short exposure to large doses of AFB_1 induced acute toxicity which may be lethal while exposure to small doses over a protracted period of time resulted in aflatoxicosis. The current major concern about aflatoxins is chronic effect at low level exposure on the general public and to workers in certain occupations (47, 48). In the chronic male reproductive toxicity testing in the present study, the dose of AFB_1 is only 50 µg/kg body weight, closer to that administered by Egbunike *et al.* (34) and much less than that those practiced by Verma and Nair (29), which is 25 and 50 mg/animal/day. Dietary exposure to human as large as 1.7mg per day has been reported (7). Intra peritoneal rather than oral route was preferred in the present study since in the latter AFB_1 is mixed in the feed, which does not ensure uniform dose to all animals.

Origin of UNGCs

The uninucleate giant cells produced in the AFB1-treated mice are typically hypertypic spermatocytes or giant spermatids. Hypertypic spermatocytes are produced when the chromosomes, in which DNA synthesis has been completed, fail to separate into daughter chromosomes. The UNGCs obtained in the present study appear to be the products of failure of paired chromosomes, in which DNA has already replicated (36, 49), to separate. Thus, the resultant cells are larger than pachytene spermatocytes. In reality, failure of the paired chromosomes to separate should produce only cells of the size as pachytene spermatocytes. As discussed *vide supra*, the resultant cells in this study were double the size of pachytene spermatocytes. It is to be inferred that the cellular machinery has completed all the biosynthetic processes towards division of the cell in meiotic division I (M1) that would contribute to an increase in the volume of the cell. Uninucleate giant spermatids are produced due to failure of cells to divide during M2. It is also to be inferred that the cell, for all practical purposes, is abnormal as far as testis is concerned and, therefore, cannot end up as spermatozoa. As the aim of the cellular changes in the male germ cell line is to produce spermatozoa, the abnormal cells do not end up as spermatozoa. Essentially, any cell which does not end up as spermatozoon is to die and be removed (50). The death as far as the UNGC is concerned involves swelling through a likely hydropic mechanism. The cells either become necrotic or loose contact with the Sertoli cells and are released into the lumen from where, through the rete testis, arrive at the epididymis. During this transit, the cells undergo nuclear pycnosis through chromatin condensation. It is an established fact that abnormal cells arriving at the epididymis are removed through phagocytic action of the luminal macrophages (51). Thus, it is suggested that the UNGCs in the present case are generated due to failure of the bivalents to separate, or failure of division at M2, both probably due to failure of the spindle mechanism (52). Apparently, one of the mechanisms of AFB_1 action is to disrupt the spindle fibers.

Origin of MNGCs

The present study has clearly established that one of the mechanisms of action of AFB_1 in the testis is generation of MNGCs. The organization of the MNGCs thus generated suggests them to be formed of round

spermatids. The maximum number of nuclei in such giant cells appears to be 16. The presence of multinucleate spermatids is not uncommon. They occur occasionally in the testis of normal animals and are generated in large numbers due to various disorders and male reproductive toxicants (50), and in transgenic mice such as sys (symplastic spermatids) (53, 54) and those deficient in BAX (55) and HSL (56). The MNGCs produced in mice due to treatment with AFB_1 resemble the symplasts produced due to cytochalasin D treatment (57, 58) and ursolic acid (59) although not with the same extent of multinucleation. Russell *et al*. (57) demonstrated for the first time that symplastic multinucleate giant cells are produced due to loss of integrity of the intercellular bridges between male germ cell clones. Since the spermatids are haploid cells, the cytoplasmic bridges are meant for equal distribution of gene products among all the cells of a clone and, thus, provide for equality of gene products between the haploid cells (60). Russell *et al*. (57) suggested that the cytoplasm left behind by the nuclei that move on to create multinucleate cells develop into large cytoplasmic bodies within the SE. Thus, it is inferred that AFB_1 is capable of disrupting the cytoplasmic bridges connecting spermatids, and generate multinucleate gaint spermatids. MNGCs are an abortive end in spermatogenesis.

