Retention of cytoplasmic droplet by rat cauda epididymal spermatozoa after treatment with cytotoxic and xenobiotic agents

M. A. Akbarsha, P. N. L. Latha and P. Murugaian

Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli-620 024, India

Spermatozoa leaving the testis contain a cytoplasmic droplet which they release during transit through the epididymis before reaching the cauda epididymidis. The cytoplasmic droplet shows P450 aromatase activity, which plays a role in synthesis of oestrogen from androgen. In the present study, 3-month-old Wistar strain male albino rats were administered with the organophosphate insecticides malathion or dichlorvos, or the phytotherapeutics andrographolide or ursolic acid. Segments of the epididymis were subjected to histopathological and ultrastructural analyses and it was found that 60–95% of the spermatozoa residing in the lumen of the cauda epididymidis retained the cytoplasmic droplet. The motility of the spermatozoa released from the cauda epididymidis was inhibited. One of the mechanisms of action of these toxicants on male reproductive function may be attributed to the retention of the cytoplasmic droplet and the resultant impairment of sperm motility.

Introduction

During spermatogenesis spermatozoa lose much of their cytoplasm from a residual body that is phagocytosed by the Sertoli cell (de Krester and Kerr, 1994). However, the spermatozoon leaves the testis with a small droplet of cytoplasm, the cytoplasmic droplet. The organization of the cytoplasmic droplet and its displacement from the head to the terminal part of the principal piece during the transit of the spermatozoon towards the distal parts of the epididymis have been reported (Bloom and Nicander, 1961; Dott and Dingle, 1968; Roberts et al., 1976; Jones et al., 1984; Kaplan et al., 1984; Temple-Smith, 1984). The cytoplasmic droplet is shed into the lumen when the spermatozoon leaves the corpus epididymidis and the droplet is fragmented and then endocytosed by the clear cells of the cauda epididymidis (Hermo et al., 1988; Robaire and Hermo, 1988). The cytoplasmic droplet is an extra load on the spermatozoon, and it has been hypothesized that it has a role in the aromatization of androgen into oestrogen, which has a role in the regulation of male reproductive tract function (Hess et al., 1995, 1997a,b; Janulis et al., 1996, 1998). The cytoplasmic droplet is shed after it has fulfilled its role and before ejaculation, when spermatozoa are required to have optimal motility.

Akbarsha and Averal (1996) suggested that the cytoplasmic droplet was the target of action of cytotoxic drugs. Male Wistar rats were administered a cytotoxic drug vincristine and several spermatozoa retained the cytoplasmic droplet after arrival at the cauda epididymidis. In a series of investigations in our laboratory, the organophosphate insecticides, malathion and dichlorvos, and the therapeutic phytochemicals, andrographolide and ursolic acid, were tested at doses known to adversely affect male reproductive function (in the case of malathion and dichlorvos) or recommended for phytotherapeutic agents (in the case of andrographolide and ursolic acid). The present study investigated whether spermatozoa exposed to such cytotoxic and xenobiotic agents fail to shed the cytoplasmic droplet before ejaculation.

Materials and Methods

Animals and treatments

Adult Wistar 3-month-old male rats were used in the experiments. The animals were provided with standard rat pellet feed and water ad libitum.

Malathion formulation (50% w/w) was obtained from Tudiyalur Co-operative Agricultural Services Ltd, Coimbatore. Dichlorvos (76% w/w) was purchased from Tamilnadu Agro Industries Ltd, Madras. Andrographolide (purity 84%) was purchased from Research Organics Ltd, Madras. Ursolic acid (purity 90%) was purchased from Sigma, St Louis, MO.

The rats were divided into groups of five each and treated as follows:

Group I: received malathion (diluted in water) at a daily dose of 100 mg active ingredient kg⁻¹ body weight by oral gavage (Ozmen and Akai, 1993) for 48 days.
Group II: dichlorvos (diluted in water) was given to the rats at a daily dose of 10 mg active ingredient kg⁻¹ body weight by oral gavage (Krasue and Homola, 1974) for 48 days.
Group III: received water (control for groups I and II).
Group IV: received andrographolide, suspended in...
propylene glycol, at a daily dose of 50 mg kg⁻¹ body weight by oral gavage (Handa and Sharma, 1990a) for 48 days.

Group V: received propylene glycol (control for group IV). Group VI: received an i.p. injection of 25 mg ursolic acid kg⁻¹ body weight per day (quantitatively dissolved in a minimum quantity of ethanol and then diluted in physiological saline, 0.9% NaCl) (Liu et al., 1994) for 48 days.

Group VII: received vehicle (control for group VI).

