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A Comprehensive Review of Male Reproductive Toxic Effects of Aflatoxin

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1. Introduction

Aflatoxins, highly oxygenated, heterocyclic, difuranocoumarin compounds that could be present in human foods and animal feedstuffs, are an important group of mycotoxins produced by the fungi *Aspergillus flavus, A. parasiticus* and *A. nomius* (Diaz et al., 2008). Other species of Aspergillus such as *A. bombycis*, *A. ochraceoroseus* and *A. pseudotamari* may also produce aflatoxins (Bennett & Klich, 2003; Klich et al., 2000; Mishra & Das, 2003). On a worldwide scale, the aflatoxins are found in stored food commodities and oil seeds such as corn, peanuts, cottonseed, rice, wheat, oats, barley, sorghum, millet, sweet potatoes, potatoes, sesame, cacao beans, almonds, etc., which on consumption pose health hazards to animals, including aquaculture species of fish, and humans (Abdel-Wahab et al., 2008; Hussein & Brassel, 2001). Health effects occur in fish, companion animals, livestock, poultry and humans because aflatoxins are potent hepatotoxins, immunosuppressants, mutagens, carcinogens and teratogens. Public health concerns center on both primary poisoning from aflatoxins in commodities, food and feedstuffs, and relay poisoning from aflatoxins in milk (Coppock & Christian, 2007). There are four major natural aflatoxins (AFs), AFB1, AFB2, AFG1 and AFG2. The hierarchy of toxicity of different aflatoxins is in the order AFB1>AFG1>AFB2>AFG2. There are two additional metabolic products of aflatoxins B1 and B2, *viz.*, M1 and M2. More than 5 billion people in developing countries worldwide are at risk of chronic exposure to naturally occurring aflatoxins through contaminated foods (Shephard, 2003; Williams et al., 2004) and more so in the tropical regions, where the climatic conditions favour luxurious growth of *Aspergillus spp*, and people rely on commodities such as cereals, oilseeds, spices, tree nuts, milk, meat and dried fruits that are potentially contaminated by aflatoxins (Strosnider et al., 2006).

Symptoms of aflatoxicosis include feed refusal, decreased feed efficiency, stunted growth, decreased milk production and impaired reproductive efficiency (Diekman & Green, 1992; Oguz & Kurtoglu, 2000; Pier, 1992; Raju & Devegowda, 2000). Aflatoxins in general, and AFB1 in particular, can induce DNA damage, gene mutation, sister-chromatid exchanges and other chromosomal anomalies, which account for their genotoxic, teratogenic and carcinogenic properties (Batt et al., 1980; International Agency for Research on Cancer

[IARC], 1993; Ray-Chaudhuri et al., 1980). AFB1 can form adducts with DNA, RNA and protein, which form the major basis of the health risks (Sun et al., 2001; Williams et al., 2004). Epidemiological and experimental studies have implicated aflatoxins in male reproductive health, and the present review is an attempt to put together the knowledge in a comprehensive perspective.

2. Aflatoxins in sperm and semen

Aflatoxins or their metabolites can reach the testis (Bukovjan et al., 1992) and be present in the semen through this route (Ibeh et al., 1994; Picha et al., 1986; Uriah et al., 2001). Aflatoxins have been detected in boar sperm (Picha et al., 1986) and human semen (Ibeh et al., 1994). In a cross sectional study, Ibeh et al., (1994) found a relationship between aflatoxin levels in serum of infertile men compared to controls: 40% of semen from infertile men had aflatoxins and 50% of spermatozoa were abnormal, whereas 8% of semen from fertile individuals had aflatoxins and only 10-15% were abnormal. The concentrations of aflatoxins detected in the semen were consistently higher among infertile compared to the fertile men. This study was supported by experiments conducted in rats, and the results were in agreement with the observations in the human samples. Uriah et al., (2001) reported translocation of aflatoxin B1 in humans from blood to semen through the blood-testis barrier. In the boars, the highest AF residues in sperm were recorded in March to May and were related with aflatoxin concentration in the feed ration. The group of boars with fertility disorder had more AF in their sperm (up to 100 pmol^{-1}), lower sperm concentration, impaired survival of spermatozoa and a large proportion of abnormal spermatozoa (Picha et al.,1986). When ram epididymal sperm were put in different concentrations of aflatoxin, on one-hour post-incubation in control group 81.25% of sperm cells were alive of which 82.88% were motile. The lowest motility (15.93%) was observed in 62.5 ppb aflatoxin-exposed sperm. Sperm viability did not change significantly after 2nd and 3rd hr incubation but significantly decreased in 4th and 5th hr post- incubation. The results of the experiment showed that aflatoxin could decrease motility of sperm obtained from ejaculation or epididymis (Tajik et al., 2007). Ibeh et al., (2000) cultured oocytes for *in vitro* fertilization (IVF) in IVF medium containing AFB$_1$ and exposed to sperm cells. Epididymal sperm capacitated in IVF medium, with or without AFB$_1$, were exposed to oocytes. AFB$_1$ exposure significantly reduced the mean number of ova fertilized. Exposure of sperm to AF caused significant reduction in their motility.

3. Some classical experimental studies on testicular effects of AFs

One of the earliest reports indicating impairment of reproductive efficiency due to AF toxicity was that of Maryamma & Sivadas, (1975) who found that continuous feeding of a diet containing 0.7 ppm AF produced testicular degeneration in male goats. Subsequently, there have been other reports of AFB$_1$ causing delay in physiological and behavioural sexual maturation (Ottinger & Doerr, 1980) and also delayed testicular development in juvenile Japanese quail (Doerr & Ottinger, 1980). Sharlin et al., (1980) found decreased semen volumes and testes weights, and disruption of the germinal epithelium in mature male white Leghorn chicks. Another study conducted by Sharlin et al., (1981) to investigate the relative importance of ingestion of aflatoxin versus decreased feed consumption led to the conclusion that even though decreased feed consumption did not produce symptoms of

aflatoxicosis, it had accounted for 60% of the effects of aflatoxin on reproduction. AFB1 toxicity leads to reduction in size and weight of testis, with mild testicular degeneration to complete disappearance of cellular components accompanied by interstitial cell proliferation and reduced estrogen concentration in rat (Gopal et al., 1980).

Ikegwuonu et al., (1980) observed some degeneration in the testis of aflatoxin treated rats, accounting for the loss of germ cells. Further, the authors provided biochemical insight into the toxicity, and postulated inhibition of testicular ribose 5'-phosphate, which in turn might lead to the impairment of testicular nucleic acid synthesis. It was postulated that prolonged intake of aflatoxin leads to the disturbance of the ensemble of transaminases, particularly GOT and GPT activities, which can adversely affect testicular protein synthesis resulting in decrease of the testicular weight. Aflatoxin impairs protein biosynthesis by forming adducts with DNA, RNA and protein, inhibits RNA synthesis, DNA-dependent RNA polymerase activity, and causes degranulation of endoplasmic reticulum (Cullen & Newberne, 1994; Groopman et al., 1996). Reduction in protein content has also been reported in the testis of aflatoxin-treated mice (Nair & Verma, 2000), which could be responsible for the reduced enzyme activities.

Piskac et al., (1982) showed that prolonged administration of aflatoxin to male rats and pigs resulted in different degrees of dystrophy leading to the destruction and atrophy of spermiogenic epithelium and oedema formation in the tissue. According to Hafez et al., (1982), aflatoxins affect sperm counts and morphology in buffalo bulls. The effect of dietary aflatoxin has also been reported to be clastogenic for meiotic chromosomes, and capable of inducing abnormalities in sperm head morphology and decreasing sperm count in mouse. In this case, the abnormal chromosomes were found to have both structural changes such as breaks, gaps, fragments, translocations, terminal associations as well as gross changes which include numerical changes, clumping and stickiness (Sinha & Prasad, 1990).