Origin of MMGCs

. This study clearly distinguishes between MNGCs and MMGCs. The nuclei in the MNGCs are as large as in the spermatids, but the multiple micronuclei are small spherical bead-like chromatoid bodies. It is true that micronucleate spermatids have been reported to be produced in several experimental conditions (61-63), but such micronuclei are in addition to a normal-sized nucleus. In other words, the micronucleus in a spermatid, already known in the literature, is an extranumerary inclusion. The micronuclei, supernumerary bodies outside the main nucleus, in spermatids are produced due to chromosome breakage and/or disruption of the spindle in the meiotic spermatocytes following exposure to mutagens (64, 65). Chromosome fragment or whole chromosome detached from the spindle lag behind during anaphase and can be identified as a small spherical body of chromatin (63). Thus, what were observed in the present study are different and are multiple micronuclei, and the cell lacks the usual nucleus. A situation closely approximating the multiple micronucleation was reported by Kallio and Lahdetie (66), who found aneuploidy induction in germ cells by treatment with merbarone, a DNA topo-isomerase II inhibitor (topo II). It is known that AFB_1 can induce sister chromatid exchanges and other chromosomal anomalies which might have resulted in breakage of the chromosomes of germ cells, particulary spermatocytes. Multiple micronuclei, in the present study, might have been produced due to these chromosomal effects of AFB_1 or due to failure of the spindle apparatus causing each pair of chromosomes to condense into a chromatoid body, the micronucleus.

Aflatoxin toxicity

In the discussion so far in respect of origin of UNGCs, MNGCs and MMGCs, the emphasis has been on disruption of microtubules of spindle fibres. However, there is a more recent report analyzing the cell cycle, DNA synthesis and expression of p21 and p53 in the cell lines HepG2 and SK-N-MC-N-SH2 (67). On exposure to aflatoxin, a significant alteration in progression of the cell cycle of these cells was observed. The observation in this study clearly demonstrates damaging effect of AFB_1 to chromatin of pachytene spermatocytes. As discussed *vide supra*, pachytene is the longest stage in meiosis, commencing with thickening and shortening of the chromosomes. It is the phase characterized by nuclear and cytoplasmic growth (36). The metabolic processing of AFB_1 by cytochrome P450 generates the reactive intermediate exo-AFB_1-8, 9-epoxide, which is capable of covalent binding to DNA to form bulky adducts (18). These adducts, if not repaired, may cause site-selected mutagenesis (68). The adduct formation occurs preferably with guanine resulting in AFB_1-N-7 guanine adduct, responsible for mutagenesis in AFB1-treated cells (69). The interaction of AFB_1 can be with the total genomic DNA (70). This can result in small to large changes in genomic DNA, including bulk lesions and sister chromatid exchanges (66). Since pachytene is the longest phases in meiosis, at the threshold level the AFB_1-metabolite, perhaps, brings about such gross changes in the DNA, resulting in fragmentation of the chromatin.

Mechanisms of cell killing

Three kinds of morphological or cytological manifestations of cell death, as caused by AFB1 in the male germ cells of mouse, were observed in the present study, namely necrosis, apoptosis, and nuclear pycnosis. Necrosis, in conventional terms, involves cytoplasmic swelling, karyolysis and rupture of cells to release the content onto the immediate surrounding, which would affect the neighboring cells. Apoptosis involves compaction of the cytoplasm and fragmentation and marginalization of the chromatin followed by formation of apoptotic bodies through budding. In apoptosis, the neighboring cells are not affected (71). Thus, the disruption caused to chromosomes, chromatin or DNA as well as microtubules

of the spindle apparatus of male germ cells ultimately leads to death of the affected cells. The dead cells either rupture and release the content (necrosis) or undergo compaction (apoptosis) and are prematurely released from the Sertoli cell. In the case of apoptosis, the cells, on being released from the Sertoli cell either from the intercalary region in the epithelium or from the luminal end, are carried onto the epididymis for apoptotic body formation and their phagocytosis by macropahages in the lumen.

