ANNEXURES

PUBLICATIONS AND CERTIFICATES
Male reproductive toxicity of vincristine: ultrastructural changes in the epididymal apical cell

M. A. Akbarsha*, H. I. Averal, R. Girija, S. Anandhi and A. Faridha Banu

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Key words: male reproductive toxicology, epididymis, apical cell, narrow cell, endocytosis, lysosomes, vincristine

Abstract

The toxic effect of vincristine on the apical cells of the rat caput epididymis was investigated. The drug was administered at 20 and 40 μg/kg body weight daily for 15 days. Light microscopy using semithin sections, and transmission electron microscopy, of the caput epididymis were undertaken. The results revealed that the basal region of the apical cell was in contact with the basement membrane and the luminal end took part in endocytosis. The apical cells reflected a dose-dependent response to vincristine (VCR) treatment. In general the changes included protrusion of the apical ends deep into the lumen, with the nucleus of the cell located in such protruded ends, and an increase in the abundance of lysosomal bodies and multivesicular bodies. These changes reflected the physiological response of the apical cell to VCR treatment rather than toxic manifestations.

Introduction

The epididymis is a novel organ, present only in the amniotes, playing a significant role in the physiological maturation of the spermatozoa (Robaire and Hermo, 1988; Cooper, 1992, 1995a,b, 1998). Such a maturation is considered essential for the spermatozoa to become motile and to fertilize the ova (Cooper, 1990, 1993). The epididymal duct originated due to the joining of the ductuli efferentes at the initial segment, and subsequently differentiated histologically into caput, corpus and cauda epididymides (Hamilton, 1975; Robaire and Hermo, 1988).

The epithelial lining of the duct also participated in this differentiation through a decrease in the height of the columnar cells and differences in the distribution and relative percentage of the various cell types designated principal, narrow, clear, basal and halo cells (Reid and Cleland, 1957; Hamilton, 1975; Robaire and Hermo, 1988; Robaire and Viger, 1993).

The epididymal duct contributes to the physiological maturation of the spermatozoa by way of secretion of several proteins, glycoproteins and small molecular weight substances. Modification of the luminal fluid through absorption and secretion of organic and inorganic ions
Male reproductive toxicity of vincristine: ultrastructural changes in the epididymal apical cell

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The epididymal duct contributes to the physiological maturation of the spermatozoa by way of secretion of several proteins, glycoproteins and small molecular weight substances. Modification of the luminal fluid through absorption and secretion of organic and inorganic ions
and fluid, and phagocytosis of dead and defective sperm and debris from the lumen (Robaire and Hermo, 1988; Hermo et al., 1988) assists in the maturation process. Moreover, through these activities the epididymal duct contributes to a changing luminal micro-environment which is considered essential for the physiological maturation of the spermatozoa (Hinton and Palladino, 1995). However, the role of the various epithelial cell types in the processing of the spermatozoa and the fluid is not fully understood. In particular there is some controversy regarding the existence of the apical cell as an exclusive cell type in the epididymal epithelium. Recently, the apical cell has been revealed as a distinct cell type in the adult rat epididymis (Adamali and Hermo, 1996).

The epididymis as a target for the toxic manifestation of xenobiotics and drugs was indicated in a few recent studies (Trasler et al., 1988; Klinefelter et al., 1990; Klinefelter et al., 1992; Averal et al., 1996; Akbarsha and Averal, 1996; Akbarsha and Sivasamy, 1998; Robaire and Fan, 1998; Hess, 1998). Such toxic manifestations appear to be different and specific to the various cell types (Trasler et al., 1988; Klinefelter et al., 1990; Akbarsha and Averal, 1998, 1999a,b).

The different patterns of toxic manifestation at the ultrastructural level of the cancer chemotherapeutic and microtubule disrupting agent, vincristine (VCR), on the principal, narrow and clear cells of the rat epididymis have been described (Akbarsha and Averal, 1998, 1999a,b). Furthermore, the apical cell is a distinct cell type with an exclusive structure, distribution and function (Adamali and Hermo, 1996).

The present work was undertaken to discover the ultrastructural changes in this cell type in response to VCR treatment in the adult male rat.

**Materials and methods**

The methodology was explained in our earlier papers (Akbarsha and Averal, 1998, 1999a,b). Briefly, 90-day-old Wistar strain male albino rats were administered vincristine sulphate intraperitoneally (Tamil Nadu Dhadha Pharmaceutical Ltd, Madras, India), at two daily doses of 20 and 40 μg/kg body weight, diluted in physiological saline, for 15 days. The control rats received the saline. At the end of the experiments the reproductive system was perfused with Karnovsky’s (1965) fluid, and slices of the proximal part of the caput epididymis were immersion-fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide. The tissues were embedded in low viscosity resin.
(Spurr's mixture; Sigma, U.S.A.). Semithin sections (1 μm thickness) were stained in toluidine blue O for light microscopic observation, and ultrathin sections were stained in uranyl acetate and lead citrate for observation using a Phillips 200 electron microscope (Hayat, 1981).

Results
In the initial segment of the ductus epididymidis the apical and narrow cells could be clearly distinguished in the VCR-treated rats, even at the light microscopic level (Figures 1 and 2). Both these columnar cell types projected into the lumen at their apical ends, but they differed tinctorially and in cytoplasmic organization. The narrow cells had a very slender basal region, whereas in the apical cell the basal region was broader than in the narrow cells though narrower than in the principal cells.

Transmission electron microscopy (TEM) of the apical cell of control rats revealed the occurrence of an apically located nucleus, a few lysosomal bodies and mitochondria in the supranuclear cytoplasm.

Figure 1  Epithelium of the initial segment of the caput epididymidis of a control rat showing principal cell (p), apical cell (a) and narrow cell (n). Semithin section stained with toluidine blue O (TBO). x1,000.

Figure 2  VCR-treated rat as in Figure 1 (semithin, TBO stained). x1,000.
In addition, numerous endocytotic vesicles occasionally enclosed an electron dense material in the apical cytoplasm. The latter was fairly abundant with smooth as well as rough endoplasmic reticulum (SER and RER). The Golgi apparatus was less prominent than in the neighbouring principal cells. Apical microvilli were sparse and, when present, were short and stumpy (Figure 3).

In contrast, in the VCR-treated rats, the apical end of the apical cell was seen to project beyond the level of the principal cells, deep into the lumen, and such ends were seen to spread out over the apical ends of the principal cells (Figure 4). In some of the apical cells the nucleus was located at a level beyond the apical end of the principal cell. Apical microvilli were totally absent. The perinuclear cytoplasm abounded with small to large, and variously shaped, lysosomal bodies of varying electron density.

Large lysosomal bodies in the basal cytoplasm were evident as shown in Figures 1 and 2. In the apical cytoplasm there were pale as well as dark multivesicular bodies (MVB), more numerous in the VCR-treated rats, and interspersed among the lysosomal bodies (Figure 4).

![Figure 3](image_url) TEM of an apical cell of a control rat. Principal cell (p); apical cell (a); endocytotic vesicle (e); lysosomal bodies (l); arrow indicates a short, stumpy microvillus. x9,800.
The initial segment of the human caput epididymidis (Yeung et al., 1991), and the rat epididymis (Adamali and Hermo, 1996) showed that the apical and narrow cells were distinct cell types. The apical cell is a separate cell type, in the rat as in man, and the initial segment is important in the concentration of spermatozoa and the modulation of the ionic and fluid composition of the luminal content (Burgos, 1974; Robaire and Hermo, 1988; Ilio and Hess, 1992).