Feeding of adult roosters with AF-contaminated diet produced several toxic manifestations which included atrophy of the testes, decrease in the diameter of the seminiferous tubules, decrease in the height of seminiferous epithelium, thickening in intertubular area of the testes, and increase in the abundance of interstitial cells. In some cases, there was no spermatogenesis in the testis (Ortatatli et al., 2002). Desquamation of seminiferous epithelium and degeneration of the desquamated or necrotic cells have been reported (Jayakumar et al., 1988; Sharlin et al., 1980). Ortatatli et al., (2002) observed focal lymphoid cell infiltration in testes in adult roosters fed AF-contaminated diet, which has already been reported to occur in other organs such as liver, kidney and pancreas due to aflatoxicosis (Dafalla et al., 1987; Esapda et al., 1997; Kiran et al., 1998).

Evidence for impairment of Leydig cell function with a resultant drop in testosterone in testis preceding disruption of spermatogenesis in rats was provided by Egbunike et al., (1980, 1982). Recently, Abu El-Saad & Mahmoud, (2009) found decreased levels of FSH, LH and testosterone in AF-treated rat. However, no significant differences were observed in testosterone production and secretion by isolated testicular cells of control or aflatoxin treated male chickens when incubated *in vitro* with different concentrations of LH (Clarke & Ottinger, 1989). This could be due to the species differences in rate of exposure of aflatoxin (dietary *vs.* intraperitoneal) or potency of aflatoxin (aflatoxin mixture *vs.* purified aflatoxin B1). However, there was an unexpected suppression of testosterone production even in the presence of 1600ng/ml LH. The similar response of isolated testicular cells from both aflatoxin-treated and control males when exposed to varying amounts of LH indicated a lack of effect of dietary aflatoxin on the steroidogenic capacity of testicular cells *in vitro*.

4. Gross histopathological changes in the testis of AFB1 treated mouse

Aflatoxin B1 was tested for male reproductive toxic effects in our laboratory, and the observations were published in a series of papers. In addition to the already known ones, several newer manifestations were reported. One of our early studies (Faridha et al., 2006) aimed at finding gravimetric, histopathological and histometric changes in the testis of Swiss mouse in response to treatment of aflatoxin B1 (AFB1) in a chronic toxicity testing over different periods of time. AFB1, suspended in corn oil and ethanol, was administered through intra-peritoneal route to 90 day old Swiss mouse at a daily dose of 50ug/kg body weight for 7, 15, 35, 45 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. Gravimetric analysis of testicles and seminal vesicles revealed duration-dependent decrease in their respective weights (Table 1). 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 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 trend was decrease in size of the seminiferous tubules (STs) (Table 2).

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)

Table 1. Weight of the paired testicles and seminal vesicles of control and AFB1-treated mice (Mean ± SD). *p<0.01. Number in parenthesis, percentage of the control value.

Duration of Treatment	Perimeter (um)		Diameter (um)	
	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*

Table 2. 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. *p<0.001

Critical observation of the individual STs revealed almost total absence of elongating spermatids (Fig. 1A-D). Spermiated spermatozoa were invariably absent in the lumen. The height of the seminiferous epithelium (SE) either increased or decreased and, correspondingly, the lumen was either almost obliterated or increased. A duration-

dependent appearance of uni- (Fig. 2A-D) and multinucleate (Fig. 3) 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. Giant cells were noticed in the epithelium as well as in the lumen, and they possessed vacuolated cytoplasm and pycnotic nuclei or nuclei with marginalized chromatin.

Fig. 1. A-D. Seminiferous tubule of control and treated mice; **A:** Control; **B:** Treated (7days). Note loss of intercalary germ cells (arrowheads). **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 C, a different tubule. Note absence of elongating spermatids and presence of uninucleate giant cells towards the lumen (arrowhead). Semithin sections, TBO staining. Scale bar 18μm.
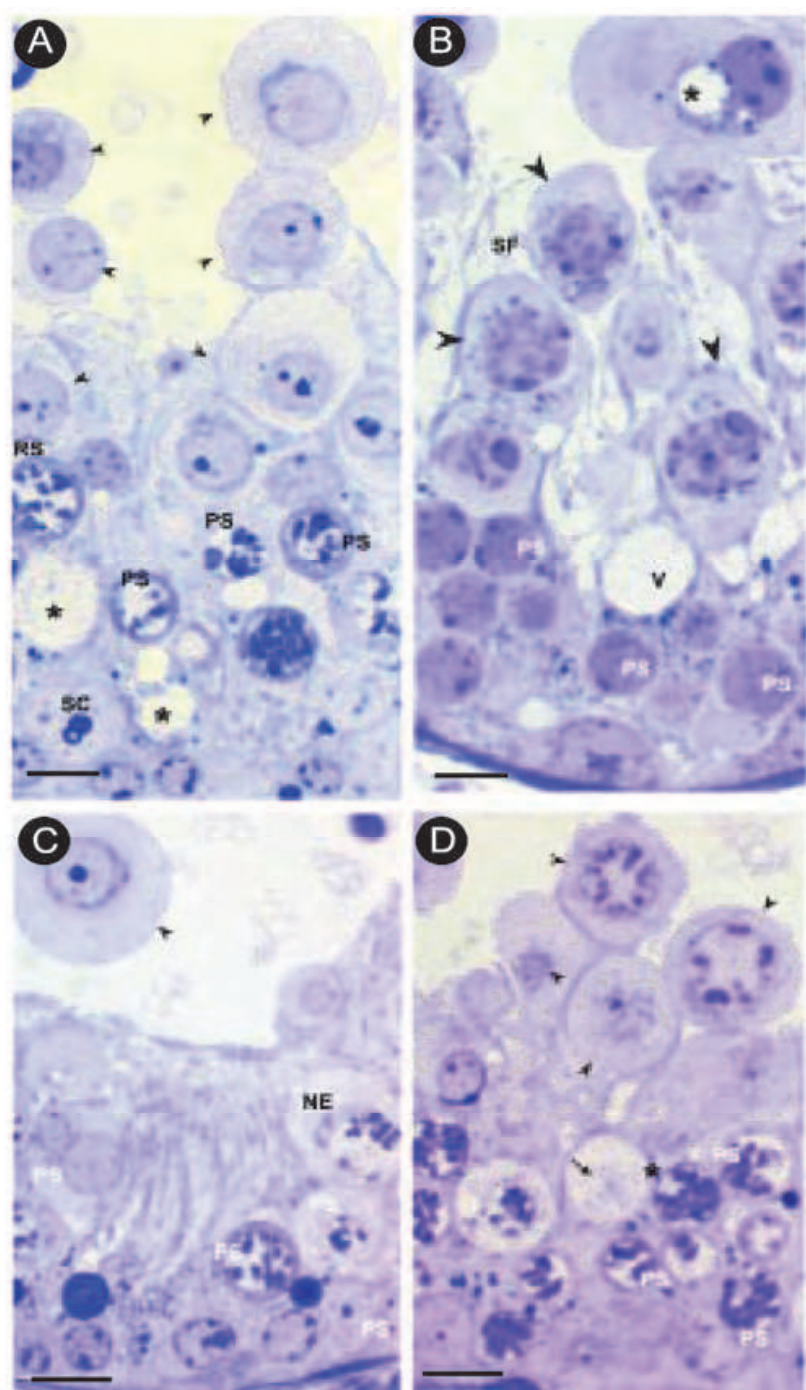
Fig. 2. A-D. Seminiferous epithelium of treated mice. **A:** Shows uninucleate giant cells
(arrowheads) (which are spermatids) in the epithelium, damage to chromatin of pachytene
spermatocytes, and loss of intercalary germ cells (asterisks). **B:** The uninucleate giant
spermatid (arrowhead) is seen 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. Semithin sections, TBO staining. Scale bar, 4μm.