At the end of the treatments, the rats were subjected to mild ether anaesthesia, the scrotal sac on the right side was incised and the tail of the epididymis was located. The capsule was incised and the cauda epididymal duct was exposed. Fluid from the duct was collected in a cannula and diluted quantitatively in PBS.

A hanging drop preparation of the dilute semen was made and the duration of motility of the sperm was assessed under a microscope according to Harrison et al. (1978) by two independent observers, and the average was obtained. The values from each group of rats were used to calculate the mean and the SEM. Paired sample t tests were conducted to determine the difference between the control and experimental rats. Subsequently, the reproductive system of the rats was perfused with Karnovsky’s fluid using a peristaltic pump (Karnovsky, 1965). Thin slices of caput, corpus and cauda (proximal and distal segments separately) epididymides were immersion-fixed overnight in 2% (v/v) glutaraldehyde and post-fixed in 1% (w/v) osmium tetroxide for embedding in low viscosity resin (Sigma). Semithin sections (1 μm) were cut using a Reichert Jung ultramicrotome and stained in toluidine blue O for observation under a microscope (Hayat, 1981). Ultrathin sections were cut using LKB-Bromma ultracut and stained in uranyl acetate and lead citrate (Hess and Thurston, 1977) and examined under a Phillips EM-200 transmission electron microscope (TEM).

Areas were chosen at random in semithin sections (at ×1000 magnification) and ultrathin sections (at ×1500 magnification) and the spermatozoa in cross-section in the field and in cross-section retaining the cytoplasmic droplet were counted separately and the percentage of sections of spermatozoa retaining the cytoplasmic droplet was calculated. Spermatozoa were counted in 20 different fields for each animal. Morphometric measurement of the length of 20 spermatozoa of control rats was made using thin smears of cauda epididymal sperm stained in Papanicolaou stain (Raphael, 1976). The mean ± SEM length of the cytoplasmic droplet was measured under the TEM in longitudinal sections passing through the axoneme of 20 spermatozoa (Fig. 1). The length of the cytoplasmic droplet was 5.84 ± 0.23 μm. Thus, the possibility of transverse sections passing through the cytoplasmic droplet was only 3.33% (6/180 × 100). Thus, if 3.33% of the sections in the field showed retention of the cytoplasmic droplet, all the sectioned spermatozoa in the field would have retained the cytoplasmic droplet.

**Results**

The spermatozoa in storage at the distal cauda epididymidis did not retain the cytoplasmic droplet in any of the control rats (Table 1; Fig. 2a). The largest proportion of spermatozoa retaining the cytoplasmic droplet was obtained in rats treated with ursolic acid (95%), followed by rats treated with andrographolide (82%; Fig. 2b), malathion (72%; Fig. 2c) and dichlorvos (65%). The droplet was confluent with the cytoplasmic sheath surrounding the axial filament (Fig. 1). Several flattened membrane-bound vesicles and lamellae were aggregated at one pole of the droplet (Fig. 3a). Most of the lamellae were C-shaped but straight, but V-shaped and circular lamellae were also found. In some C-shaped lamellae, the terminal ends were dilated, and the lumen contained heterogeneous material. Some of the V-shaped lamellae were apposed closely to each other, forming stacks. Small-to-large membrane-bound vesicles containing dense outer material with a less dense core were also seen among the C-shaped lamellae. There were fewer lamellae in the cytoplasmic droplets of spermatozoa collected from the cauda epididymidis of the treated rats (Fig. 3b) than there were in the cytoplasmic droplets collected from the spermatozoa of the corpus epididymidis of control rats (Fig. 3a). However, there were more membrane-bound vesicles in the cytoplasmic droplets of treated compared with control spermatozoa, and the content of these more abundant vesicles appeared to be more dense and heterogeneous.

The spermatozoa of malathion- and andrographolide-treated rats were immotile and the motility of spermatozoa in dichlorvos- and ursolic acid-treated rats was impaired (Table 2).

**Discussion**

Malathion and dichlorvos are organophosphate insecticides the principal action of which is the inhibition of acetylcholinesterase. Malathion and dichlorvos, like several other...
organophosphate insecticides, are severely toxic to non-target organisms. Krause and Homola (1974) studied the toxic effect of dichlorvos and reported severe disturbance of spermatogenesis, damage to Sertoli cells and increased size of Leydig cell complexes.