The overall shape and organization of the apical cell in respect of the nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum, lysosomes, multivesicular bodies and endocytotic vesicles were in accord with earlier work (Yeung et al., 1991; Adamali and Hermo, 1996; Dutt, 1999). However, whereas Adamali and Hermo (1996) were not sure whether apical cells really contacted the basement membrane, the present investigation has clearly established such a contact.

In the apical cell the VCR treatment produces a projection of the cell deep into the lumen with an increase in the abundance of lysosomal bodies and multivesicular bodies (MVB). In the control rats there were a few lysosomal bodies distributed only in the supranuclear cytoplasm. However, in the VCR-treated rats the lysosomal bodies were found throughout the cytoplasm and even at the apical extremities of the cell. Endocytotic vesicles, with particulate material in the lumen, were present in the apical cytoplasm of the apical cells in the control rats.

In the VCR-treated rats there was an increase in lysosomal bodies and MVB, which indicated a distinct endocytotic/absorptive role for the apical cell (Adamali and Hermo, 1996). Thus, treatment with VCR appeared to be solely physiological. Moreover, the principal, clear and narrow cells reflected pathological manifestations in response to VCR treatment (Akbarsha and Averal, 1998, 1999a,b).

In conclusion, we suggest that the apical cell does make contact with the basement membrane and that it responds to VCR treatment with increased endocytotic/absorptive activity.

Acknowledgements

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et al., 1987; Feuchter et al., 1987; Tingari, 1989; Goyal and Williams, 1991; Palacios et al., 1991; Palacios et al., 1993; Dutt, 1999).
In addition, endocytotic vesicles were found in the apical cytoplasm, but fewer than in the control. The manifestations in the apical cell were dependent on the dosage of the VCR treatment.

**Discussion**

Reid and Cleland (1957) were the first to identify the apical cell as a separate cell type in the rat epididymis. However, subsequent studies on several mammals including man did not provide a clear-cut distinction between the apical cell, narrow cell and apical mitochondria-rich cell (Martan and Risley, 1964; Martan and Allen, 1964; Cohen et al., 1976; Nicander and Malmqvist 1977; Ramos and Dym, 1977; Moore and Bedford, 1979; Prakash et al., 1979; Brown and Montesano, 1980; Jones et al., 1984; Abou-Haila and Fain-Maurel, 1984; Goyal, 1985; Connell and Donjacour, 1985; Burkett
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Curative property of *Withania somnifera* Dunal root in the context of carbendazim-induced histopathological changes in the liver and kidney of rat

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Summary

The liver and kidney of rat underwent severe histopathological lesions when treated with a single bolus dose of carbendazim, a fungicide, particularly affecting the hepatocytes and the renal corpuscles, respectively. The effects appear to be manifestations of the microtubule-disrupting activity of carbendazim. Treatment of carbendazim-treated rats with the powder of tuberous root of *Withania somnifera* (Ashwagandha) for 48 days resulted in complete cure of these organs. The results indicate that *Withania somnifera* would be an effective curative for carbendazim-induced histopathological changes in the liver and kidney.

Key words: liver pathology, kidney pathology, carbendazim toxicity, *Withania somnifera*, Ashwagandha.

Introduction

Carbendazim (MBC) (Methyl 2-benzimidazole carbamate) is a widely applied fungicide. It is a derivative of another fungicide *viz.* benzomyl. The fungicidal property of MBC resides in its ability to cause depolymerization of microtubules; it also binds to tubulin monomers and prevents their polymerization to form microtubules (De Brabander et al., 1976; Davids et al., 1977; Havercroft et al., 1981; Ireland et al., 1979; Dustin, 1984; Burland and Gull, 1984; Foster et al., 1987; Can and Albertini, 1997). It is known that during industrial manufacture or through food and environmental contamination it could access man and domestic animals and bring about toxic effects, particularly affecting male reproduction (Burland and Gull, 1984). The various manifestations of the male reproductive toxic effects of MBC have been established through investigations on laboratory animals (Carter and Laskey, 1982; Carter et al., 1987; Goldman et al., 1989; Rehnberg et al., 1989, Gray et al., 1990, Hess et al., 1991; Nakai and Hess, 1994; Nakai et al., 1992, 1995, 1998; Lim and Milik, 1997a, b, Tiama et al., 1998). However, there is little information on the effects of MBC on the liver and kidney, though it is known that these organs can accumulate MBC (Hellman and Laryea, 1990) and, hence, the present work was undertaken to find the histopathological changes in these organs in response to a single bolus dose of MBC.

An attempt was also made to find a herbal curative in *Withania somnifera* Dunal (Family: Solanaceae), commonly known as Ashwagandha, for the hepatic and renal pathologies induced by MBC. Ashwagandha is one of the major ingredients in a variety of Siddha, Ayurveda and Unani formulations prescribed for common diseases of the respiratory, circulatory and digestive tracts, and for skin diseases; it is also an ingredient in the formulations prescribed for the relief of pain, diabetes, rheumatism and epilepsy and for improvement of general health (Tripathy et al., 1996).
Materials and methods

- **Standardization of Withanta root powder:**
  The total withanolide (steroid)-content in the root powder was estimated as 1.13 mg% according to a modified Liebermann-Burchard method (J. Biol. Chem. 195: 357, 1952). In short: The powder was extracted with petroleum ether, the extract evaporated to dryness and after addition of the reagent the withanolides spectrophotometrically estimated at 620 nm using cholesterol as reference compound.

- **Experimental animal studies:**
  Three months old Wistar strain male albino rat was used as the test animal. MBC (BAVISTIN) was obtained from BASF India Ltd., Mumbai. Root tuber of *Withanta somnifera* was purchased from a local herbal drug house.

  Rats were divided into two groups and treated as follows:

  **Group I**
  Experimental group, consisting of 15 rats, and received a single bolus dose of MBC (400 mg/kg body weight), suspended in sunflower oil, through an oral gavage.

  **Group II**
  Control group for MBC treatment, consisting of five rats and received the sunflower oil only. 48 hr after the treatment, five rats in the experimental group were dissected to remove the liver and right kidney. The remaining 10 rats in group I were divided into two subgroups of five each and treated as follows:

  **Group Ia**
  Experimental group for *Withania* treatment; the rats received dry powder of *Withania* tuber, at a daily dose of 250 mg/kg body weight, suspended in unadulterated cow’s milk, through an oral gavage, for 48 days.

  **Group Ib**
  Control group for *Withania* treatment (also formed the recovery group), and received cow’s milk only.

  At the end of the treatments rats were dissected to remove the liver and right kidney. Slices of these organs were fixed in Hollande-Bouin fixative (Humason, 1979). After paraffin embedding, sections were cut at 3 μm thickness in a Leica (Germany) microtome, using disposable blades. The sections were stained in Harris hematoxylin and eosin, and mounted in DPX mountant. Photomicrographs were obtained using a Leitz (Germany) diplan microscope.

Results

In general, both liver and kidney underwent histopathological changes on MBC treatment, and *Withanta* treatment to MBC-treated rats resulted in complete recovery.