Fig. 3. Seminiferous epithelium of a treated mouse showing a multinucleate giant cell (GC). The MNGC, with nuclei containing marginalized chromatin, lies towards the lumen. Note spermatocytes arrested in M2 (asterisk). Note abnormality in the entire adluminal compartment. The basal compartment is intact. Semithin section, TBO staining. Scale bar 4μm.

Critical observation of the STs of AFB1-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. 2A-D). Such cells are designated as uninucleate giant cells (UNGCs). They were present in the epithelium along the luminal profile (Figs. 1D, 2A-D), some projecting into the lumen but still adherent to the Sertoli cells or lying loose in the lumen. In several cases the UNGC possessed highly vacuolated cytoplasm, and the nucleus was altered in morphology.

Another observation made in several of the STs of the AFB1-treated mice belonging to 15, 35 and 45 day treatment groups was occurrence of multinucleate giant cells (MNGCs) or symplasts (diameter, 40-52 μm) (Fig. 3). Such cells possessed two to 16 nuclei. The nuclei were either intact or had marginalized chromatin. The cytoplasm indicated little to extensive vacuolation. One of the observations was appearance of large cells (diameter 20-30 μm) containing several micronuclei (Fig. 4). Such cells are designated as multiple (or meiotic) micronucleate giant cells (MMGCs). 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 are 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. In a few tubules UNGCs, MNGCs and MMGCs coexisted. Loss of germ cells in a few tubules was so acute that hardly any germ cell was present in the ad-luminal compartment, with the epithelium manifesting small to large vacuoles. In some of the tubules the Sertoli cells themselves, from above the level of the ectoplasmic specialization, *i.e.*, the tight junctions of the 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. 5). The immature germ cells thus lost from the STs could be traced to the rete testis.

Fig. 4. 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). Semithin section, TBO staining. Scale bar, 4µm.
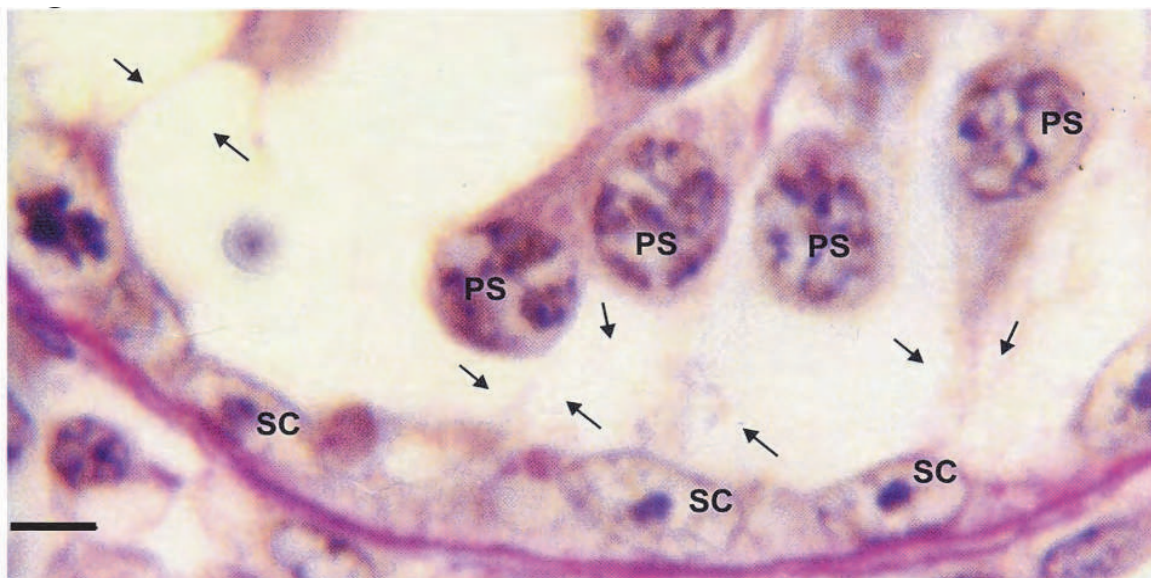
Fig. 5. Pachytene spermatocytes including portions of Sertoli cells are being lost (arrows). But the body of the Sertoli cell and the few basal compartment germ cells are intact. Paraffin section, hematoxylin and eosin staining. Scale bar, 4 μm.

5. Multinucleate giant cells (symplasts) and their origin

Whereas the seminiferous tubules of control mice did not contain any multinucleate giant spermatid or symplastic spermatid (hereinafter referred to as symplasts), the 50 sections of seminiferous tubules of the treated mice counted for symplasts, 28 had 1–17 symplasts (Faridha et al., 2007). The symplasts possessed two to several nuclei (Fig. 3), and the maximum number of nuclei in a symplast was 16. The symplasts, mostly spherical, measured a diameter of 12–20 μm as compared to 6–8 μm of the normal step1 round spermatids. The nuclei of both the normal round spermatids and symplasts measured the same diameter, 5–7 μm. Though the nuclei had normal appearance in a few symplasts, the chromatin was either marginalized (Fig. 6A) or fragmented (Fig. 6B) in the others. In the symplasts, which possessed nuclei with normal morphology, the cytoplasm was intact whereas in those which possessed nuclei with pathological manifestations, the cytoplasm was mostly vacuolated (Fig. 6B, C). The constituent spermatids of the symplasts progressed in spermiogenesis only up to step 8, as seen in the development of acrosome, which indicated that the cells did not progress beyond this step (Fig. 6D) and were released from the Sertoli cells.

