Spermatotoxic effect of aflatoxin B1 in rat: extrusion of outer dense fibres and associated axonemal microtubule doublets of sperm flagellum

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Abstract

Male Wistar rats were treated with aflatoxin B1 (AFB1). Live as well as methanol-fixed cauda epididymal spermatozoa were stained with acridine orange (AO) and ethidium bromide (EB) and observed under a fluorescence microscope. Giemsa-stained smears were observed in a bright field microscope. Unstained smears were observed with phase contrast illumination. The axoneme of more than 10% of the spermatozoa of treated rats had the outer dense fibres (ODFs), in varying numbers, and the associated axonemal microtubule doublets of the flagellum extruded either at midpiece–principal piece junction or connecting piece. This could be perceived in all light microscopic preparations, but AO–EB staining offered an advantage of the assessment of the viability as well. TEM observation of sections of the testis and cauda epididymidis also revealed ODF extrusion, as seen in the transverse sections of sperm flagella missing one or more ODFs and the associated axonemal microtubule doublets. In a few such sections, the extruded elements were seen in the cytoplasm, outside the mitochondrial sheath or peripheral sheath. Marginal to severe mitochondrial pathologies were observed in the spermatozoa and elongated spermatids, suggesting a link between AFB1-induced sperm mitochondrial pathology and extrusion of ODFs. However, the possibility that AFB1 treatment would disrupt the cytoskeletal proteins of the flagellum, resulting in the extrusion of ODFs, cannot be excluded. This sperm abnormality is reported for the first time as produced by a dietary toxin. Dietary aflatoxins, therefore, could also be contributory factors for the deterioration of the reproductive health of men.

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Introduction

There has been serious concern in the recent past regarding the deterioration of human and animal male reproductive health (Carlsen *et al.* 1992, 1993, Sharpe 1992, Sharpe & Skakkebaek 1993, Veeramachaneni 2000, Handelsman 2001). The contributory factors for this deterioration have been traced to environmental, industrial and occupational chemicals, therapeutics, dietary toxins, lifestyle factors, etc. (Skakkebaek *et al.* 1998, Herath *et al.* 2004, Aruldas *et al.* 2006, Slama *et al.* 2006, Winters *et al.* 2006). Some of the manifestations of this deterioration include oligozoospermia, azoospermia, teratozoospermia, asthenozoospermia and oligoteratoasthenozoospermia. In these cases there is a defective spermatogenesis at the level of the testis and/or incomplete epididymal processing of spermatozoa (Yanagimachi 2005, Sharpe 2006). Such spermatozoa are abnormal in morphology and/or motility, which are pathophysiological attributes, and the ejaculates contain these sperm in varying abundance (Sharpe 2006). This affects not only *in vivo* reproduction

in animals and men but also the outcomes of *in vitro* fertilization as well as intracytoplasmic sperm injection. A few reports have shown that such pathological spermatozoa are acquired by the epididymal epithelial cells and processed, suggesting a newer role to epididymal epithelial cells (Seiler *et al.* 2000, Sutovsky *et al.* 2001a, 2001b, Holschbach & Cooper 2002, Aruldas *et al.* 2006).

Aflatoxins are toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* and are naturally occurring contaminants of food. Aflatoxin poisoning is a recurrent public health problem. More than 5 billion people in developing countries worldwide are at risk of chronic exposure to naturally occurring aflatoxins, aflatoxin B1 (AFB1), AFB2, AFG1 and AFG2, through contaminated foods (Shephard 2003, Williams *et al.* 2004) and more so in the tropical regions, where 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). Aflatoxins can produce serious health effects including carcinogenesis (Williams *et al.* 2004, Preston & Williams 2005, Abnet

2007), mutagenesis (Wang and Groopman 1999, Peters & Teel 2003), growth retardation (Gong *et al.* 2002, 2003, 2004, Williams *et al.* 2004) and immune suppression (Turner *et al.* 2003, Williams *et al.* 2004). Although a great deal is known about aflatoxins, little is known about aflatoxin exposure and the resulting health effects in developing countries (Strosnider *et al.* 2006). Thus, though male reproductive toxic effects of aflatoxins in humans have not yet been investigated, such an effect cannot be eliminated in the light of reports coming from domestic and experimental animals (Faridha *et al.* 2007 and the references therein) and also from a human study correlating aflatoxin content of semen and semen parameters of infertile men (Uriah *et al.* 2001).