Liver

In the control rats for MBC treatment the hepatocytes were normal, polygonal in shape, with smooth surface. There was little intercellular space. The cytoplasm had a particulate appearance and was marginally vacuolated. The nucleus was almost spherical, highly heterochromatic and contained one or more nucleoli. Canaliculi and blood capillary spaces were abundant and prominent (Fig. 1). In the MBC-treated rats the hepatocytes appeared swollen and oedematous. The cytoplasm contained large vacuoles, and the nucleus appeared suspended on strands of cytoplasm. In the nucleus, chromatin appeared condensed. Canaliculi and blood capillary spaces appeared reduced (Fig. 2). In the MBC-treated rats control for *Withania* treatment there was partial recovery as seen from the less oedematous nature of the hepatocytes though their nuclei still appeared pyknotic, and the canaliculi and the blood capillary spaces were empty or had the content withdrawn (Fig. 3). In the MBC-treated rats cured with *Withania*, the histoarchitecture of the liver was much improved over even the control for MBC treatment, and several binucleate hepatocytes as well as mitotic figures were evident (Fig. 4).

Kidney

In the cortex of the kidney of control rat for MBC treatment, the renal corpuscles were prominent. Each corpuscle was found to possess a vascular pole and an urinary pole. The capsular space between visceral and parietal epithelium was found to be continuous with the lumen of the proximal convoluted tubule at the urinary pole. Nuclei of endothelial cells and visceral epithelial cells were found in the glomerulus. The proximal convoluted tubules were seen to form a bulk of the cortex. Distal convoluted tubules were fewer in number (Fig. 5).

In the MBC-treated rats the following major changes were noticed (Fig. 6):

- In the entire kidney, irrespective of zones, the nuclei of the cells appeared pyknotic and the cytoplasm oedematous.
- In certain areas of the cortex, the renal corpuscles were totally missing.
- Wherever the renal corpuscles were present, they appeared retracted and lobate.
Curative property of *Withania somnifera*

Fig. 1. Section of liver of a rat, control for carbendazim treatment. a, ×100; b, ×1000.

Fig. 2. Section of liver of a carbendazim-treated rat. a, ×100; b, ×1000.
Fig. 3. Section of liver of a carbendazim-treated rat, control for Withania treatment. a, $\times 100$; b, $\times 1000$.

Fig. 4. Section of liver of a carbendazim-treated rat cured with Withania. a, $\times 100$; b, $\times 1000$. 
Fig. 5. Section of kidney of a rat, control for carbendazim treatment. Arrows point to renal corpuscles. a, ×100; b, ×1000.

Fig. 6. Section of kidney of a carbendazim-treated rat. Arrow points to renal corpuscle. a, ×100; b, ×1000.
7a

7b

8a

8b
• The cells lining the proximal and distal convoluted tubules appeared to have undergone cytolyis, releasing the nuclei.

In the MBC-treated rats, control for *Withania* treatment, there was partial recovery in respect of kidney pathology, though nuclear pycnosis of the cells in general, lobate and withdrawn nature of the renal corpuscles and exfoliation of nuclei from the cells lining the distal convoluted tubules were still evident (Fig. 7). In the MBC-treated rats cured with *Withania*, there was not only complete recovery but the histological picture of the kidney reflected improvement over the initial control (Fig. 8). Nuclear pycnosis of the cells was no more evident. The renal corpuscles increased in size as well as vascularization.

### Discussion

It is known that the cells in liver and kidney, concerned with the respective functions, are epithelial. It is also known that all epithelial cells possess a cytoskeletal framework consisting of microtubules. Microtubules constitute a principal component of the tissue matrix system of the epithelial cells. Cytomatrix, containing microtubules, terminate in the centre of the cell by direct attachment to the base of the cell and the extracellular matrix (Getzenberg et al., 1990). Thus, tubulin, ubiquitous in all epithelial cells as microtubular structures, anchors many cellular structures and is a critical factor in determining the shape of the cell (Luke and Coffey, 1994). Studies have clearly established that MBC disrupts the microtubules of the Sertoli cell of the testis, which has been indicated as one of the most fundamental bases of the male reproductive toxicity of this fungicide (Nakai and Hess, 1994). Disruption of microtubules by MBC is known in the elongating spermatids with special reference to the manchette (Nakai et al., 1997). Therefore, hypothetically, MBC would affect the microtubule-based cytoskeletal structures of the hepatocytes and kidney cells, and the hypothesis is proved in the affirmative in the present study. It could be suggested that the changes in the cytoplasm of these cells is a direct consequence of MBC treatment, and the nuclear changes are indirect, consequent upon the response in the cytoplasmic microtubules. The MBC-induced histopathological changes are reflective of impairment of liver and kidney function.

Absence of mitotic figures in the hepatocytes of MBC-treated rats even after 48 days of recovery period clearly indicates that the residual effect of MBC persists up to 48 days or even more. Several studies have confirmed that in addition to affecting microtubules of the cellular cytoskeletal framework, MBC affects the spindle assembly (Eon-Gerhardt et al., 1981; Hummler and Hansmann, 1988; Zuelke and Perreault, 1995; Spencer et al., 1996; Can and Albertini, 1997; Elhajouji et al., 1997). As liver cells are capable of accumulating MBC (Hellman and Laryea, 1990), the fungicide would disturb spindle assembly in addition to disrupting microtubule of the cytoskeleton.

The tuberous root powder of *Withania somnifera* is considered as a general health care food supplement (Tripathy et al., 1996). Though *Withania* is generally considered as a sexual stimulant and curative of spermatorrhoea, alcoholic extract of leaf of this plant is recommended as a liver protectant (Nadkarni, 1954). The present study indicates that *Withania* tuber would be an effective curative of liver and kidney pathologies caused by MBC through microtubule disruption. The cells appear to be cured to such an extent that in *Withania*-treated rats mitotic figures were fairly abundant in the hepatocytes, and binucleate cells, common among the hepatocytes of initial control rats, were also abundant MBC-treated rats cured with *Withania*, indicating that *Withania* treatment cures MBC-induced pathological changes to an extent of recovery of cell cycle and spindle assembly. Thus, it could be suggested that *Withania* acts to restore the microtubule status of the cytoskeletal framework of the cells as well as the spindle apparatus affected by MBC.

It is known that *Withania* root is rich in a variety of steroidal lactones and alkaloids, one or more of which may be responsible for the curative property. As already recommended by Tripathy et al. (1996), further work is in progress to correlate specific chemicals in *Withania* root with its curative property in microtubule-based liver, kidney and reproductive tissue histopathologies.

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**Fig. 7.** Section of kidney of a carbendazim-treated rat, control for *Withania* treatment. Arrows point to renal corpuscles a, × 100; b, × 1000.

**Fig. 8.** Section of kidney of a carbendazim-treated rat cured with *Withania*. Arrow points to renal corpuscle. a, × 100; b, × 1000.
References


Curative property of *Withania somnifera* (Solanaceae): a status report


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Carbendazim generates symplasts in rat spermatogenic clones

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In order to find non-microtubular targets in the seminiferous epithelium for the fungicide and reproductive toxicant carbendazim, it was administered to 90 days old male Wistar rat in a single bolus dose of 400 mg/kg body weight through an oral intubation. A parallel control group was maintained. Rats were sacrificed 48 days after the treatment and the testes were analysed for histopathological changes adopting routine histological methods when symplasts were localised. The maximum diameter of five largest symplasts was measured, and the number of nuclei in these symplasts was also determined. As it is known that symplasts of spermatogenic cells are produced due to opening up of the intercellular bridges between cells in a clone consequent upon disruption of actin microfilaments, the present study shows that actin microfilaments would also be targets in the seminiferous epithelium for carbendazim toxicity.

