

# SPERM STRUCTURAL AND MOTILITY CHANGES DUE TO AN ANTI-CANCER DRUG CYCLOPHOSPHAMIDE IN MALE SQUIRREL *FUNAMBULUS PENNANTI* (WROUGHTON)

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## KEY WORDS

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## ABSTRACT

The effect of an anticancer drug, Cyclophosphamide on the sperm count, motility and morphology has been evaluated. Cyclophosphamide in two different doses i.e. 6mg for 30 days and 12mg for 15 days was administered whereas same amount of normal saline was given to control animals. The low dose treatment resulted into a condition between normospermia and oligospermia whereas the high dose treatment resulted into oligospermia. Treatment also resulted into either immotility, "rotational" or "forward progressive movement", however, high dose treatment showed hampered motion due to different types of coiling in the tail. The head abnormalities included pyriform, amorphous, compressed, smallness, lateral displacement of head, loss of DNA or vacuolation or stippling in the nucleus, loss of acrosome or its vacuolation or its throwing into bulb-like structure as well extreme enlargement of head indicating diploidy. Low dose treatment mainly resulted into bifurcation or multifurcation of tails but high dose treatment resulted into specialized types of tail coiling.

## INTRODUCTION

The chemotherapeutic drug Cyclophosphamide (N, N- bis (2-chloroethyl)-2-oxo-1-oxa-3-aza-2 $\lambda$ {5}-phosphacyclohexan-2-amine) is a widely used anticancer and immunosuppressive agent commonly used in the reproductive age range. Its cytotoxic effects are the result of chemically reactive metabolite that creates DNA adducts, DNA-DNA and DNA-protein cross links, sister chromatid exchanges, chromosomal aberration and DNA strand breaks in many cell types, including germ cells (Sotomayor and Cumming, 1975; Bishop *et al.*, 1997; Condrington *et al.*, 2004). Alteration of male fertility is one of the serious side effects of Cyclophosphamide (Shetty and Meistrich, 2005; Vaisheva *et al.*, 2007). A perusal of literature showed that extensive research is done on toxicity of Cyclophosphamide on male reproductive organs (Toppari *et al.*, 1990; Matsumoto *et al.*, 2000 and Anguilar-Mahecha *et al.*, 2002) but little is known about its toxic effect on sperm count, motility and morphology. Therefore, the present study was undertaken since all these parameters are obligatory steps for critical evaluation of fertility.

## MATERIALS AND METHODS

A total 12 adult male squirrels (*Funambulus pennanti*) weighing between 100 to 150g were trapped alive in and around Nagpur City during the breeding period from January to July 2007 (Reddi and Prasad, 1968). They were fed in the morning and in the evening daily with the soaked grams, chappatis, breads, cooked rice and dal, fruits, vegetables, ground nuts and water. The animals were housed at constant temperature (28  $\pm$  2°C)

and relative humidity (60  $\pm$  10%) with a 12 hr light: 12 hr dark cycle.

## Treatments

One week after arrival, male squirrels were assigned to one of the two schedules of Cyclophosphamide treatment i.e. chronic low dose of 6mg/kg BW for 30 days and chronic high dose of 12mg/kg BW for 15 days dissolved in saline. The controls also received the same amount of saline (Tables 1 and 2). All doses were given intraperitoneally.

## Sperm analysis

The animals were sacrificed using chloroform 24 hr after the last day of each experiment. The spermatozoa present in the cauda epididymidis were collected after mincing/slicing the tissue in a cavity block containing 1mL of physiological saline centrifuged at 600rpm for one minute and a drop of 5% aqueous eosin (WHO, 1999).

## Sperm count, motility and morphology

The percent counts of cauda epididymal sperms were determined in a Neubauer's haemocytometer chamber. Motility of sperms in the experimental and control animals were determined by putting a drop of saline sperm solution on the cavity slide covered with a thin glass slip. Cavity slide was used since depths less than 20 $\mu$ m constraints the rotational movement of the spermatozoa. The freshly made wet preparation was left to stabilize for approximately one minute. The saline solution of caudal epididymidis prepared for studying the sperm concentration was directly observed several times for assessing the sperm morphology and

**Table 1: Experimental Design for chronic low dose Cyclophosphamide treatment**

Number of animals and sex	Treatment	Dose(mg/KgBW/day)	Route	Duration
3 males (Experimental)	Cyclophosphamide	6 mg chronic	I.P.	30 days
3 males (controls)	Saline	Equal volume	I.P.	30 days

**Table 2: Experimental Design for chronic high dose Cyclophosphamide treatment**

Number of animals and sex	Treatment	Dose(mg/KgBW/day)	Route	Duration
3 males (Experimental)	Cyclophosphamide	12 mg chronic	I.P.	15 days
3 males (controls)	Saline	Equal volume	I.P.	15 days

I.P. = Intraperitoneal, BW = Body Weight

photographed.