Our laboratory has been concerned with male reproductive toxicological evaluation for several years and we found disruption of spermatogenesis (Faridha *et al.* 2006, 2007) and production of defective spermatozoa (Agnes & Akbarsha 2001, 2003) when Swiss mice were treated with AFB1, the most potent and potentially lethal metabolite. Susceptibility to aflatoxins can differ between test animals (Bennett & Clich 2003). Therefore, in order to find if the male reproductive toxic effect of AFB1 would differ between species, and if pathophysiological spermatozoa thus produced are ubiquitinated for acquisition by epididymal epithelial principal cells (Seiler *et al.* 2000, Sutovsky *et al.* 2001a, 2001b, Holschbach & Cooper 2002, Aruldas *et al.* 2006), we repeated the investigation in the Wistar rats. We practiced a single dose (20 µg/kg body weight) at one time point based on the standardization done in our earlier work (Agnes & Akbarsha 2001, 2003, Faridha *et al.* 2006, 2007). We found several sperm abnormalities, and most spermatozoa were not motile, as was the case in the mouse (Agnes & Akbarsha 2001, 2003). In addition, we found evidence for the extrusion of outer dense fibres (ODFs) along with the associated axonemal microtubule doublets from the flagella of more than 10% of the sperm, either at the connecting piece or at the mid-piece–principal piece junction. Though this abnormality has been reported as caused by a few other etiological factors, this is the first report to suggest that it is due to treatment with a dietary toxin. Transmission electron microscopy (TEM) evidence points to a link between this sperm pathology and mitochondrial defects, although disruption of cytoskeletal polymeric proteins cannot be excluded.

Results

Unlike the control rats, in which $95 \pm 5\%$ of the sperm had normal morphology (Fig. 1A) and $94 \pm 4\%$ (concentration $62 \pm 6 \times 10^6/\text{ml}$ of the diluted cauda epididymal fluid) made forward progressive motility for an hour and more, in the AFB1-treated rats, as observed in bright field, dark field and/or phase contrast illumination, the sperm concentration was significantly

lower ($20 \pm 4 \times 10^6/\text{ml}$ of the diluted cauda epididymal fluid) and $63 \pm 8\%$ of these sperm had morphological abnormalities. The most predominant sperm abnormality was detachment of head from the flagellum ($32 \pm 9\%$ in the treated, as against $3 \pm 1\%$ in the control; Fig. 1B). Red fluorescence of the detached heads indicated these, and several intact spermatozoa, as not viable. In addition, more than $11 \pm 1\%$ of spermatozoa exhibited abrupt narrowing of the flagellum at the mid-piece–principal piece junction and subsequent extrusion of ODFs and the associated axonemal microtubule doublets at these narrow regions, which we did not observe in any of the spermatozoa of control rats. In the earliest appearance, this region measured 1–2 µm long (Fig. 1C and G) but could measure up to 9–10 µm (Fig. 1D–F and H–O). The ODFs and the associated axonemal microtubule doublets were extruded from this region in the form of one or more arches, each to various degrees (Fig. 1G–X). These 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 stopped. At more advanced stages, the entire fibrous sheath (FS) at the mid-piece–principal piece junction was flexed to one side such that all the ODFs and the associated axonemal microtubule doublets flayed out (Fig. 1V). These manifestations were also present in spermatozoa, which were fused in various numbers and to various lengths (Fig. 1S).

The connecting piece also had this manifestation and, ultimately, ended up with one or more ODFs and the associated microtubule doublets of the axoneme extruding as individual fibres (Fig. 2A–F). The observations made of the methanol-fixed sperm stained with Giemsa and unstained sperm viewed with phase contrast illumination were the same as above (not shown).