Carbendazim (methyl-2-benzimidazole carbamate, MBC) is a fungicide widely used in the agricultural and warehousing practices. Its fungicidal property resides in its ability to disrupt the microtubules. Through environmental contamination as well as occupational exposures, man could be exposed to MBC and, thereby, suffer through microtubule disruption. Studies in the laboratory animals have revealed that on exposure to MBC spermatogenesis is impaired through disruption of the microtubules of the spermatogenic cells in division and the Sertoli cell, causing sloughing of the apical parts of the Sertoli cell along with the associated spermatids. In the present study yet another mechanism of action of MBC in causing impairment of spermatogenesis, namely generation of symplasts in the spermatogenic clones, is reported. Symplasts are formed due to disruption of actin microfilaments of intercellular bridges between the cells in a germ cell clone.

MBC (Bavistin, BASF India Ltd, Bombay) was purchased from a local agrochemical supplier. Three months old Wistar strain male albino rats (20) used in the experiment were fed on standard pellet feed and water ad libitum. The experimental group consisted of 10 rats and each rat received a single bolus dose of 400 mg MBC/kg body weight through an oral intubation, according to Nakai et al. who have standardized this as the optimal dose for bringing about testicular effects. The control rats of equal number received the oil. The rats were sacrificed 48 days after the treatment and slices of testes were fixed in Bouin’s fluid, embedded in paraffin and serial sections at 3 μm thickness were obtained for staining in Harris haematoxylin and eosin. The seminiferous tubules were observed under a Leitz diaplan microscope (Germany) for deformities, and wherever symplasts were found the sections were analyzed critically. The maximum number of symplasts in a section of the seminiferous tubule were counted and the maximum diameter of each of the symplasts was measured using a calibrated ocular micrometer at ×450 magnification. The number of nuclei in the symplast was determined according to Ren and Russell. The total number of nuclei in each of the five largest symplasts was recorded. The data were used to calculate the mean and the standard deviation.

The seminiferous tubules of the control rats had no deformities and did not contain any symplast. On the other hand, seminiferous tubules of the MBC-treated rats reflected several deformities. The observation relevant to the present study was occurrence of symplasts in several seminiferous tubules of four of the treated rats (Fig 1). The maximum number of symplasts in section of a tubule was seven. The symplasts were either lying in the lumen or partially embedded in the epithelium, in either case held in position by processes of the Sertoli cell (Fig 1). The diameter of the largest symplast was 43 μm. The maximum number of nuclei in a symplast was 82 (Table 1). The nuclei, though generally appeared distributed throughout the symplast without any
specific pattern of spatial distribution, in some of the symplasts they were oriented around the periphery of the symplastic mass (Fig. 2). The morphology of the nuclei was typically spermatidic. Sometimes large areas of cytoplasmic continuity were observed between adjacent symplasts (Figs 1 and 2).

MBC is known to inhibit mitosis in fungi and a variety of eukaryotic organisms through its ability to bind β-tubulin and disrupt the normal formation of microtubule\(^1\). The latter property of MBC is considered responsible for various testicular defects observed in laboratory studies\(^2\).

Testicular damage caused by MBC includes inhibition of testicular microtubule assembly\(^8,13\), failure of poleward movement of chromosomes during spermatocytic division resulting in necrosis of meiotic spermatocytes in Stage XIV tubules, sloughing of immature spermatids\(^7,8,14\) and seminiferous tubular atrophy\(^6,12,14,16\). MBC induces various morphological abnormalities in round and elongating spermatids, including abnormally formed acrosome\(^6,9,17\). Origin of large round spermatids (megaspermatozoa and binucleate spermatids) on MBC treatment has also been reported\(^4\). Megaspermatozoa are spermatids with aneuploidy caused due to failure of the microtubule of the spindle\(^1,18\).

Sertoli cell, with its abundant microtubules, also is a target for MBC action, and due to microtubule disruption in the body region the apical portions of the Sertoli cell slough off and such sloughed off Sertoli cell fragments, along with the associated elongating spermatids, arrive at the ductuli efferentes and ductus epididymis and occlude the lumen of these ducts\(^7\). Such an occlusion brings about secondary manifestations in the seminiferous tubules resulting in long-term effects, like germinal epithelial cell necrosis and severe inflammatory responses leading to fibrosis of testis\(^9\), to be followed by permanent atrophy of seminiferous tubules and male infertility\(^12,14,16,19\). In trying to find an interpretation for increased testis weight and accumulation of fluid in seminiferous tubules leading to swelling of the testis after MBC-induced efferent duct occlusion, Nakai et al.\(^12\) suggested that non-microtubule targets may also be involved in the MBC mechanism. It was

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Largest symplast</th>
<th>Mean ± SD</th>
</tr>
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<tbody>
<tr>
<td>Total number of nuclei</td>
<td>82, 80, 79</td>
<td>77.8 ± 7.6</td>
</tr>
<tr>
<td>Maximum diameter (µm)</td>
<td>42.98, 36.84, 33.77</td>
<td>35.61 ± 8.59</td>
</tr>
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also pointed out that MBC-based disruption of cytoskeletal elements other than microtubules may be important for understanding the effects of MBC\(^\text{7}^\text{,10}\). Nakai and Hess\(^\text{7}\) observed bi- and multinucleate spermatids in MBC-treated rats, and assumed that they are formed by failure of cytokinesis of secondary spermatocytes following nuclear division.

The present study provides evidence for disruption of cytoskeletal elements other than microtubules in the seminiferous epithelium by MBC, as envisaged by Nakai and Hess\(^\text{7}\). The symplasts produced in the seminiferous tubules of MBC-treated rats compare with those produced on treatment with cytochalasin-D\(^\text{11}\) and ursolic acid\(^\text{20}\). Spermatidic symplasts are multinucleate cells and formed due to nuclei of all spermatids of a clone joining together as a result of opening of the intercellular bridges\(^\text{21,22}\). The intercellular bridges are large areas of cytoplasmic continuity, 1 to 3 \(\mu\)m in diameter\(^\text{23}\). The cells thus connected are considered to be a clone. Presence of intercellular bridges is meant to allow distribution of gene products to all cells of a clone\(^\text{24}\). The intercellular bridges are maintained due to actin microfilaments\(^\text{12}\). Symplasts are formed due to rapid opening up of the intercellular bridges in the germ cell clones in view of actin microfilament disruption\(^\text{11,22,24}\). As individual bridges open the nuclei of all spermatids, at certain stages of the spermatogenic cycle, go together to form a large multinucleate mass called symplast\(^\text{21,22}\).

Therefore, the present study indicates that cytoskeletal elements other than microtubules may also be targets in the spermatogenic compartment of the testis for MBC toxicity, and as seen in the generation of spermatidic symplasts actin microfilaments are one such target.

The photomicrography facility of National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, is gratefully acknowledged. The interaction of Dr. Rex A. Hess, Department of Veterinary Biosciences, University of Illinois, Urbana, IL 61801, USA, is also heartily acknowledged.

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Spermatotoxic effect of carbendazim

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Carbendazim, suspended in sunflower oil, was administered to Wistar male rats through an oral intubation at a daily dose of 25mg/kg body weight for 48 days, and the cauda epididymal sperm were analysed on day 49 for counts, motility and abnormalities. The study indicates that carbendazim affects the cauda epididymal sperm as seen in decreased sperm counts, inhibition of motility and increased incidence of abnormalities.