The origin of symplasts was traced to the opening of cytoplasmic bridges connecting spermatids (Fig. 7). The bridge connecting normal spermatids measured 0.1–0.2 μm diameter and its lining had an electron-dense plaque extending to a short distance into the cells connected by the bridge. Towards the origin of symplasts, the perimeter of the bridge increased. In the constituent spermatids, the nuclear chromatin underwent marginalization, indicating apoptotic morphology. Subsequently, the cytoplasm of one of the constituent cells in the case of a prospective binucleate symplast or all the constituent cells excepting one in the case of a prospective multinucleate symplast was squeezed into the remaining cell. This resulted in one of the constituent cells becoming larger than the other(s), thus becoming cytoplasm-rich. This was followed by the entry of the nucleus/nuclei of the cytoplasm-poor cell(s) into the cytoplasm-rich cell. Even at this stage, the widened cytoplasmic bridge
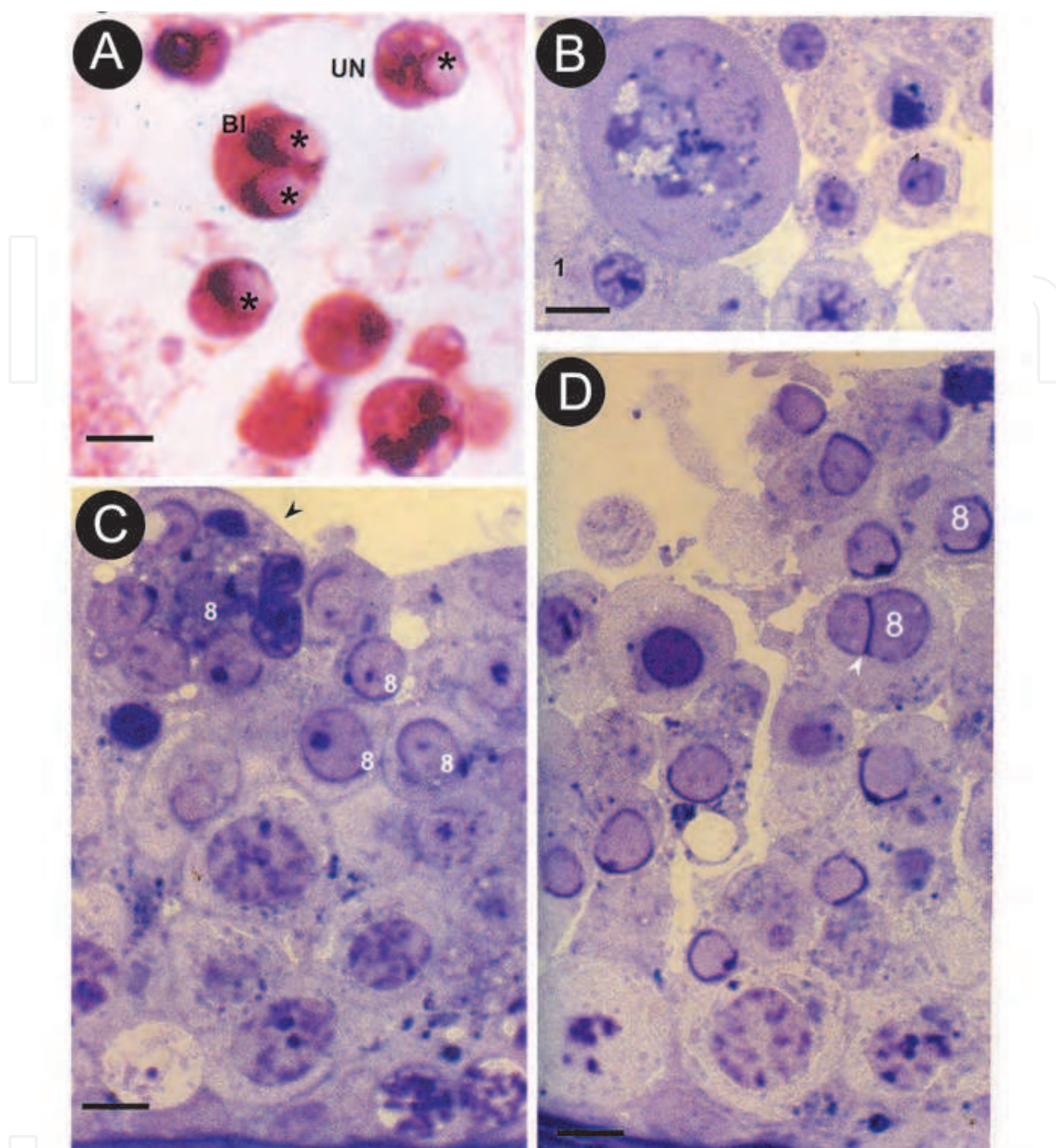
Fig. 6. Aspects of multinuceate giant cell of aflatoxin treated mice. A: A binucleate symplast (BI) with nuclei containing crescentic / marginalized chormatin (asterisks); and uniculceate giant cell (UN) also with nucleus containing marginalized chromatin (asterisks) and vacuolated cytoplasm. B: A multinucleate giant cell with the nuclei surrounded by compact cytoplasm. The normal round spermatids are in step 1 of spermiogenesis (1). C: A multinucleate giant cell (arrowhead) with pycnotic nuclei and vacuolated cytoplasm. The spermatids are in step 8 of spermiogenesis (8). D: Uninucleate and binucleate (arrowhead) giant spermatids at step 8 of spermiogenesis (8). A: Paraffin section, hematoxylin and eosin staining; B-D: Semithin section, TBO staining. Scale bar, 4 μm.

persisted, and did not collapse totally. Since the perimeter of the widened cytoplasmic bridge was not large enough for the nucleus/nuclei of the cytoplasm-poor cell(s) to pass through, it/they responded with change to a thimble shape. During this penultimate stage of origin of symplast, the cytoplasm of the cytoplasm-poor cell(s) was almost bereft of organelles whereas that of the cytoplasm-rich cell was not only rich in organelles but vacuoles too. It was only

after or consequent upon the cytoplasm-rich cell having become bi- or multinucleate, the symplast was established to its final spherical shape, with no trace of the cytoplasmic bridge(s). Though loss of integrity of the intercellular bridges between male germ cell clones has been suggested as the mechanism underlying the generation of symplastic spermatids induced due to cytochalasin D (Russell et al., 1987) and *sys* insertional mutation (MacGregor et al., 1990), this report was the first to provide unambiguous evidence for opening of the cytoplasmic bridges to lead to the formation of multinucleate spermatids, and substantiates the mechanism proposed earlier (MacGregor et al., 1990; Russell et al., 1987).

Fig. 7. Transmission electron micrograph showing symplast formation. Note the widened cytoplasmic bridge (arrowheads). A cell on top is cytoplasm poor and the one at bottom is cytoplasm rich. Prominant mitochondria (MI), endoplasmic reticulum (arrows). Scale bar, 1 μm.

Though the essential components of the vertebrate germ cell intercellular bridge have not been until now described, cytoskeletal proteins actin (Russell et al., 1987) and tubulin (MacGregor et al., 1990) have been demonstrated in the walls of the cytoplasmic bridges. Though both these proteins could be targets of agents that disrupt cytoplasmic bridges between spermatids, since cytochalasins, like AFs, are also of fungal origin, the target for AFB1 in the seminiferous epithelium could be actin microfilaments as has been proposed for cytochalasin D (Russell et al., 1987). Alternatively, AFB1 treatment would bring about oxidative damage to the cells (Abu El-Saad & Mahmoud, 2009) and the disruption of the cytoskeletal element in the cytoplasmic bridge would be a consequence of this damage (Lin et al., 2006).

6. Multiple/meiotic micronucleate giant cells (MMGCs) and their origin

The origin of MMGCs could be traced to meiotic metaphase I (M1) and metaphase II (M2) cells (Faisal et al., 2008a). It occurred due to failure of separation of chromosome bivalents in the case of M1 or failure of splitting of the centromere of replicated univalents in the case of M2, in both cases accompanied by or caused due to failure of the spindle apparatus. Delay in meiotic progression was indicated in the thorough asynchrony of the stages in the cycle of seminiferous epithelium. MMGCs invariably appeared detached from Sertoli cells. With the failure of spindle apparatus, the bivalents (in the case of M1) and the replicated univalents (in the case of M2) were arrested from progression towards completion of meiotic division (Fig. 8).

Fig. 8. Failure of meiotic chromosomes (M2) to move to the poles due to problem in spindle fibers, resulting in meiotic microculei. Semithin section, TBO staining. Scale bar, 4 μm.

Ultrastructural evidence for disruption of spindle fiber as the cause of micronucleation was also obtained. In the control mice, cells in early metaphase of second meiotic division had the chromosomes aligned in the equatorial plate and the spindle fibers appeared in the vicinity of the centrioles. Subsequently, the different chromosomes were closely aligned along the metaphasic plate, and the spindle fibers established connection with the centromeres. The separation of bivalents resulted in the univalents arriving at the poles, marking the telophase. In several of the M1 and M2 cells of AFB1-treated mice, not only the spindle fibers were absent, but the bivalents in the case of M1 and the replicated univalents in the case of M2 tended to disaggregate, each becoming a micronucleus. Each micronucleus was formed from a bivalent of M1 cell or a replicated univalent of M2 cell (Figures, in Faisal et al., 2008a).

Meiotic micronuclei are produced in the testicular germ cells by clastogenic or aneuploidogenic agents. Sinha & Prasad, (1990) provided evidence confirming the clastogenic property of AFB1. AFB1 is presumed to be aneuploidogenic also, and the concept of failure of spindle apparatus leads to the generation of meiotic micronuclei is further strengthened by the observation of intact kinetochore in the chromosomes. It was suggested that AFB1 affects assembly of tubulin into microtubules and/or brings about tubulin depolymerization, which would ultimately cause failure of pole-ward movement of the chromosomes.