Andrographolide is a diterpenoid lactone isolated from Andrographis paniculata Nees (Acanthaceae), a plant recommended for treatment of the common cold (Caceres et al., 1997). This compound is also used as a hepatoprotective agent for paracetamol and CCl4 toxicities (Handa and Sharma, 1990a,b). Administration of a suspension of the leaf powder of A. paniculata to male rats has been reported to affect spermatogenesis (Akbarsha et al., 1986), although another study reported that the dried extract of A. paniculata did not produce any subchronic testicular toxicity in male rats (Burgos et al., 1997). Ursolic acid is a triterpene isolated from the leaves of plants such as Arctostaphylos uva-ursi, Vaccinium macrocarpon and Rhododendron hymenanthes (Budavari, 1983), and has been recommended for use as an anti-ulcer drug (Farrina et al., 1994) and as a hepatoprotective agent against CCl4, acetaminophen and CdCl4 toxicities (Liu et al., 1994). Ursolic acid inhibits interferon γ-induced nitric oxide production in mouse macrophages (Honda et al., 1997). Akbarsha et al. (1998) have reported disruption of spermatogenesis in Wistar rats treated with ursolic acid.

The aim of the present study was not specifically to examine the impact of treatment of these compounds on the cytoplasmic droplet of cauda epididymal spermatozoa but rather to investigate the histopathological changes in the testis and epididymis in response to treatment of these compounds (these histopathological findings will be published separately). However, in the course of the study, it was noted that treated spermatozoa did not shed the cytoplasmic droplet. Since the cytoplasmic droplet was not the original subject of the study, its retention was not assessed using whole mount preparation. Nevertheless, an objective indirect quantitative assessment was made of the retention of the cytoplasmic droplet by the cauda epididymal spermatozoa from the histological preparations. In making this assessment, it has been assumed that when the spermatozoa in the epididymal duct are sectioned at random the probability of a spermatozoon being sectioned through the cytoplasmic droplet is 3.33%.

It has been clearly established that mammalian spermatozoa leaving the testis contain a cytoplasmic droplet. On leaving the testis, the cytoplasmic droplet is located in the region rear to the head of the spermatozoon. Subsequently, it moves posteriorly along the midpiece until it reaches the point of annulus (Kaplan et al., 1984; Hermo et al., 1988) and then is shed when the spermatozoon leaves the corpus epididymidis (Hermo et al., 1988; Janulis et al., 1996). The loss of the cytoplasmic droplet from the spermatozoon represents an additional reduction in cell volume, occurring extratesticularly and a considerable amount of time after the release of the spermatozoon from the Sertoli cell (Gist et al., 1992).

The membrane-bound vesicles and lamellae of the cytoplasmic droplet of spermatozoa residing in the cauda epididymidis of rats treated with malathion, dichlorvos, andrographolide and ursolic acid are generally comparable with those of spermatozoa residing in the caput epididymidis. The vesicles were once considered hollow (Bloom and Nicander, 1961; Gist et al., 1992), but studies have also shown that the cytoplasmic vesicle encloses some content (Hermo et al., 1988). It has been clearly established that mammalian spermatozoa leaving the testis contain a cytoplasmic droplet. On leaving the testis, the cytoplasmic droplet is located in the region rear to the head of the spermatozoon. Subsequently, it moves posteriorly along the midpiece until it reaches the point of annulus (Kaplan et al., 1984; Hermo et al., 1988) and then is shed when the spermatozoon leaves the corpus epididymidis (Hermo et al., 1988; Janulis et al., 1996). The loss of the cytoplasmic droplet from the spermatozoon represents an additional reduction in cell volume, occurring extratesticularly and a considerable amount of time after the release of the spermatozoon from the Sertoli cell (Gist et al., 1992).

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unable to attribute any structural or cytochemical resemblance of vesicles to the elements of Golgi apparatus, and could find no evidence for their derivation from the endoplasmic reticulum. Hermo et al. (1988) concluded that the vesicles represent a unique membrane-bound element of unknown origin. The occurrence of high concentrations of lysosomal and other degradative enzymes in the cytoplasmic droplet has been reported (Dott and Dingle, 1968; Harrison and White, 1972; Moniem and Glover, 1972; Roberts et al., 1976). The results of the present study are in agreement with the contention that the membrane-bound vesicles of the cytoplasmic droplet enclose some contents, and showed that the treatments resulted in an increase in the abundance of the membrane-bound vesicles and an increase in the density and heterogeneity of their contents.