Several papers have reported that treatment of experimental animals with the fungicide carbendazim (MBC) brings about disruption of spermatogenesis. Also, Nakai et al. found that treatment of rats with a single bolus dose of 400 mg MBC/kg body weight of carbendazim resulted in decrease in the total testicular sperm head counts between days 8 through 16, and increase in the incidence of abnormal sperm in the epididymis with 10% of the spermatozoa having the head separated from the flagellum, the number of motile sperm decreased but there was no effect on sperm concentration in the cauda luminal fluid. The authors suggested these as effects of the fungicide on spermatogenesis in the testis and/or occlusion caused in the ductuli efferentes. Thus, to-date, there is no report of MBC toxicity to cauda epididymal spermatozoa when the compound is administered in several smaller doses. Therefore, the present preliminary study was undertaken to find the toxic effect of carbendazim on cauda epididymal spermatozoa when administered in several smaller doses for 48 days, the duration of one complete spermatogenic cycle.

The methodology was as previously reported. Briefly, Bavistin containing carbendazim at 50% strength (w/w) (BASF India Ltd., Bombay) was suspended in refined sunflower oil and administered to 90 day old rats (5) through an oral intubation, at daily dose of 25 mg/kg body weight for 48 days. Control rats (5) received the vehicle alone. The rats were sacrificed on day 49, the cauda epididymides were dissected out and, washed thoroughly in saline. The organ was incised at several places so as to allow the semen to ooze out. The semen was sucked into a capillary tube upto 0.5 ml mark. On being transferred to an eppendorf tube, the semen was diluted in 99.5% of phosphate-buffered saline. Sperm counts were made using an improved Neubauer's chamber. Duration of motility of the last motile sperm was determined using hanging drop preparations. Sperm abnormalities were observed at x450 or x1000 magnification using smears stained in Papanicolaou's stain. Data were used to calculate the respective group mean and standard error. Photomicrographs were obtained using a Leitz Diaplan (Germany) microscope in bright field or phase contrast illumination.

MBC treatment resulted in significant decrease in the epididymal sperm counts (control, 21.88 ± 0.78 x 10^6 per ml, treated, 13.14 ± 0.11 x 10^6 per ml), and inhibition of motility of the spermatozoa. In the control rats 93-95% of the spermatozoa possessed normal morphology (Fig 1), whereas in the MBC-treated rats only 55-61% had normal morphology (Table 1). Following various abnormal morphologies were observed. In about 15% of the spermatozoa the head was flexed in such a way that the pointed tip of the head was either facing away from the flagellum (Fig 2) or facing the flagellum (Fig 3). About 10% of the spermatozoa had the flagellum curved at various points to result in a wavy appearance and several other spermatozoa had the tail coiled in a manner highly varied (Fig 5-6). A large percentage of the spermatozoa remained agglutinated or entangled in loose epithelial cells (Fig 7) or in a large epithelial cell mass (Fig 8). Another major abnormality was sticking or fusion of the spermatozoa.
Fig. 1 — A typical sperm of rat from a control animal, × 1000. Figs 2-11— Sperm from carbendazim-treated rats; 2— Sperm with the head flexed, tip of the head facing away from the flagellum, × 450. 3— Sperm with the head flexed, tip of the head facing the flagellum, × 450, phase contrast. 4— Detached head, × 450. 5— Coiling of the flagellum, × 450, phase contrast. 6— Twisting and coiling of the flagellum, × 450. 7— Sperm in admixture with loose germinal epithelial cells, × 450. 8— Sperm in entangled in a compact mass of germinal epithelial cells, × 100. 9— Fusion / attachment of sperm, × 450. 10— Retention of cytoplasmic droplet, arrow, × 450. 11— 'V'-shaped head, × 450.
Table 1 — Percentage of normal and abnormal sperm (values are mean ± SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal sperm (%)</th>
<th>Abnormal sperm (%)</th>
<th>Predominant abnormalities</th>
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<tbody>
<tr>
<td>Control</td>
<td>94 ± 2</td>
<td>6 ± 1</td>
<td>Head alone, Curved tail</td>
</tr>
<tr>
<td>Carbendazim-treated</td>
<td>55 ± 4 (P&lt;0.001)</td>
<td>45 ± 3 (P&lt;0.001)</td>
<td>Flexed head, Curved / Coiled tail, Detached</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>head, Attached sperm, Agglutination</td>
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at various points and over short to long distances (Fig 9) A few spermatozoa retained the cytoplasmic droplet (Fig 10) and a few others possessed 'V'-shaped head (Fig 11).

The decreased sperm counts in the epididymis may be attributed to the impairment of spermatogenesis by carbendazim through inhibition of meiotic division of spermatocytes^1^, sloughing of immature spermatids^2^, and occlusion of ductuli efferentes^2^. Impairment of motility of the cauda epididymal spermatozoa may be due to either or both of the following:

(i) Motility of the spermatozoa is due to the flagellar beat which in turn is dependent on the microtubular apparatus of the flagellum^1^. As MBC is a microtubule poison, the chemical, through testicular and epididymal routes, would affect the sperm flagellar microtubules, thereby impairing sperm motility.

(ii) Spermatozoa, while leaving the tests, are not motile and are initiated into motility only during their epididymal transit. Epididymis contributes to initiation of sperm motility by providing unique microenvironments along the length for the spermatozoa to reside, and secreting proteins and small molecular weight compounds which in one way or the other are concerned with the initiation of sperm motility.

(iii) Agglutination and attachment of sperm may be explained in the light of imminent changes in the surface proteins. It has been conclusively shown that the spermatozoa, during their epididymal maturation, are altered in respect of the surface proteins. The epithelium of the epididymis, particularly the principal cells of the initial segment and caput, secrete several proteins some of which get translocated onto the spermatozoa. Also, the changing luminal microenvironment along the ductuli efferentes and ductus epididymidis contributes to the change in the sperm surface proteins. Therefore, it is reasonable to speculate that MBC affects the epididymal epithelium towards secretion of proteins and/or the luminal microenvironment, effecting change in the sperm surface proteins rendering them to stick together in small to large numbers and over short to long distances. It is already known that change in the sperm surface proteins and pH of the medium are causative of sperm agglutination.
(iv) Cytoplasmic droplet (CD) is a small mass of cytoplasm which the spermatozoa carry while leaving the seminiferous tubules. The droplet is shed when the spermatozoa leave the corpus epididymidis, spermatozoa in storage at the cauda are devoid of the droplet. Spermatozoa which retain extra cytoplasm are inhibited in motility. The retention of CD by Cauda epididymidal sperm of MBC-treated rats could be speculated as due to MBC treatment impairing the process of shedding of the CD.

It is concluded that continuous treatment of small doses of carbendazim is toxic to spermatozoa in storage at the cauda epididymidis and results in decrease in the sperm counts, impairment of the motility of spermatozoa and appearance of several sperm abnormalities.

The photomicrography facility of National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappalli, is gratefully acknowledged.