7. Manifestations in the epididymis to AFB1 treatment

Little is known about the extent of the damaging effect of aflatoxins on the male reproductive tract, particularly the epididymis. Epididymis being the critical organ where in the spermatozoa arrive from the testis and undergo physiological maturation so as to become motile and fertilizable, any toxic manifestation here will explain why spermatozoa become morphologically abnormal and/or physiologically defective and unviable; alternatively, the epididymis would play a protective role so as to safeguard the spermatozoa. Agnes & Akbarsha, (2001) made a pioneering study on the effect of aflatoxin in mouse epididymis. Treatment of male mice with aflatoxin B1 through intra-peritoneal route, in a chronic toxicity testing, resulted in several histopathological changes in the epididymis. Light as well as transmission electron microscopic observations of the sections of epididymis of AFB1-treated mice revealed the presence of small or large vacuoles in the epithelial lining of all segments of the epididymis (Fig. 9). These vacuoles were enclosed in large pale epithelial cells which were quite different in organization from the other epididymal epithelial cell types (principal, clear, narrow, apical and basal cells, and intra-epithelial leucocytes). These cells were designated pale vacuolated epithelial cells (PVECs). The lumen of the vacuole contained spermatozoa and debris or an amorphous to dense PAS-positive material (Fig. 9), or all three materials. There were short microvilli extending from the cell into the vacuole. The vacuole appeared to arise as a result of the degeneration of a principal cell that led to fistula formation, during which the content of the ductal lumen and the principal cell fistula merged and spermatozoa from the ductal lumen entered into the fistula. The neighbouring intact principal cells bent over the degenerating principal cell, cutting off its continuity with the ductal lumen. The basal cell flanking the principal cell apparently developed into a PVEC and enclosed the disintegrating principal cell, including the spermatozoa that had entered it.
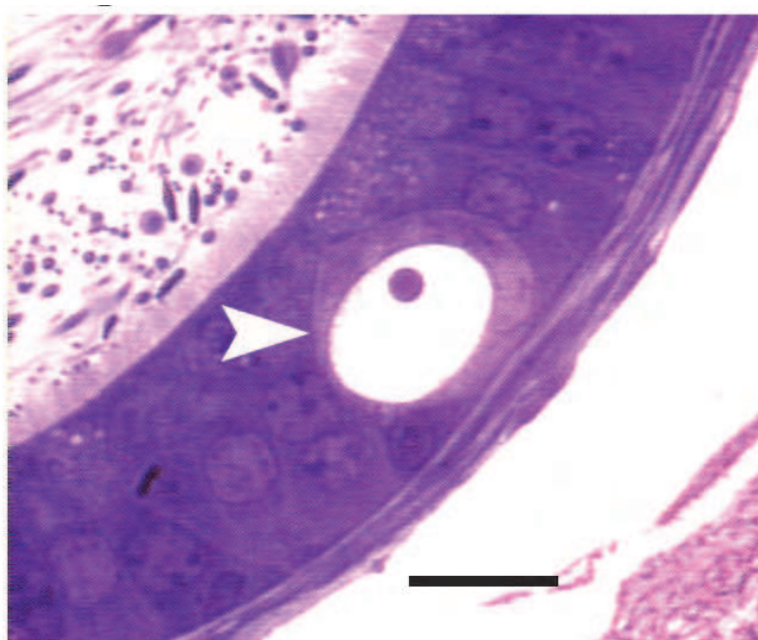

Fig. 9. Section of epididymal duct at caput of a treated mouse showing a pale vacuolated epithelial cell (arrowhead) with a large vacuole containing a dense PAS positive material. Paraffin section, PAS and hematoxylin staining. Scale bar, 4 μm.

Presumably, the PVEC acts upon the material enveloped, through digestion in the vacuole, followed by endocytotic uptake, lysosomal digestion and absorption. Hence, it was proposed that the PVEC develops from the basal cell as a protective device against the autoimmune response to spermatozoa in the context of pathological changes in the principal cells. Though the underlying mechanism of development of PVEC may be either due to androgen deprivation or direct toxicity of AFB1 to the epididymis, the onset of the development of PVEC is due to the pathological change in one or more of the principal cells.

Subsequently, Faisal et al., (2008b) reported the presence of epididymosomes in the AFB1 treated rats (Fig. 10). Epididymosomes, the apocrine secretions from the epithelium of epididymis, are found to be associated with a complex mixture of proteins and play a critical role in the transfer of proteins to sperm surface towards their post-testicular maturation (Frenette et al., 2006; Saez et al., 2003; Thimon et al., 2008). Two or more epididymal spermatozoa embedded in a dense matrix were observed. Such spermatozoa underwent disintegration to varying degrees (Fig. 11) starting with the outer membrane and then the mitochondrial sheath/fibrous sheath, microtubule doublets and ODFs, in that order. From the transmission electron micrographs, it was seen that when the lumen abounded with the defective spermatozoa, there was profuse discharge of epididymosomes. This was further strengthened by the observation of abundant matrix-entangled spermatozoa in the epididymal lumen (Fig. 11). Thus, it was suggested that the epididymosomes in this context are concerned with contributing the dense matrix and the enzymatic mechanism for degradation/dissolution of the defective spermatozoa, thereby excluding the normal sperm from the enzymatic degradation, which is an aspect of versatility of epididymis.
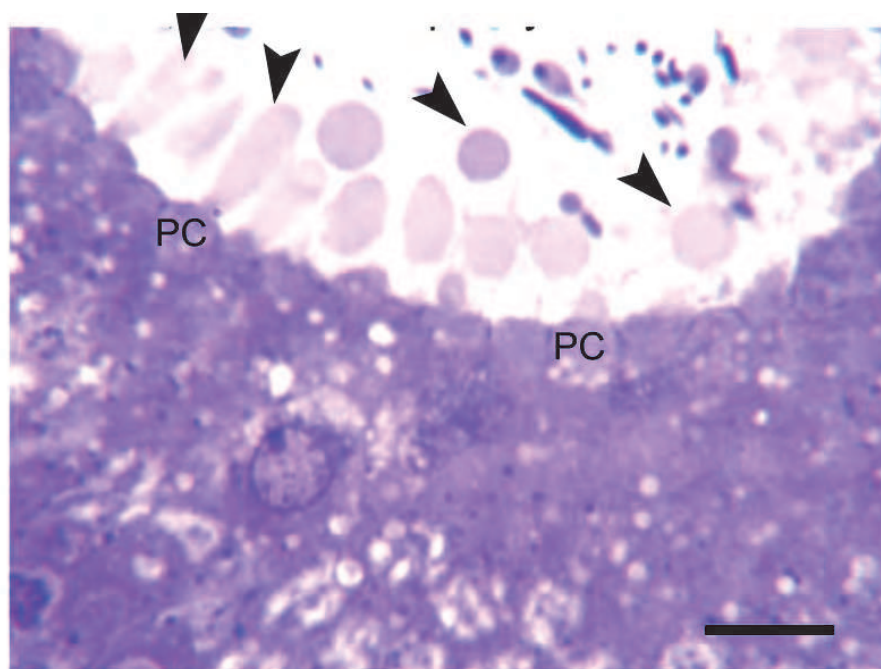