The cytoplasmic droplet was once considered to be rather inert (Robaire and Hermo, 1988), but is now thought to play a role in oestrogen biosynthesis in the lumen of the male reproductive tract. Janulis et al. (1996, 1998) demonstrated the activity of P450 aromatase, the enzyme that converts androgen to oestrogen, as well as immunostaining for P450 aromatase in the cytoplasmic droplet of mouse spermatozoa. A decrease in P450 aromatase activity in the cauda epididymal spermatozoa has been related to the shedding of the cytoplasmic droplet by the spermatozoa (Eiler and Graves, 1977; Free and Jaffe, 1979; Claus et al., 1987, 1992; Janulis et al., 1996). In view of this observation, it is worth investigating whether the cytoplasmic droplet retained by spermatozoa in the cauda epididymis of rats treated with the two insecticides and the two phytochemicals in the present study serves a physiological role in the synthesis of oestrogen. The retention of the cytoplasmic droplet by several spermatozoa during cauda epididymal storage in adult male rats treated with vincristine was reported by Akbarsha and Averal (1996). This finding and the results of the present study indicate that retention of the cytoplasmic droplet by the spermatozoa in the cauda epididymis is a manifestation of toxicity to spermatozoa of the chemicals acting at the testis or the epididymis. The spermatozoa carrying extra load in the form of cytoplasmic droplets during ejaculation can be correlated with altered epididymal function and reduced fertility (Cummins and Glover, 1970; Cummins, 1973; Bedford, 1976; Akbarsha and Averal, 1996).

### Table 2. Duration of motility of sperm of rats treated with organophosphate insecticides and phytotherapeutics

<table>
<thead>
<tr>
<th>Group</th>
<th>Duration of motility (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control for malathion and dichlorvos</td>
<td>28.3 ± 3.2</td>
</tr>
<tr>
<td>Malathion-treated</td>
<td>Nil*</td>
</tr>
<tr>
<td>Dichlorvos-treated</td>
<td>12.4 ± 2.1*</td>
</tr>
<tr>
<td>Control for andrographolide</td>
<td>23.3 ± 4.6</td>
</tr>
<tr>
<td>Andrographolide-treated</td>
<td>Nil*</td>
</tr>
<tr>
<td>Control for ursolic acid</td>
<td>23.4 ± 3.8</td>
</tr>
<tr>
<td>Ursolic acid-treated</td>
<td>9.2 ± 3.15*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

*P < 0.001, compared with the corresponding control.

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Fig. 2. (a) Light micrograph showing spermatozoa in the lumen of the cauda epididymis of a control rat; none of the spermatozoa in section appear to retain the cytoplasmic droplet. Scale bar represents 10 μm. (b) A low power transmission electron micrograph showing the epithelium and lumen of the cauda epididymis of an andrographolide-treated rat. Several spermatozoa in section retain the cytoplasmic droplet (arrowheads). Scale bar represents 2 μm. (c) Light micrograph showing spermatozoa in the lumen of the cauda epididymis of a malathion-treated rat. Several spermatozoa in section retain the cytoplasmic droplet (arrowheads). Scale bar represents 10 μm.
1988) or any other extra cytoplasm (Keating et al., 1997) would have inhibited motility and, hence, may not fertilize the ova. This contention is substantiated by the finding in the present study that the cauda epididymidal spermatozoa, in all experimental treatments, demonstrated reduced motility in vitro. Thus, treatment of rats with malathion, dichlorvos, andrographolide or ursolic acid results in retention of the cytoplasmic droplet by cauda epididymidal spermatozoa and a reduction in their motility.

A question raised by the results of the present study is what is the cause for spermatozoa to shed their cytoplasmic droplets? Three possibilities are suggested. First, a specific secretory product of the corpus epididymidis may cause the shedding of the cytoplasmic droplet, in which case the toxicants may impair secretion of this substance. Reports that several male reproductive toxicants affect the secretion of proteins from the epididymal epithelium are in agreement with this hypothesis (Akbarsha and Averal, 1997; Klinefelter, 1997). Second, the role of cytoplasmic droplets in the aromatization of androgen is confined to the proximal segments of the epididymis (Janulis et al., 1996; Hess et al., 1997), where the functional exhaustion of the cytoplasmic droplet may result in its shedding, in which case the toxicants may cause the cytoplasmic droplet to continue to aromatize androgen actively and hence the cytoplasmic droplet is not shed. Third, the different segments of the ductus epididymidis provide unique microenvironments critical for the physiological maturation of the spermatozoa (Hinton and Palladino, 1995), in which case the toxicants may alter the microenvironments such that they are no longer optimal for the spermatozoa to shed the cytoplasmic droplet. Investigations are underway in our laboratory to find the target of action of the toxicants that results in the spermatozoa retaining their cytoplasmic droplets.

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Fig. 3. (a) Transmission electron micrograph of a corpus epididymidal spermatozoon of a control rat showing the profile of the vesicles and lamellae in the cytoplasmic droplet. Scale bar represents 0.6 μm. (b) Transmission electron micrograph showing the profile of the vesicles and lamellae in the cytoplasmic droplet of a cauda epididymidal spermatozoon of a ursolic acid-treated rat. Scale bar represents 0.55 μm.

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