TEM analysis of spermatozoa in transverse sections through the mid-piece or principal piece revealed that several of them were missing one or more ODFs and the associated axonemal microtubule doublets (Fig. 3A–D). In a few sections of the principal piece, the ODF–axonemal microtubule doublet complexes were seen between the FS and plasma membrane (Fig. 3D). In the spermatozoa with normal flagella, the mitochondrial sheath (MS) was intact (Fig. 4A). On the other hand, the MS under formation of several elongated spermatids in the seminiferous tubules (Fig. 4B) and the cauda epididymal luminal spermatozoa (Fig. 4C) of AFB1-treated rats indicated pathological changes of different kinds, the most prevalent being swelling of several mitochondria and their missing, irregular or discontinuous spacing. In the normal spermatozoa, the connecting piece (Fig. 5A) as well as midpiece–principal piece junction (annulus; Fig. 5C) had the respective characteristic organizations. However, in several abnormal elongated spermatids and differentiated spermatozoa, the MS ended abruptly either subadjacent to the connecting

Figure 1 AO-EB stained spermatozoa of AFB1-treated rat observed in a fluorescence microscope. (A) A normal viable sperm; (B) detached heads, non-viable; (C)–(F) spermatozoa showing a narrow region at the mid-piece–principal piece junction (indicated by an arrowhead or square bracket); (G)–(X) extrusion of one or more ODFs and the associated axonemal microtubule doublets to varying degrees (arrowheads; note the extrusion of ODFs from the flagella of fused spermatozoa in S, and sideways flexing of the peripheral sheath, exposing all ODFs and the associated axonemal microtubule doublets, in V). Scale bar: 15 μ m.

piece (Fig. 5B) or ahead of the midpiece–principal piece junction (Fig. 5D), resulting in a short length of axoneme at these critical positions, from where the ODF–axonemal microtubule doublets would extrude. Further, while there was dense accumulation around the striated columns and capitulum of the connecting piece, in several steps 15–19 spermatids were not properly developed (Fig. 5A and B).

Discussion

Aflatoxins are among the serious food contaminants (Williams *et al.* 2004, Strosnider *et al.* 2006) and disruptors of male reproductive structure and function through the dietary route (Egbunike *et al.* 1980, Sharlin *et al.* 1980, Egbunike 1982, Hafez *et al.* 1982, Ibeh *et al.* 1994, Ibeh & Saxena 1998, Agnes & Akbarsha 2001,

Figure 2 AO-EB-stained spermatozoa of AFB1-treated rat observed in a fluorescence microscope, showing extrusion of one or more ODFs and the associated axonemal microtubule doublets, to varying degrees, at the connecting piece (arrowheads). Scale bar: 15 μ m.

Figure 3 TEM of transverse sections of principal piece of spermatozoa of AFB1-treated rats. (A) Normal organization; (B) slight disruption of the axoneme and ODF; (C) one axoneme and the associated axonemal doublet are missing (arrowhead); (D) ODFs and associated microtubule doublets on one side are missing (arrowheads) and are seen in the cytoplasm between the fibrous sheath and sperm membrane (wide arrows). AD, axonemal doublet; FS, fibrous sheath; PM, sperm membrane; ODF, outer dense fibres. Scale bar: 0.24 µm.

2003, Faridha *et al.* 2006, 2007). Focused studies on the spermatotoxic effect of aflatoxins in animal models have revealed that the exposure would affect the morphology as well as physiology of spermatozoa (Hafez *et al.* 1982, Agnes & Akbarsha 2003). The various sperm abnormalities reported in AFB1-treated mice consisted of head without the hook, several unusual shapes, vacuolation of the head, incomplete head, bent or coiled tail, detachment of the head from flagellum, fusion of flagella of two or more sperm over short to long distances and retention of cytoplasmic droplets (Agnes & Akbarsha 2003). This paper reports, in addition, extrusion of one or more ODFs along with the respective microtubule doublets of the axoneme at the mid-piece–principal piece junction and/or connecting piece of rat sperm.