### Statistical analysis

The data was analyzed statistically following the method adapted by Delgaard (2008). Standard Deviation (SD) and Probability test *i.e.* 't test' were employed to know the levels of significance. A significance level of  $p < 0.05$  was accepted.

## RESULTS

Both the treatment resulted in the suppression of body and cauda epididymis weights being significant for higher dose ( $p < 0.05$ ) as well as remarkable histopathological alteration in testis, epididymis and accessory glands. The other behavioral changes were sluggishness, loss of appetite, withdrawn mood, however, mortality rate was zero percent.

### Vehicle-treated control

#### Sperm count

As the animal was in breeding period the epididymidis of vehicle-treated group showed swarms of spermatozoa and hence a condition of normospermia (Fig.1).

#### Sperm motility

Rapidly fast moving sperms were observed.

#### Sperm morphology

The head was oval, acrosomal region was well defined, occupying 40-70% of the head area. Mid-piece region was slender, about one and half times the length of the head and attached axially to the head. The tail region was straight, uniform, thinner than the mid-piece and uncoiled (Figs.2 and 3).

### Chronic low dose treatment (6mg/kgBW/day)

#### Sperm count

Decline in the number of sperms was observed in the aqueous saline solution when compared to the control (Fig.1). Thus a condition in between oligospermia and normospermia was observed.

#### Sperm motility

Some sperms were immotile, some showed non-progressive movement and remaining others showed either random forward or "rotational movement".

#### Sperm morphology

The observed morphological changes in head abnormalities included pyriform, curved, amorphous, compressed, smallness, lateral displacement of head, loss of DNA or vacuolation or stippling in the nucleus, loss of acrosome or its

vacuolation or its throwing into bulb-like structure, pin-head condition etc., extreme enlargement of head indicated diploidy. Loss of mid-piece, partial separation of mid-piece and tail or swollen mid-piece. Chronic low dose treatment mainly resulted into bifurcation or multifurcation and bent tails (Figs. 4 to 26).

### Chronic high dose treatment (12mg/kgBW/day)

#### Sperm count

A condition of oligospermia to oligozoospermia was observed. Beside the sperms undifferentiated immature germinal cells were frequent in the aqueous medium (Fig.1).

#### Sperm motility

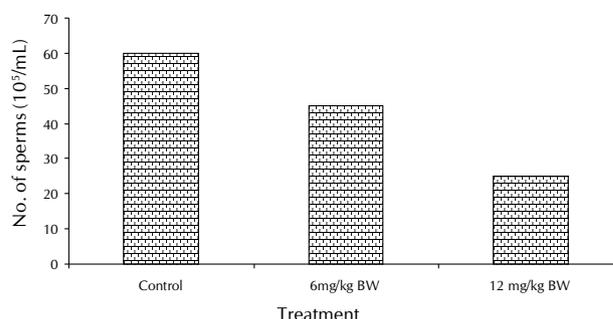
Sperms with straight tails were noted to move fast. Type - D coiled (the end piece, the principal piece and the mid-piece) were stagnant. Type - B coiled (the end piece and distal principal piece) were sluggish. Type 'C' (the end piece and the entire principal piece coiled) were also immotile.

#### Sperm morphology

The chronic high dose 12mg/KgBW/day for 15 days resulted into following types of abnormalities, the morphological defects due to treatment induced head, middle piece, tail and retention of protoplasmic or cytoplasmic droplets along with specialized types of tail coiling involving different segments of tail such as Types 'A', 'B', 'C' and 'D' (Figs. 27 to 33).