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Male reproductive toxic effects of carbendazim: Hitherto unreported targets in testis

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Carbendazim (MBC), a widely used fungicide, is toxic to male reproductive mechanisms. Various cellular targets in the testis for MBC action are being understood only recently and still more targets have been conceived. The present study was aimed at finding such newer targets. Male rats were administered through oral route a single dose of carbendazim (400mg /kg) and the testis was studied adopting routine histological technique. It has been observed that pachytene spermatocytes could also be targets for MBC action in the testis. The study also reports selective loss of step 14 spermatids, asynchrony of the stages in the spermatogenic cycle and development of Sertoli cell fibrosis of the seminiferous tubules of carbendazim-treated rats. From the different kinds of responses seen in the seminiferous tubules in the same testis to MBC, particularly in the Sertoli cell, MBC action in the testis appears dependent on the stage in the spermatogenic cycle at first exposure.

Materials and Methods
For animal experimentation towards male reproductive toxicity testing of MBC, previously described procedure was adopted. Briefly, MBC (Bavistin, BASF India Ltd., Bombay) was administered to 90 day old Wistar strain male albino rats at a single oral dose of 400 mg/kg body weight in oil base as per Nakai et al. Control rats received oil only. Rats were sacrificed 48 days after treatment (the minimum duration of one complete spermatogenic cycle) and the testis was processed for routine histological analysis adopting paraffin embedding and haematoxylin and eosin staining. The sections (5μm thick) were critically analysed for changes hither-to not reported in respect of MBC toxicity. Stages in the spermatogenic cycle were identified according to Clermont and Hess. The cell type along the seminiferous epithelium were identified based on the morphological and cytological characteristics of each, according to de Krester and Kerr and Hess.

Results
Four hitherto unreported observations in respect of MBC toxicity to testis were made:
(i) Missing of the pachytene spermatocytes in the Stage III-V tubules (Fig. 1a and b). The affected seminiferous tubules, when observed at higher magni-
fications, revealed that spermatocytes in pachytene stage were missing resulting in the formation of lacunae. Such lacunae appeared always closer to Sertoli cell, and spermatogonia were seen projecting into such lacunae.

(ii) Unique loss of elongating spermatids in several Stage XIV tubules. As against the abundant step 14 spermatids in the Stage XIV tubules of control rats (Fig. 1c), in the MBC-treated rats step 14 spermatids were depleted (Fig. 1d). In some such Stage XIV tubules, we found co-existence of meiotic figures characterizing Stage XIV and large diplotene spermatocytes with prominent nucleoli characterizing Stage XIII in the same tubule reflecting asynchrony of spermatogenesis (Fig. 1e).

(iii) A stage-relatedness in the sloughing of the Sertoli cell. In a few tubules reflecting asynchrony with the co-existence of step 9 spermatids characterizing the Stage IX and step 17 spermatids characterizing the Stage V, it was seen that the apical
portion of the Sertoli cell was detached from above the tier of round spermatids (Fig. 2a), whereas in the tubules at the Stages VII and VIII, not reflecting any asynchrony of the Stages in the spermatogenic cycle, the Sertoli cell in which the sloughing took place was at a level below the tiers of round spermatids, i.e., between the round spermatids and the spermatocytes (Fig. 2b).

(iv) Thorough fibrosis of some of the seminiferous tubules. In such tubules there was almost total loss of germ cells, and the Sertoli cells were retained in a manner more prominent and were extended as narrow

Fig. 2-a,b—Sloughing of Sertoli cells in the seminiferous tubules of carbendazim - treated rats (arrows). (a) Asynchrony in having step 17 (17) and step 9 (9) spermatids in the same tubule; sloughing affects the Sertoli cells beyond the step 19 (19) spermatids [lumen formed due to killing of the pachytene spermatocyte (asterisk)]; (b) Tubule at Stage VII containing step 19 (19) and step 7 (7) spermatids; sloughing of Sertoli cell occurs beneath the round spermatids (a, b×1000).

Fig. 3a—Section of seminiferous tubules of a carbendazim- treated rat showing atrophy of the seminiferous tubules (AT) and fibrosis (FS). b: Part of a fibrotic seminiferous tubule in Fig. 3a enlarged. Note abundance of Sertoli cell fibrotic extensions and absence of germ cells [pycnotic nuclei of germ cells (arrowheads); lumina produced due to killing of pachytene spermatocytes (asterisks)]. (a, ×100; b, ×1000).
thread-like processes into the lumen to obliterate the latter (Fig. 3a). There was also clear evidence of pycnosis of the nuclei of a few left out germ cells (Fig. 3b).

Discussion

The damage caused to the pachytene spermatocytes in the Stage III-V tubules defies explanation from the point of view of microtubule disruption, the established mechanism of action of MBC in the testis\(^9\). Pachytene is the longest stage in the spermatogenic wave and it commences with the completion of synopsis of the homologous chromosomes and is associated with thickening and shortening of the chromosomes. There is exchange of chromosomal material between maternal and paternal chromosomes through crossing over. Pachytene is characterized by nuclear and cytoplasmic growth resulting in the cells becoming the largest of the male germ cell lines\(^9\). The selective loss of pachytene spermatocytes from the seminiferous epithelium, as observed in the present study, probably, reflects a direct effect of MBC toxicity to the germ cells. Perhaps, synopsis formation is the specific target. Once the cells are killed, they are lost from the epithelium due to phagocytic activity of the Sertoli cell\(^9\). It is rather interesting that in such tubules there was no other histological manifestation of MBC toxicity. Perhaps, pachytene spermatocyte effect is the earliest impact of MBC treatment in the seminiferous tubules. Nakai et al.\(^7\) are of the opinion that spermatocytes just before and during meiotic division at Stage XIV and step 1 spermatids are likely to be the germ cell targets of MBC and it is unlikely that pachytene spermatocytes are susceptible to MBC. Thus, the present observation indicates for the first time that pachytene spermatocytes also as the target for MBC action. Validation of the opinion of Nakai et al.\(^7\) is still possible because MBC could have acted during mitosis of A\(_n\) towards B spermatogonia, thus killing the cells and the consequent loss of B spermatogonia would result in absence of pachytene spermatocytes.

An explanation for the loss of step 14 spermatids in the Stage XIV tubules is to be sought only in the impact of MBC on the microtubules of the Sertoli cell. In the spermatogenic compartment Sertoli cell is the principal target for MBC action\(^4\). MBC particularly affects microtubules in the body region of the Sertoli cell resulting in the apical portion of the Sertoli cell sloughing off and such sloughed off Sertoli cell fragments carry with them the germ cells. Perhaps, targeting the Sertoli cell does not occur very precisely at the Stage XIV but could occur even earlier\(^10\). The co-existence of diplotene spermatocytes characterizing Stage XIII and meiotic spermatocytes characterizing Stage XIV, with the apical portions of elongating spermatids missing, may be explained in terms of the already proposed asynchrony. The Stage-dependent manner in which MBC acts is very much reflected in the present results since in some of the tubules Sertoli cell sloughing took place beyond the level of the spermatids whereas in certain other tubules it took place below the tiers of the round spermatids. However, the present results are interesting in that on administration of a single bolus dose of MBC the spermatocytes which have survived continue to engage in meiotic division even 48 days after the treatment whereupon the spindle fibres are clearly developed in the meiotic spermatocytes but elongating spermatids are missing. Perhaps, the microtubules of the Sertoli cell are more vulnerable to MBC than those of the spindle fibres. An explanation to this hypothesis may be sought in the peculiar association of the microtubules with vimentin fibrils and the microtubule-associated proteins\(^4\).