Fig. 10. Section of epdididymal duct at initial segment of a treated mouse showing release of epididymosomes (arrowheads) from the principal cells (PC). The lumen contains epididymosomes (arrowheads) and a few sperm. Semithin section, TBO staining. Scale bar, 4 μm.
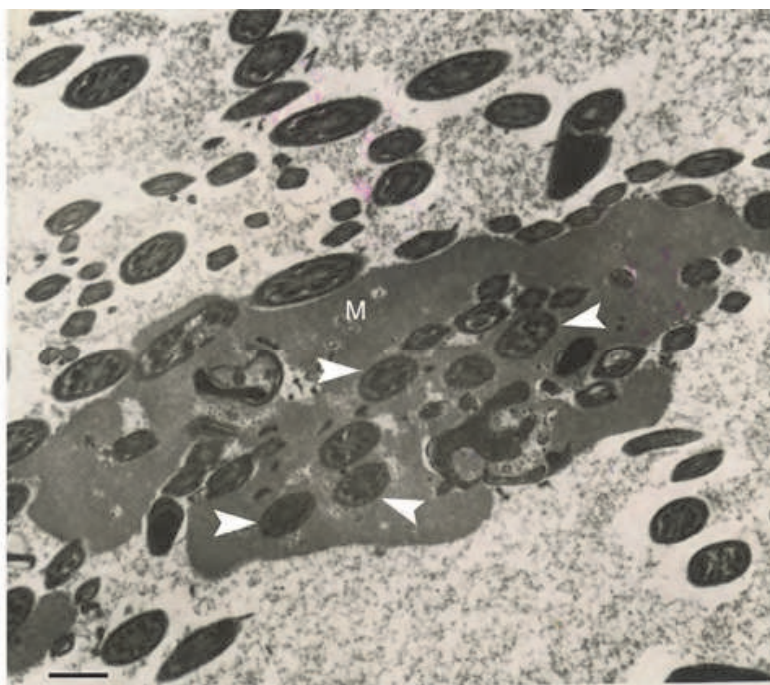
Fig. 11. A transmission electron micrograph showing corpus epididymidal spermatozoa (arrowheads) embedded in a dense matrix (M). Note the disintegration of spermatozoa to various degrees. The dense matrix is surrounded by normal epidiymal plasma, in which normal spermatozoa are found. Scale bar, 1 µm.

Epididymal epithelial cells are, by and large, terminally differentiated cells and do not usually divide unless in case of induction into mitosis. Under this background, we found in about 60% of AF treated mice and rats the principal cells of the initial segment of the epididymis were provoked into mitosis (Fig. 12A, B). Thus, AF could be a potent mitogenic agent, and potentially carcinogenic agent in respect of epididymis (Agnes, Faisal and Akbarsha, unpublished observation).

8. Manifestations in sperm

Agnes & Akbarsha, (2003) assessed the changes in the sperm. There was little change in the sperm concentration of mice treated AFB1 for 7 and 15 days, whereas in the mice treated for 35 and 45 days there was a drastic reduction in sperm concentration. In the mice treated for 35 days, this decreased to about 32% of the control and in those treated for 45 days it decreased to 19%. Sperm motility also displayed the same trend, again in a duration-dependent manner.

The percentage of sperm with abnormal morphology increased on AFB1 treatment in a manner dependent on the duration of the treatment. The various head abnormalities included head without the hook, unusual head shapes, vacuolation of the head and incomplete head. The major tail abnormality was bent or coiled tail. In each treatment group 20–40% of the mice had sperm head detached from the flagellum. Also, a considerably high percentage of sperm had sticky flagellum. Several sperm remained fused in varying numbers over short to long distances and several sperm were agglutinated. More recent studies also reported low sperm concentration, reduction in sperm motility, increased sperm abnormal morphologies and, additionally, decrease in the viability of spermatozoa of mice treated with aflatoxin (Abu El-Saad & Mahmoud, 2009; Mathuria & Verma, 2008).
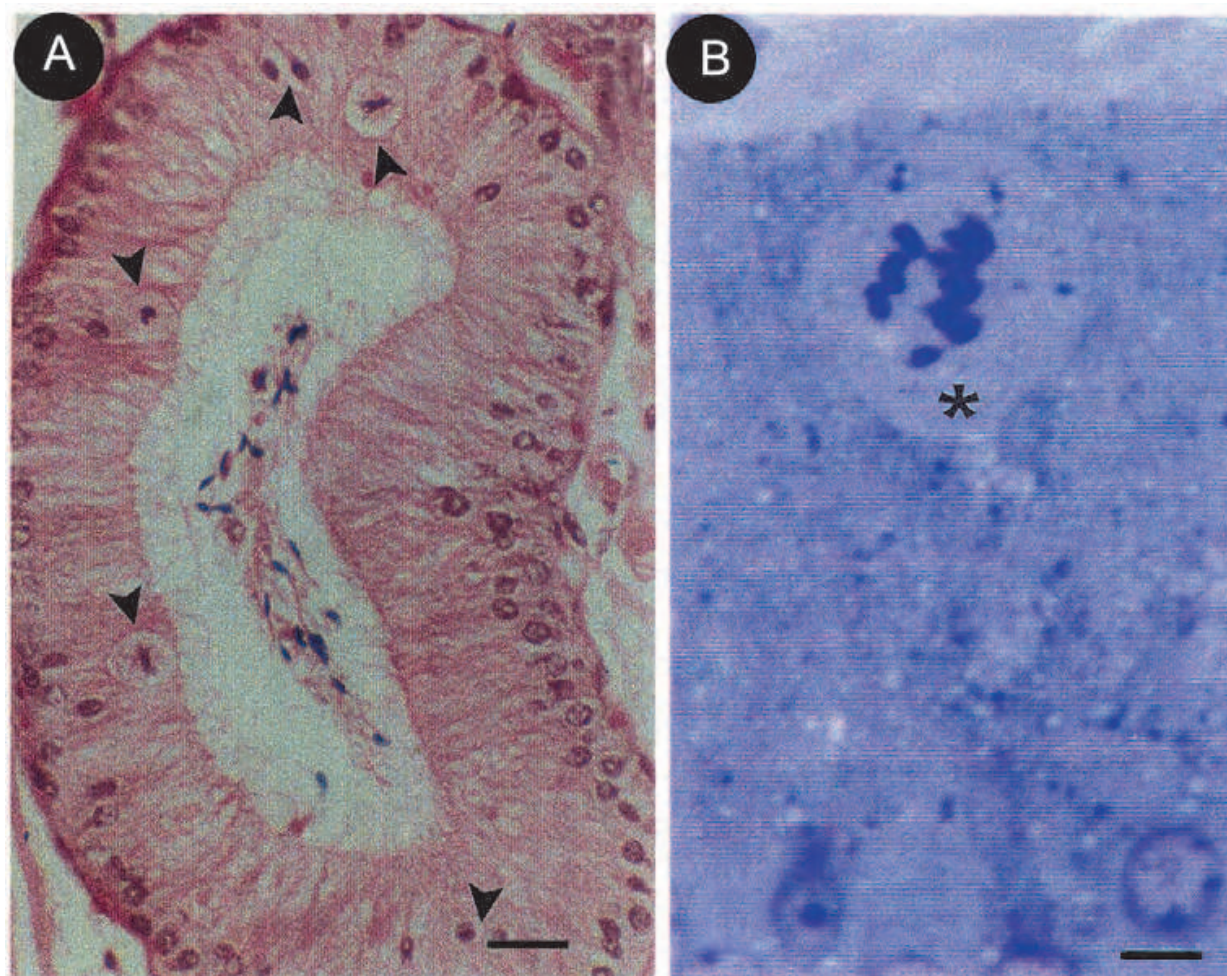
Fig. 12. Sections of the initial segment of the epididymis of treated mice. A: Many mitotic cells (arrowheads) are shown. B: One of the cells in mitosis clearly shown. A, paraffin section, PAS and hematoxylin staining. Scale bar, 10 µm; B, semithin section, TBO staining. Scale bar, 4 µm.

Another major observation was retention of predominant cytoplasmic droplet (CD) by the cauda epididymidal sperm of AFB1-treated mice (Fig. 13A). The quantitative assessment of retention of CD revealed that it increased in the duration-dependent manner of the treatment. In several such spermatozoa, large highly electron dense inclusions were found in the CD (Agnes & Akbarsha, 2003). Spermatozoa with two axonemes in a common cytoplasm were observed among which, in few cases, the axonemes contained the lamellar and vesicular elements of the CD (Fig. 13B).