Aflatoxins or their metabolites can reach the testis (Bukovjan *et al.* 1992) and be present in the semen through this route (Picha *et al.* 1986, Ibeh *et al.* 1994, Uriah *et al.* 2001). Infertile men with high concentrations of aflatoxins in semen had decreased sperm counts and increased incidence of sperm abnormalities (Uriah *et al.* 2001). Thus, there is an imminent link between chronic exposure of men to aflatoxins and male reproductive health. Therefore, generating data from human studies in this regard and understanding the manifestations and the mechanisms of action from animal studies are highly relevant in the light of poor understanding of the health

effects of aflatoxins (Strosnider *et al.* 2006). Thus, this study contributes to the understanding of the male reproductive health effects of aflatoxins, particularly the manifestations in the affected sperm.

A thorough screening of literature revealed that this kind of sperm abnormality has been reported only in very few instances. Olson & Linck (1977) reported extrusion of axonemes from demembranated rat spermatozoa in the presence of ATP. Cooper & Hamilton (1977) found that in several spermatozoa caught in a dense material occurring in patches in the lumen of epididymis and vas deferens, the MS was no longer intact and there was complete disruption and dissolution of the ODFs and axonemes. Olson *et al.* (2004) found similar abnormal spermatozoa produced in selenium-deficient rats. In these spermatozoa, the MS terminated prematurely, producing gaps in the most proximal region of the MS accompanied by separation and/or extrusion of the underlying fibrils of the ODF–axonemal microtubule doublet complex. The extrusion of flagellar fibres was noticed in the connecting piece also. Olson *et al.* (2005) generated selenoprotein P (SEPP1)-null male mice, which also produced sperm of this kind. Andersen *et al.* (2003) found sperm with morphological abnormalities produced in apoER-2 deficient mice. Several sperm produced by these mice had defects comparable to those in the present report (TG Cooper, personal communication). Thus, relying on

Figure 4 TEM of longitudinal sections of the middle piece of spermatozoa of AFB1-treated rats. (A) Normal sperm from the cauda epididymidis showing intact and organized mitochondrial sheath; (B and C) two adjacent elongated spermatids in the seminiferous epithelium showing swelling (wide arrows) and aberrant spacing (arrowheads) of mitochondria in the mitochondrial sheath. AX, axoneme; MS, mitochondrial sheath. Scale bar: 0.33 µm.

Figure 5 TEM of longitudinal sections of spermatozoa of AFB1-treated rats. (A) Normal cauda epididymidal sperm showing highly organized connecting piece; (B) defective elongated spermatid in the seminiferous epithelium showing abrupt ending of the mitochondrial sheath with vacuolated mitochondria and disruption of the dense material around the striated columns (arrowhead); (C) midpiece–principal piece junction of a normal sperm showing the annulus (arrowhead) and the complete mitochondrial sheath (arrow); (D) A defective sperm in which the mitochondrial sheath has ended abruptly ahead of the annulus (arrowhead) such that the axoneme is not wrapped around by the mitochondrial sheath (arrow), at the annulus, outside the axoneme. AX, axoneme; FS, fibrous sheath; MS, mitochondrial sheath; NU, nucleus; SC, striated columns. Scale bar (A and B): 0.43 μm ; (C and D): 0.40 μm .

the interpretation of Olson *et al.* (2004, 2005) and our TEM observations in respect of the pathological features of the MS, we presume that this flagellar defect could be related with abnormal development of the MS during spermiogenesis. The evidence further suggests that the AFB1-induced flagellar defect has a testicular origin as in the case of selenium-deficient rat (Olson *et al.* 2004) and SEPP1-null mice (Olson *et al.* 2005). Interestingly, the nutrition of both zinc and selenium is affected by aflatoxins in the diet (Kalorey *et al.* 1996, Mocchegiani *et al.* 1996). Thus, selenium deficiency caused due to AFB1 could be one of the possible mechanisms in generating this kind of abnormal spermatozoa, a point worth investigating. The other possibility is that aflatoxin-induced defect in the late maturation phase in the spermiogenesis results in a missing segment (gap) of the MS as has been observed in the spermatozoa of rat treated with gossypol (Hoffer 1982, Oko & Hrudka 1982, Xue *et al.* 1983, Agarwal 1989, Barth & Oko 1989, Bhiwgade & Nair 1989, Swan *et al.* 1990).