The present results relating to fibrosis of the seminiferous tubules are interesting. The well-established mechanism of action of MBC in the spermatogenic compartment is the sloughing of the Sertoli cell as discussed vide supra, ultimately resulting in complete atrophy of the seminiferous tubules\(^11\). If this is the only and indiscriminate mechanism, the kind of fibrosis observed will not be possible. In the fibrotic seminiferous tubules the entire Sertoli cell was not only intact but its processes were more ramified and filled the entire seminiferous tubule leaving little lumen. Perhaps, the present results signify that the ultimate reflection in the seminiferous tubule in response to MBC is based on the Stage at first exposure of the seminiferous tubule to MBC. First exposure at certain Stages may result in sloughing of apical portion of the Sertoli cell, that too at different levels along the height in a manner stage-dependent, whereas that at some other Stages may result in retention of Sertoli cell but cause premature exfoliation of the germ cells resulting in Sertoli cell fibrosis. Thus, the present report expounds newer targets in the seminiferous epithelium to MBC toxicity and hitherto unreported mechanisms of action in the already established cellular targets.
Acknowledgement

The photomicrography facility of National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, is gratefully acknowledged.

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**P43 NOVEL RESPONSES IN THE EPIDIDYMAL EPITHELIAL CELL TYPES TO TOXICITY: LIGHT- AND ELECTRON MICROSCOPIC STUDY**

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In a study to find the extent to which epididymis could be a target to aflatoxins, aflatoxin B1 (AFB1) was administered intraperitoneally to 90 day old male Swiss mouse at a daily dose of 50 μg/kg body weight for 7, 15, 35 & 45 days. The phytotherapeutics andrographolide (AG) and ursolic acid (UA) were also tested for epididymal toxicity and administered to 90 day old male Wistar rat through intragastric route at a daily dose of 25 and 5 mg /kg body weight, respectively, for 55 days. Different segments of the epididymis of control and treated mice/rats were subjected to light- and transmission electron microscopic analysis. Several exciting observations were made, some of which are as follows:

1. Fortunate sections cutting the entire narrow cell in the right longitudinal plane revealed that the apical expanded portion and the basal peduncle possessed separate nuclei. When the sections were out of plane, the basal peduncle, cut off from the stalk, matched the basal cell. In the treated animals the narrow cell increased in counts up to 7% and the apical nucleated portions became densely vacuolated, protruded deep into the lumen and were seen pinching off into the lumen. Such cell fragments were comparable to luminal macrophages. It appears that the narrow cell bears developmental, anatomical and functional relationships with the basal cell.

2. The basal cell increased in counts as well as lipofuscin content. Such basal cells could be traced into the intertubular connective tissue. Occurrence of sperm heads and tails in endocytic vesicles of the principal cells and their processing towards formation of lipofuscin-like inclusion were also evident. The basal cell, perhaps, acquires lipofuscin inclusions from the principal cells.

3. The basal cell at its post-natal recruitment is first established as plumpy cell to mature into the basal cell. Plumpy basal cell has not been reported in the adult animal. In the present study plumpy basal cells appeared in all the treated animals. This observation, combined with that *vide supra*, suggests that in the adult animal basal cells, loaded with the lipofuscin inclusions, exit from the epithelium and fresh basal cells are recruited through plumpy basal cells.

4. The establishment of plumpy basal cells was preceded by accumulation of leucocytes resembling monocytes closer to the basal aspect of the epithelium, and their migration into the epithelium. It is conceived that these cells are precursors of the plumpy basal cells in the adult animal.

5. Retraction of the plumpy basal cells from the overlying principal cells and taking the shape of macrophages was also noticed. It may also imply that the monocytes first develop into macrophages, which then become the plumpy basal cells, to finally establish as basal cells.

6. In all the epididymal segments of AF- treated mice appearance of cells with pale-staining cytoplasm was noticed. Each cell possessed a large lumen, containing cell debris and spermatozoa, bounded by membrane which produced tall microvilli. The cell possessed a single nucleus mostly in a basal peduncle. We conceive these pale vacuolated epithelial cells to enclose and process the spermatozoa arriving from the lumen, through certain disintegrating principal cells and, thus, avoid an autoimmune response to spermatozoa.

Thus, the paper throws newer insight into the relationships between the monocytes, intraepithelial macrophages, plumpy basal cells, mature basal cells, pale vacuolated epithelial cells and narrow cells of rodent epididymis.
Dietary aflatoxins can damage chromatin and DNA of male germ cells

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Abstract
In the background that aflatoxins are mutagenic and carcinogenic in the liver, and can also affect the aspects of spermatogenesis, the present study was undertaken to find the specific effect of dietary aflatoxins, at a subchronic dose, on DNA and chromatin of the male germ cells in the mouse model. The treatment resulted in generation of hypertypic giant germ cells, lacking the nuclear envelope, or pycnosis of the male germ cell nuclei. The chromatin of the hypertypic giant cells appeared fragmented implying damage to DNA. Agarose gel electrophoresis of DNA of testis further reflected on damage caused to the DNA by aflatoxins. Thus, the present study provides direct and histological evidence of damage to male germ cell DNA by aflatoxins.

Key words: Aflatoxin B₁, DNA damage

Introduction
Aflatoxins (AFs) are produced as secondary metabolites by the moulds Aspergillus flavus and A. parasiticus, which are commonly found as contaminants of rice, maize, corn, peanut, treetnuts, soy beans, dry beans, cotton seed, oil seeds, etc., and can access the human and animal systems through the dietary route (1). Among the various AF types, AFB₁ inflicts serious damage and its acute toxicity LD₅₀ value ranges from 1 to 17.9 mg/kg body weight in the laboratory animals (2). Dietary AFB₁ undergoes cytochrome P450-dependent methylation to generate AFB₁-8, 9 exoepoxide which is the toxic metabolite of AFB₁ (3). Among the various tissues liver is the immediate target where the AFB₁-8, 9 exoepoxide is a potent genotoxic and mutagenic agent (3). As regards the male reproductive toxic effects of AFs, the few reports indicate testicular degeneration including sloughing of germ cells and concomitant drop in the rate and efficiency of sperm production in rats treated with sublethal doses of AFB₁ (4-8). HPLC analysis of DNA of testis of rat treated with AFB₁ showed formation of adducts of DNA with AFB₁ (9), but to the best of our knowledge there is no direct histological evidence for the genotoxic effect of AF on the male germ cell lines. The present paper reports on the damage caused to chromatin and DNA of mouse male germ cells by dietary AFs.

Materials and methods
Aspergillus parasiticus 'NRRL 2999', obtained from National Culture for Utilization Research, University of St Peoria, USA, was cultured on rice medium. The mouldy rice was powdered and the total AFs and the AFB₁ content were determined adopting HPLC. The experimental design consisted of a control group with five Swiss albino male mice fed the formulated feed (Lipton India Ltd., Bangalore) for 35 days and an experimental group with five mice fed with the same feed with which powdered rice containing AFs was mixed. The daily intake of AF was 34.3 ± 8.4 μg/kg body weight. After the experimental period, thin slices of the right testis were fixed in Bouin's fluid, embedded in paraffin and 3-5μm thick sections were obtained. The sections were stained in haematoxylin and eosin. Photomicrography was done under bright light in Olympus Research Microscope (Japan). The left testis was decapsulated and the DNA was extracted and quantified spectrophotometrically (10). The DNA was electrophoresed using calf thymus DNA (Sigma, USA) as the standard, on agarose gel, and observed in an UV illuminator. Densitometric scanning was conducted using UV-visible spectrophotometer.