Apart from the impact on the sperm count, another important factor to be accounted for determining fertility status in the male is the motility of the sperm. In this study, sperm motility was found to be impaired. The factors affecting motility are to be looked at among the endogenous and exogenous factors namely machinery for motility and contribution of the epididymis towards the physiological maturation of the sperm, respectively (Cooper et al., 1998). Considering these histopathological changes, it was speculated that AFB1 treatment through a direct effect on epididymis or indirectly through the Leydig cells, affects the epididymal function of physiological maturation of sperm, leading to an impairment of sperm motility. However, a direct effect of AFs on the epididymal sperm count can not be ruled out,

since Ibeh et al., (1994) have shown AF to be present in the human semen, and Picha et al., (1986) reported high levels of AFB1 residues in the seminal plasma of boars.
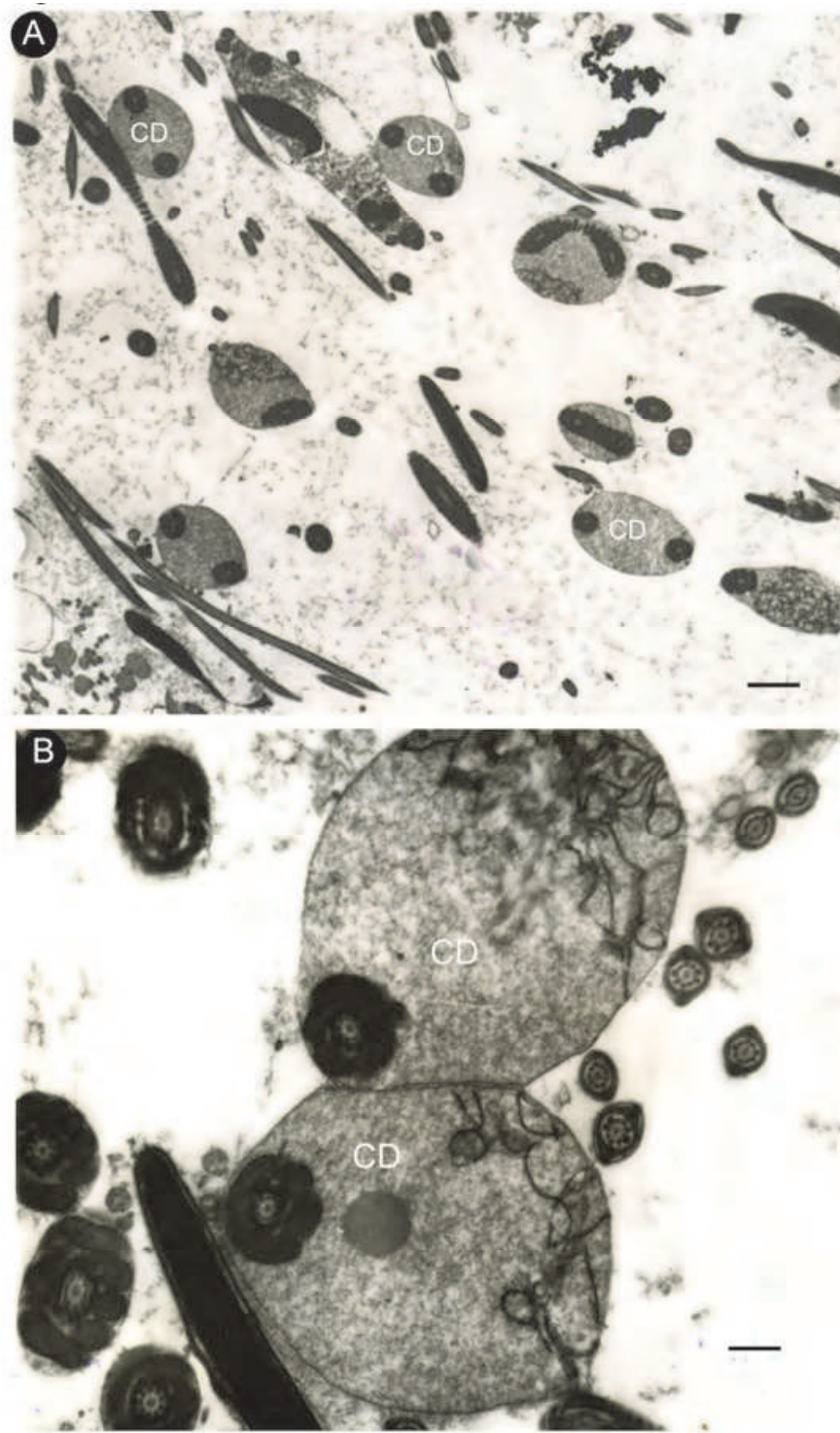

Fig. 13. Transmission electron micrographs showing cauda epididymidal spermatozoa retaining the cytoplasmic droplet (CD). A: Almost all the sperm retaining the cytoplasmic droplet. B: Two seprmatozoa retaining CD, which contains lamellar and vesicular elements, charateristic of CD. A: Scale bar, 2 μm; B: 0.2 μm.

Another intriguing observation was extrusion of one or more outer dense fibres (ODFs) along with the respective microtubule doublets of the axoneme at the midpiece –principal piece junction and/or connecting piece of rat sperm (Fig. 14) (Faisal et al., 2008b). The ODFs took either of two following courses. In one course the ODFs were disorganized and lost their connection with the axoneme. In the second course ODFs underwent slow disintegration such that in some sections there was no trace of ODFs on one side but those on the other side were intact. The affected spermatozoa did not exhibit forward progressive motility but a few exhibited sideways lashing of the flagellum at the very early stages but, subsequently, the lashing also stopped. There were also sperm mid-piece sections without any trace of plasma membrane, mitochondrial sheath and even axoneme in some cases, leaving only the ODFs intact. In many spermatozoa ODFs, in varying numbers, also disintegrated. These sections were not revealing identity as belonging to sperm mid-piece (Faisal et al, 2008b).
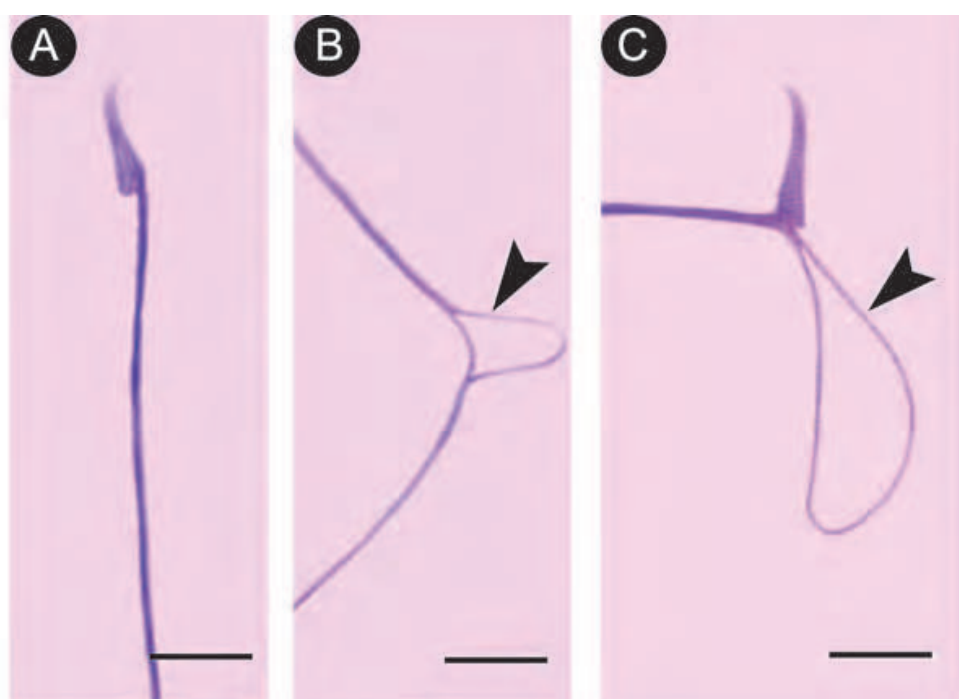

Fig. 14. Eosin and nigrossin stained spermatozoa of rat. A: Normal sperm, B: ODFs on one side are extruded (arrowhead) at the midpiece-principal piece junction, C: ODFs on one side extruded (arrowhead) at the connecting piece. Scale bar, 4 μm.