The affected spermatozoa travel through the epididymis with structural weakness in the sheath leading to protrusion of axial fibres at this level, accompanied by retraction of the fibres from the parts of the tail below the gap (Oko & Hrudka 1982). In this case, the problem is with the MS which is apparently affected by gossypol (Barth & Oko 1989) or aflatoxin treatment (present study). Both gossypol and aflatoxin possess a phenolic ring in their chemical structures and this could be the basis of the induction of defects in the mitochondria by uncoupling oxidative phosphorylation, eventually causing the spermatid mitochondria to swell (Tso & Lee 1982).

However, an effect on the cytoskeletal structures of the motility apparatus of sperm should also be considered seriously. Si & Okuno (1993) treated activated mouse spermatozoa with Triton X-100 and dithiothreitol to remove the plasma membrane and MS of sperm flagella. When the mitochondria-free demembranated flagella were perfused with Mg-ATP and trypsin, the microtubule doublet of the axoneme and the FS were pulled proximally to the principal piece from the annulus. This FS sliding and the order of doublet ODF extrusion were trypsin concentration dependent (Si & Okuno 1995). Kinukawa *et al.* (2004) found microtubule extrusion in demembranated sperm of hamster and mouse treated with huge concentrations of protease inhibitor and dithiothreitol or 2-mercaptoethanol (2-ME). Sperm microtubule extrusion was also reported in elastase-treated human spermatozoa (Ishijima *et al.* 2002) and Triton X-100-treated bovine spermatozoa (Kanous *et al.* 1993). These studies linked microtubule extrusion to protease digestion. Aflatoxins can adduct with albumin and other proteins (Dash *et al.* 2007). This adduction can affect the protein structure and function. The structural integrity and normal functioning of sperm depend greatly on the several cytoskeletal proteins such as actin, tubulin, vimentin, tektin, septin, spectrin, ankyrin, etc. of flagella (Irons 1983, Kann *et al.* 1993, 1998, Paranko *et al.* 1994, Schalles *et al.* 1998, Inaba 2003, Ihara *et al.* 2005, Iida *et al.* 2006, Azamar *et al.* 2007, Roy *et al.* 2007, Touré *et al.* 2007, Vaid *et al.* 2007, Xiao & Yang 2007). Aflatoxins are potential culprits of disrupting cytoskeletal protein dynamics (Koo *et al.* 1987, Wirth 1994, Albertini *et al.* 1988). There is indirect evidence for the disruption of actin microfilaments at the cytoplasmic bridges connecting male germ cell clones in the mouse (Faridha *et al.* 2007).

In making this observation, we adopted several light microscopic techniques in addition to TEM. The application of AO–EB staining has the advantage of revealing not only the morphological abnormalities but also the sperm viability. This technique has been applied for the spermatozoa in two earlier studies, Le Lannou & Blanchard (1988) and Sivashanmugam & Rajalakshmi (1997), though not for the detection of sperm abnormalities and viability.

The cause for the loss of viability of spermatozoa of AFB1-treated rat is another area worthy of investigation. The morphological abnormalities, including breaking away of the head from flagellum and extrusion of ODFs and associated axonemal doublets, could be one of the immediate causes. However, the AO–EB staining pattern of sperm nuclei indicates DNA strand breaks, as revealed in the AO staining, as well as the loss of membrane integrity and chromatin condensation, as revealed in the EB staining, and suggests targets including DNA. Aflatoxins can adduct with the DNA of the testis and, thus, bring about DNA damage of germ cells (Sotomayor *et al.* 1999).