## DISCUSSION

The low dose treatment resulted into a condition between normospermia to oligospermia whereas the high dose treatment resulted into oligozoospermia. A perusal of literature shows that Cyclophosphamide treatment resulted also into



**Figure 1: Effect of 6mg and 12mg/kgBW Cyclophosphamide treatment on spermatozoal counts in the caudal epididymidis after last injection. Values are expressed as mean  $\pm$  S.D., n=3 in each group. There was a significant decrease in the sperm count when compared with the vehicle. \* $p < 0.001$**

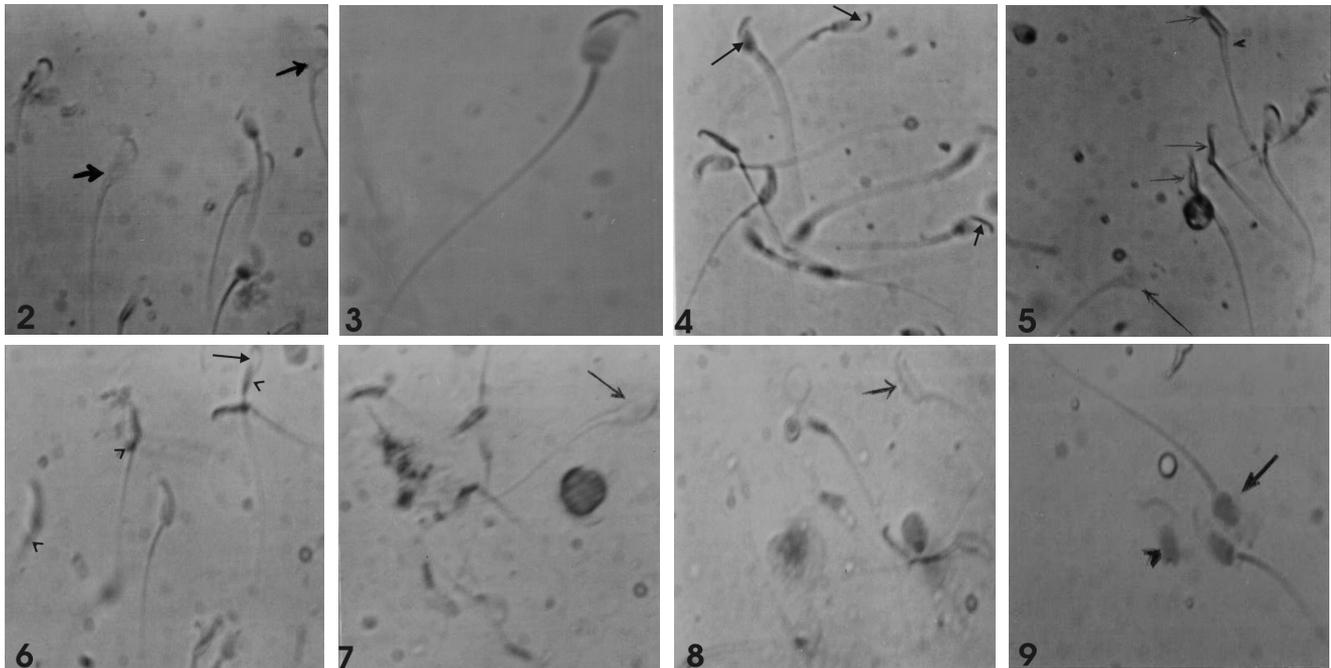


Figure 2 to 9: (2) Few sperms photographed from the vehicle-treated control squirrel. Note oval head, slender axially attached midpiece and thin straight tail X 1000; (3) A single sperm photographed from vehicle-treated control X 1000; (4) Few sperms from chronic low dose treated group. Note increase in the size of the head but simultaneous loss of nuclear material from some and vacuolation in the acrosome (arrow) X 1000; (5) Note extreme elongation of the nuclear material, pinching of the acrosome (arrow), swelling of mid-piece (arrow head) and deformed head (long arrow) from the same regimen X 1000; (6) Bulb-like vacuolated acrosome (arrow), retention of cytoplasmic droplet at mid-piece (arrow head) from low dose Cyclophosphamide treatment X 1000; (7) Chronic low dose treatment: Extreme increase in the size of (diploidy) sperm head (arrow); (8) Chronic low dose treatment: Loss of DNA material (arrow) X 1000; (9) Note normal sperm from low dose treated group (arrow) and one broken head (arrow head) a "pin-head" condition x 1000.