The manifestations in the principal piece were different from the above. Here, the fibrous sheath and the plasma membrane were lifted off from the ODFs - axoneme complex. With the fibrous sheath (FS) remaining intact, the ODFs and the axonemal doublets on one side or all around the circumference disintegrated, and in the latter case the principal piece in transverse section appeared as an empty vesicle. In some transverse sections of spermatozoa, the ODFs on one side were missing but such missing ODFs were found outside the fibrous sheath, i.e., between the fibrous sheath and the sperm plasma membrane.

9. Effect of AFB1–treatment on Leydig cells

In the mice treated AFB1, two trends were noticed. In the mice treated AFB1 for 7 days the Leydig cells underwent hypertrophy, and dark dense vesicles accumulated in the cytoplasm

(Fig. 15). 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. Histometric analysis of Leydig cells of AFB1-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 3) (Faridha et al., 2006).
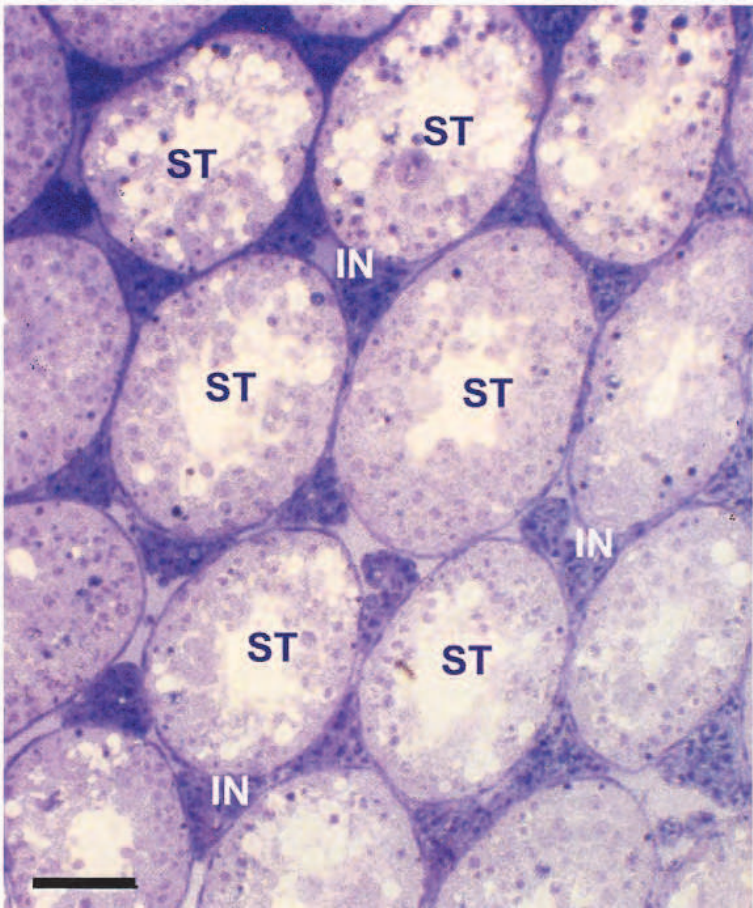

Fig. 15. Section of the testis of a treated mouse showing seminiferous tubules (ST), wtih various histopathological changes, and the interstitium (IN) showing Leydig cells which are dense, hypertrophied and densely vacuolated. Semithin section, TBO staining. Scale bar, 20 µm.

Duration of treatment	Counts per 10^3 um^2 area		Leydig cell perimeter (um)		Leydig cell nuclear diameter (um)	
	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 days	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*

Table 3. Leydig cell counts, perimeter and nuclear diameter of Leydig cells of AFB1-treated mice. Each value is mean ± SD of 25 measurements made at x400 with sections from the right testis of 5 animals. *$p<0.001$

10. Effect of AFB1 on fertility in the male

There was no change in the litter size of female mice mated with male mice treated AFB1 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 4) (Faridha et al., 2006). It was earlier reported that a number of young pups had abnormalities such as stumpy tail and blindness of one eye, and there was greater mortality of the pups (Agnes & Akbarsha, 2003).

Duration of AFB1 treatment	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*

Table 4. Results of fertility test of treatment group.

11. Conclusions

It has long been suspected, based on epidemiological studies on humans and animals, and experimental studies on fish, poultry, cattle, ram, boar, rat, mouse, etc., that dietary aflatoxins, on chronic exposure at small doses, could be causing disturbance to male reproductive mechanisms. In this background, a series of investigations were undertaken by the authors of this chapter and their students where in Swiss mouse and Wistar rat were treated with aflatoxin B1 through intra-peritoneal route, at a concentration of 20 μg per kg body weight per day (50 μg per kg bw in one study), in chronic male reproductive toxicity testing, for selected durations in relation to the duration of one spermatogenic cycle of the respective animals. The investigations led to the conclusion that aflatoxin B1 is severely toxic to male reproductive mechanisms. The manifestations include severe histopathological changes in the testis, affecting both spermatogenic and androgenic compartments. In the spermatogenic compartment the seminiferous epithelium is severely disrupted resulting in loss of germ cells to various degrees. This loss is preceded by hampering of division (mitotic as well as meiotic) of germ cells, resulting in uninucleate and symplastic giant cells. Meiotic micronucleate giant cells are also produced in large numbers. Tubulin of microtubules of the spindle apparatus appears to be the immediate target to aflatoxin in this case. The affected germ cells are prematurely released from the Sertoli cell. Thus spermatogenesis is severely hampered, resulting in decrease of sperm counts. Motility and viability of the spermatozoa are also impaired. Spermatozoa end up with a variety of abnormal morphologies. Leydig cells undergo hypertrophy and/or hyperplasia, and thorough cytoplasmic vacuolation, which indicate impairment of androgen secretion. The epididymis also undergoes histopathological changes, the most important of which is degeneration of principal cells of the epithelium, access of spermatozoa into these cells, and development of pale vacuolated epithelial cells to deal with such spermatozoa so as to circumvent an autoimmune response to the sperm antigens. Aflatoxin could also be mitogenic in the principal cells of initial segment of the epididymis, suggesting carcinogenic potential of aflatoxin in the epididymis. The fertility of the treated animals is highly compromised. Thus, chronic exposure of

humans and animals to aflatoxins, which is possible through dietary contamination, particularly in the tropical climate of developing countries, can bring about deterioration of male reproductive health.

12. Acknowledgments

13. References

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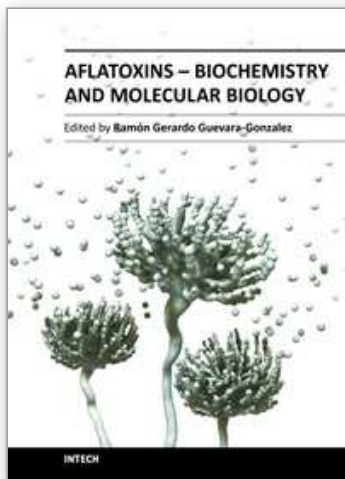
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Aflatoxins - Biochemistry and Molecular Biology

Edited by Dr. Ramon G. Guevara-Gonzalez

Aflatoxins â€" Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

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