Thus, our paper reports extrusion of one or more ODFs and the associated microtubule doublets of the axoneme of spermatozoa of AFB1-treated rats at the midpiece–principal piece junction and/or connecting piece; aflatoxin therefore could be a potential risk factor affecting reproductive health in men in developing countries chronically exposed to aflatoxins through the dietary route.

Materials and Methods

Institutional Animal Ethics Committee (IAEC), established under the auspices of Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, approved the experiment. Ninety-day-old Wistar strain male rats, raised from a stock obtained from the Indian Institute of Science, Bangalore, India, were used. AFB1 was obtained from Sigma Chemical Company and was dissolved in olive oil at 20 µg/ml concentration. The toxin was injected into ten rats (three months old) through i.m. route at a daily dose of 20 µg/kg body weight i.e. 0.2–0.25 ml/day, depending on the weight of the animal (Agnes & Akbarsha 2001, 2003) for 55 days, the duration of one spermatogenic cycle (Clermont 1962, Hess 1990) and the control animals in equal number received the oil alone. At the end of the treatment, five rats in each group were subjected to cervical dislocation and dissected to expose the testicles and epididymides. After a thorough wash in normal saline, 0.5 µl fluid from the cauda epididymidis was collected in a cannula and diluted with 99.5 µl PBS, pH 7.4, according to Akbarsha *et al.* (2000). The sperm numbers were assessed with a Neubauer counting chamber and expressed as number per ml of the original cauda epididymidal fluid. The duration and extent of motility of the diluted spermatozoa were assessed in a hanging drop preparation observed in phase contrast and/or dark field illumination in a Carl Zeiss Axioscope 2 Plus research microscope (Jena, Germany) at ×400 magnification. Giemsa-stained smears were observed in bright light at ×400 or ×1000. Unstained fresh sperm smears were observed in phase contrast illumination at ×400.

In order to find the viability of spermatozoa, fresh sperm were stained with acridine orange (AO) and ethidium bromide (EB), according to Spector *et al.* (1997). To a glass slide, 50 µl dilute semen was transferred using a micropipette and 10 µl each of AO (Sigma; 100 µg/ml in normal saline, pH 7.4) and EB (Bio-Rad; 100 µg/ml in normal saline, pH 7.4) were added

separately, according to Spector *et al.* (1997). A cover slip was placed on the drop and the edges were sealed with fingernail polish. Then the smears of sperm, fixed in methanol, were also stained. The preparations were observed in the same microscope, now with epifluorescent attachment. In all cases the images were captured in a Pentium III computer via a Sony DXC-151AP CCD camera (Tokyo, Japan) using Carl-Zeiss Axiovision image-analysis software. In all cases of counts of spermatozoa with morphological abnormalities, 200 randomly selected spermatozoa from each slide were observed and assigned to the categories viz., normal, head alone and flagellar defect of interest in this study (Mortimer & Mortimer 2005). The data were expressed as per cent of the total and used to calculate the mean ± s.d.

Thin sections of the testis and cauda epididymidis, collected from the remaining control and treated rats (five each) perfused with Karnovsky's (1965) fluid, were fixed in glutaraldehyde and post-fixed in osmium tetroxide (Hess & Thurston 1977). After a thorough wash in buffer (Hayat 1981), the sections were dehydrated in ethanol and cleared in propylene oxide. Infiltration was carried out in propylene oxide and Spurr's mixture (Sigma Chemical Co). The tissues were embedded in Spurr's mixture and semithin sections (1 µm thickness) were stained with toluidine blue-O (TBO) for selecting the areas for ultrathin sections. The latter were obtained in a Leica ultramicrotome (Jena, Germany) and stained in Reynold's lead citrate and 6% aqueous uranyl acetate. The sections were examined and those of elongated spermatids from the testis and spermatozoa from the cauda epididymidis were photographed with a transmission electron microscope (Phillips 201C; Phillips, Amsterdam, The Netherlands). The TEM images were scanned into the computer and the images, in all cases, were processed with Adobe Photoshop 7.0 software to clean the background and adjust the brightness and contrast.

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