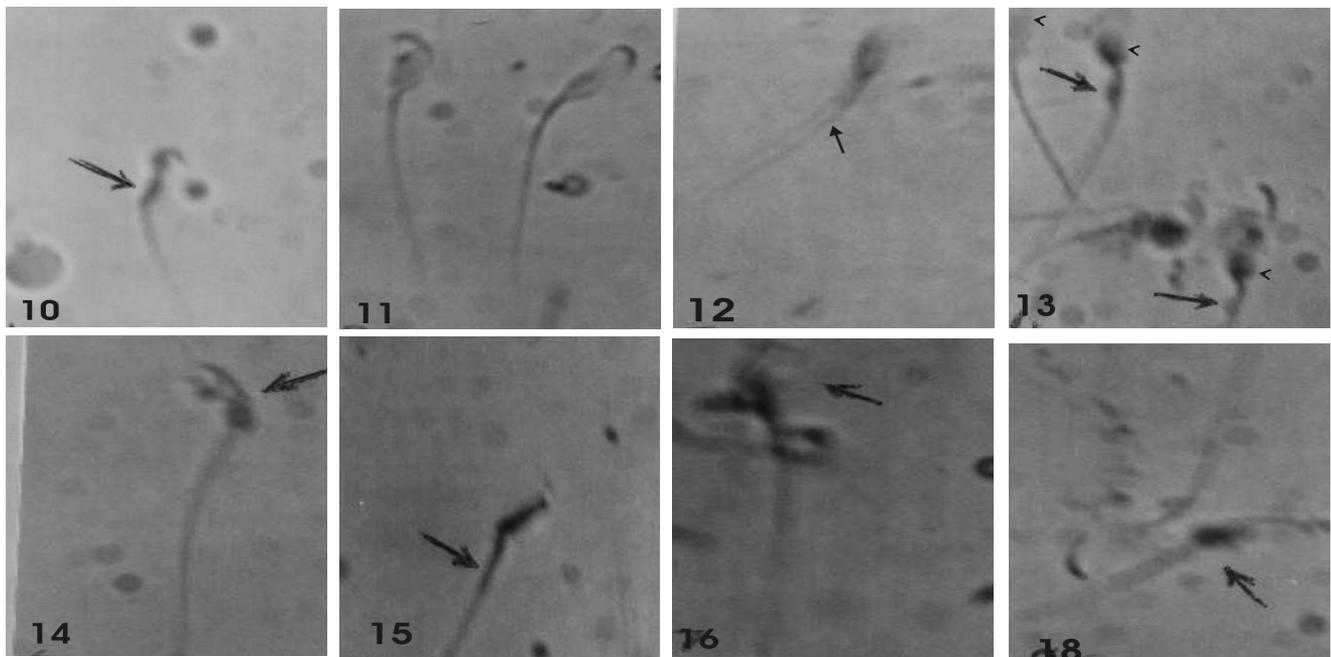


Figure 10 to 18: (10) Highly compressed nucleus, curved acrosome and complete curved head (arrow) from 6mg/kgBW/day for 30 days Cyclophosphamide treated X 1000; (11) Large head (diploid condition), acrosome vacuolated (6mg/kgBW/day for 30 days) x 1000; (12) Small head with bifurcated tail (arrow) (6 mg/kgBW/day for 30 days) X 1000; (13) Amorphous head with cytoplasmic droplet at the mid-piece (arrow), with deformed heads (arrow head) from low dose treated group X 1000; (14) Deformed head (arrow) from low dose treated group X 1000; (15) Abnormal spermatozoa from low dose group. Head is extremely shrunk, tail bent from mid-piece and short (arrow) X 1000; (16) Amorphous head from low dose treated group (arrow) X 1000; (17) Pin-head condition as the detached tail is seen (arrow) and highly swollen head (arrow head) from low dose treated group X 1000; (18) Loss of mid-piece from the low dose group (arrow) X 1000.