Premature release of germ cells

AFB1-treatment brings about depletion of germ cells in the SE. UNGCs, MNGCs and MMGCs do not constitute the normal cells of the SE, and such abnormal cells are bound to be removed through death and degeneration. Retardation of spermatogenesis is an abnormality that eventually leads to cell degeneration. The cells may be phagocytosed by Sertoli cells just before or at the time of their expected release into the tubular lumen (50). Such cells, by disengagement of Sertoli cell-germ cell junctions, would be sloughed off individually or as a group. This condition will frequently occur when degeneration of germ cells is massive (50). However, the observations in the present study do not support phagocytosis of aberrant immature germ cells by Sertoli cells. On the contrary, the cells, in the process of degeneration or apoptotic body formation, arrive at the lumen of the STs and through the rete testis reach the epididymis. This is understandable because under normal circumstances macrophages do not have access into the STs.

Leydig cell response

The impact of AFB1-treatment on testosterone secretion has already been reported in a few papers. It was reported that in the male rat treated with 50mg aflatoxin on three occasions at intervals of 48 h, Leydig cell function was impaired, as reflected in the extremely reduced responsiveness of Leydig cells of the aflatoxin-pretreated rats to hCG stimulation (24). Aflatoxin treatment suppressed both onset of production and final concentration of male hormone in aflatoxin-treated Japanese quail (22). In another study it was observed that while the LH secreting capacity of the anterior pituitary was not diminished in birds receiving aflatoxin, the testicular response to LHRH was altered during aflatoxicosis (72). Aflatoxin treatment may affect cholesterol concentration in the Leydig cells and serum levels of testosterone (29, 44-46). Particularly, the study of Verma and Nair (29) revealed that there could be significant dose-dependent reduction in the key enzymes of androgen biosynthesis, namely 3-beta and 17-beta hydroxysteroid dehydrogenases. These and the present observations reveal Leydig cells also to be target to aflatoxin. The original contribution in the present study in this regard relates to the pathological changes in the Leydig cell. In aflatoxin-treated mice the androgenic machinery, consisting of smooth endoplasmic reticulum and mitochondria, are thoroughly affected (data not presented), understandably, hampering androgen synthesis. The impairment of androgen synthesis is further substantiated in two opposing situations *viz.*, (i) accumulation of lipid, and (ii) depletion of lipid droplets. Cholesterol and its esters are the raw material for synthesis of androgens, and their depletion or accumulation reflects impairment of androgen synthesis. Though the circulating levels of androgens were not determined in the present study, the pathological changes, combined with the decrease in the weight of the seminal vesicles, clearly indicate hypo-androgen status of the AFB_1-treated mice. It would be pertinent to relate the hypo-androgen status with the derangements in the aspects of spermatogenesis, since androgens are known to play a pivotal role in the regulation of spermatogenesis (36).

Recovery

At the dose of AFB_1 and the duration of treatment practiced, the animals recovered since cessation of the treatment. A partial recovery occurred in 35 days and an almost complete recovery in 70 days. The observation in respect of histopathology showed that the spermatogonia are invariably spared from any pathological changes. Though Sertoli cells appeared to be a target of AFB_1 toxicity, the manifestations were mostly loss of adherence to germ cells and breaking away of only the apical portions of Sertoli cells. The former appears more because of the pathological changes in the germ cells concerned, rather than in the Sertoli cells. Breaking away of apical portions of Sertoli cells is due to damage caused to microtubules in the body of the Sertoil cells, sparing the basal portion of the cell containing the nucleus (73). Thus, the intact spermatogonia and Sertoli cells provide scope for recovery of fertility. Thus, the paper provides anatomical, histological and histometric evidence of severe impact of AFB1 on both spermatogenic and androgenic compartments of testis, which is dependent on the duration of treatment. The study also reports a newer kind of giant male germ cell, multiple micronucleate giant cells, produced due to AFB_1 treatment. However, the manifestations appear to be only temporary, as the animals recovered in course of time since exposure to AFB_1.

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