Results
The following observations were made:
1. in several of the seminiferous tubules of AF-fed mice, the germ cells closer to or in the lumen appeared like giant cells (Fig. 1). Critical examination of the affected cells revealed them to be hypertypic (i.e., the single cell was 2-4 times larger). In such cells the chromatin was highly particulate and appeared fragmented. Further, the chromatin did not appear as surrounded by a nuclear envelope (Fig. 1). In such seminiferous tubules several other germ cells had the nuclei highly pycnotic.

Fig. 1. The seminiferous epithelium of an AFB₁-treated mouse showing the hypertypic giant cells with reflections of fragmentation of the chromatin (GC) and cells with pycnotic nuclei (PY) x 1000
Whereas the DNA of the testis of control mouse appeared as a clear band exactly matching the band of the control calf-thymus DNA, in the AF-fed mice the DNA moved further down and appeared diffuse (Fig. 2). Densitometric scanning of the DNA bands of the testis of control and AF-fed mice reflected damage to DNA.

Fig. 2. Electropherogram of DNA separated on agarose gel. Lane 1, control calf-thymus DNA; Lane 2, control testis; Lane 3, treated testis.

Discussion

In as much as AFB₁ can target the testis also and impair spermatogenesis, the manifestations so far known are germ cell depletion, depression of the activity of alkaline phosphatase, inhibition of glutamic oxaloacetate and 5' nucleotidases, enhancement of pyruvate transaminase and lactic dehydrogenase and impairment of Leydig cell function (4-8). There is only one report on the molecular mechanism of action of AFB₁ in the testis, which indicates that AFB₁ can access the mammalian testis and form adducts with the DNA, but the methodology consisted of HPLC analysis of AFB₁ adducts, and lacked support of histological evidence (9). The present paper fills this gap and provides histological evidence for damage to DNA of male germ cells caused due to AF treatment.

In this evidence, there is an interesting observation of generation of hypertypic giant cells among the male germ cell lineage. Hypertypic giant cells are produced due to replication of DNA accompanied by failure of the nucleus to divide, resulting in double or four-times the DNA of the affected cells but the cell has, perhaps, failed to divide. The underlying mechanisms in the generation of hypertypic giant male germ cells on AF treatment are worth investigating.

Thus, the present study provides histological evidence for generation of hypertypic giant male germ cells due to AF treatment and the subsequent damage to DNA as revealed in the fragmentation of chromatin or pycnosis of the nuclei.

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References

CERTIFICATE OF MERIT

This is to certify that Ms. A. Faridha Banu, Department of Animal Sciences, Bharathidhasan University, presented a scientific paper entitled, “Aflatoxin B1 treatment brings about generation of multinucleate giant spermatids” (Abstract no RT-06 page 40) at the XIX National Symposium of the Society for Reproductive Biology and Comparative Endocrinology and UGC DSA Conference held at the Department of Zoology, M.S. University of Baroda, Vadodara – 390 002, India during January 17-19, 2001. The paper was awarded Third Prize for the best Oral presentation.

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Secretary, SRBCE

Prof. M. Michael Aruldhas
Treasurer, SRBCE
17th National Conference of Society for Reproductive Biology & Comparative Endocrinology

This is to certify that Dr./Mr./Ms. A. Hariha Banu
Irulrapalli actively participated in the Conference. He/She presented a paper

Dr. A. V. Ramchandran
President
SRBCE

Dr. N. C. Shah
Chairman
17th National Conference of SRBCE

Dr. Nandita Arbatti
Organising Secretary
17th National Conference of SRBCE
SRBCE 2000
18th National Symposium on Reproductive Biology and Comparative Endocrinology

"Endocrine Update Towards The Next Millennium"

The Society for Reproductive Biology and Comparative Endocrinology
Dr. ALMPGIBMS, Univ. of Madras
Chennai - 600 113

CERTIFICATE

Certified that Farida Banu. A

has attended and presented a paper in the 18th National Symposium on Reproductive Biology and Comparative Endocrinology held from December 27 to 29, 1999 at Madras Christian College (Autonomous), Tambaram East, Chennai - 600 059, India

Dr. A.V. Ramachandran
President

Dr. P. Govindarajulu
Secretary

Dr. R. Moses Inbaraj
Organising Secretary
FOURTH INTERNATIONAL
SIXTEENTH NATIONAL SYMPOSIUM ON
RECENT TRENDS IN LIFE SCIENCES

DEPARTMENT OF AQUATIC BIOLOGY & FISHERIES
UNIVERSITY OF KERALA, TRIVANDRAM

&

INDIAN SOCIETY OF LIFE SCIENCES, KANPUR

7-9 FEBRUARY 2000

CERTIFICATE

Certified that Ms. Fazleha Banu

has attended / presented a scientific paper in the symposium held at Trivandrum.

[Signatures]

Prof. C. M. ARAVINDAN
ORGANISING SECRETARY

Dr. A. K. SAXENA
SECRETARY, ISLS

Prof. P. NATARAJAN
SYMPOSIUM DIRECTOR
INTERNATIONAL CONFERENCE ON
PROBING IN BIOLOGICAL SYSTEMS

February 7-11, 2000

Organised by

Department of Zoology
THE INSTITUTE OF SCIENCE
15, Madam Cama Road, Mumbai - 400 032.

This is to certify that

Dr./Mr./Mrs./Ms. A. FARIDHA BANU

has participated / presented research paper / chaired session / delivered plenary lecture in the conference.

Prof. S. A. Suryawanshi
Convener and Director

Dr. B. G. Kulkarni
Organising Secretary
NATIONAL SYMPOSIUM ON MALE REPRODUCTION

10-12\textsuperscript{TH}, NOVEMBER, 2000
COIMBATORE - 641 046.

CERTIFICATE

This is to certify that Ms. FARIHA BANU A. has participated in the National Symposium on Male Reproduction held at the Department of Zoology, Bharathiar University, Coimbatore, India as a delegate / chairperson / invited speaker. He / She presented a paper in the scientific session.

President, ASI
(P.R. Reddy)

Secretary, ASI
(N.S. Ujwal)

Head, Department of Zoology, B.U.
(R. Manavalaramanujam)

Organizing Chairman, B.U.
(G. Vanithakumari)

Dr. G Vanithakumari
Professor of Zoology,
Bharathiar University,
Coimbatore 641 046.
CERTIFICATE

This is to certify that

Prof./Dr./Mts./Miss. A. Farida Bans

participated in the proceedings of the Symposium and delivered a Lecture

Presented paper / Chaired / Co-chaired a session

N.J. Chinchoy
PROF. N.J. CHINCH
President - SRBCE

PROF. P. GOVINDRAJULU
Secretary - SRBCE

PROF. A.V. RAMACHANDRAN
Org. Secretary
This is to certify that Prof./Dr./Mr./Miss./M/s./Miss Faridha Banu participated and presented a paper / delivered an invited lecture / chaired a session at the XX National Symposium on Reproductive Biology and Comparative Endocrinology, held at the Department of Animal Science, Bharathidasan University, Tiruchirappalli-620 024, India, on January 7-9, 2002.

President, SRBCE
(Prof. Miss. N. J. Chinoy)

Secretary, SRBCE
(Prof. P. Govindarajulu)

Organizing Secretary
(Prof. M. A. Akbarsha)