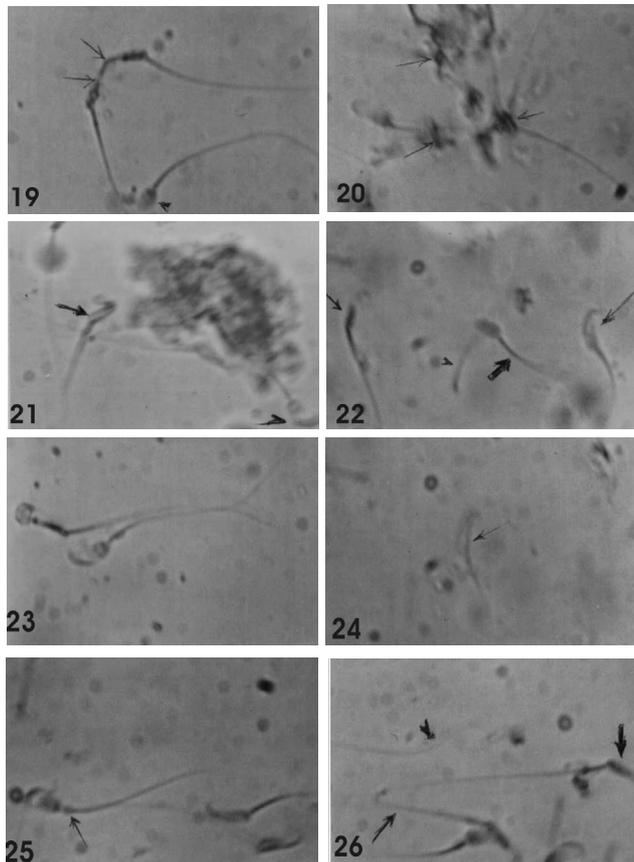


Figure 19 to 26: (19) Sperms from chronic low dose treatment. Please note extreme compression and lateral displacement of head and elongation of head with highly compressed acrosome (arrow). Small head directly attached to tail (loss of mid-piece) as well as loss of acrosome (arrowhead) X 1000; (20) Many dead sperms with decondensed nuclei (arrow) (6mg/kgBW/day). One sperm with large head but with extreme loss of DNA (arrow head) X 1000 (21) Extremely curved head with reduced tail in length (arrow). Large vacuolated pyriform head (arrow head) from low dose Cyclophosphamide treated animal X 1000; (22) Sperms from 6mg/kgBW/day Cyclophosphamide treated for 30 days. Nuclear elongation, acrosome bulb-like (arrow), pin head condition, only tail is seen due to loss of head which gets absorbed (arrow head), swollen head due to increase in nuclear material but multiple tails (thick arrow), vacuolation in the acrosome (long arrow) X 1000 (23) Chronic low dose Cyclophosphamide treated. Note asymmetrical insertion of tail into mid-piece (arrow). Decondensation of nuclear material and retention of cytoplasmic droplet at the end of mid-piece (arrow head) X 1000; (24) Note extreme vacuolation in the sperm head may be due to loss of nuclear material (arrow) from the same regimen X 1000 (25) Please note retention of cytoplasmic droplet (arrow) at the mid-piece from 6 mg/kgBW/ day for 30 days X 1000; (26) Note bent tail (arrow), pin-head condition (arrow head) and elongated head (thick arrow) from the same treatment X 1000.

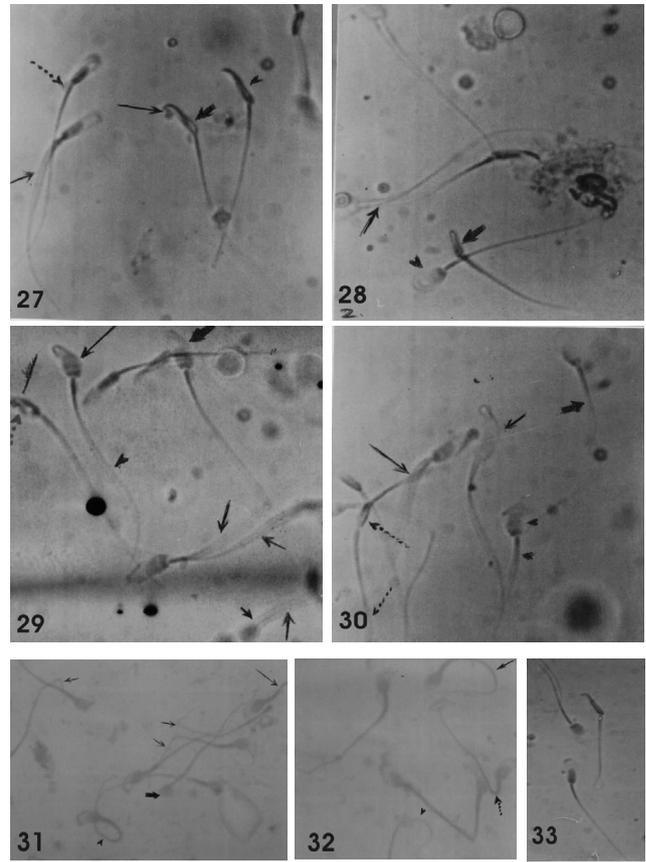


Figure 27 to 33: (27) Note partial splitting in the tail (arrow). Unusual compression and therefore elongation of nucleus (arrow head), vacuolation due to loss of DNA in the head nucleus (thick arrow), unexceptional curvature of the acrosome (long arrow), acrosome thrown into bulb and protoplasmic droplet at the end of the midpiece (broken arrow). All from chronic high dose treated group X 1000; (28) "Pin-head" condition due to loss of head (arrow), loss of nucleus in the head (arrow head), loss of acrosome (thick arrow) from chronic high dose treated group (12mg/kg BW/day for 15 days) X 1000; (29) Sperm from high dose group (12mg/kgBW/day for 15 days). Note multiple tails (arrow), double tails (arrow head), vacuolated head due to loss of DNA (broken arrow), stippling in the chromatin (long arrow), small and compressed head with no midpiece (short arrow) X 1000; (30) Sperms from chronic high dose treated group (12mg/kgBW/day for 15 days). Head exceptionally large with less DNA (arrow), cytoplasmic droplet at principle piece and bifurcated tail. Amorphous head and bifurcated tail (arrow head). Multiple tails directly attaching to basal plate (long arrow), head compressed with bent tail (broken arrow) and tail bifurcated at principle piece (thick arrow) X 1000; (31) Sperm from 12mg/kgBW/day for 15 days showing different types of coiling of the tail. 'C' type of coiling (arrow), 'A' type of coiling (arrow head) and sharp curve at the principle piece (broken arrow) X 1000 (32) Note type 'B' coiling from (arrow) 12mg/kgBW/day for 15 days X 1000; (33) 'A' type coiling of tail (arrow) and 'D' type coiling of tail (arrow head), loss of acrosome (thick arrow) and wavy tail (long arrow) from 12mg/kgBW/ day for 15 days X 1000.

azoospermia (Devita *et al.*, 2005; Selvakumar *et al.*, 2006), however, not with the present regimens. Furthermore, occurrence of severe oligospermia or oligozoospermia in the present study may be correlated to its maximum effect on elongating spermatids and spermatozoa (post-meiotic germ

cells) because these cells lose the ability to repair DNA and hence undergo apoptosis (Trasler *et al.*, 1987; Cai *et al.*, 1997; Higuchi *et al.*, 2001; Bieber *et al.*, 2006; Elangovan *et al.*, 2006; Condrington *et al.*, 2007; Delbes *et al.*, 2009).

Beside a decline in sperm count low dose treatment also

revealed “immotility”, “rotational” as well as “forward progressive” movement of sperms, however, the percentage of rotational movement was more. In the high dose group the numbers of sperms were highly reduced as well as most of them had undergone a specialized type of coiling of their tails, the coilings were of A, B, C and D types involving different segments of tail, thereby hampering the motility. Our observations are supported by manual motility studies by Qiu *et al.*, 1992; Kaur *et al.*, 1997; Barton *et al.*, 2003, however, Higuchi *et al.*, 2001 contradicted that such abnormalities of tail affected no sperm motion when observed by CASA system, furthermore, it can be interpreted that damaged sperm could exhibit no change in the motion when their nuclei are affected. They further emphasized that sperm motion parameters are not always altered when spermatozoa are toxically damaged. Because the development and maturation of the sperm and acquisition of critical functional characteristics are highly regulated by the biochemical environment in the testis and epididymidis, perturbation of this balance may produce alterations in sperm properties such as motility. Similar to our observations a significant decrease in motility was observed with various drug treatments (Kaur *et al.*, 1997; Elangovan *et al.*, 2006; Selvakumar *et al.*, 2006).

The morphological defects due to Cyclophosphamide treatment have also induced head, middle piece, tail, retention of protoplasmic or cytoplasmic droplets and specialized types of tail coiling involving different segments of tail such as Types A, B, C and D with the high dose treatment probably due to an alteration in epididymal function resulting in inhibition of sperm maturation (retention of cytoplasmic droplet) as well as by decreasing Dihydrotestosterone (DHT) and hence an increase in the number of spermatozoa with coiled tails and the degree of coiling (A, B and C type of coiling) as described by Rajalakshmi *et al.*, 1990 and A, B, C and D type by Sastry and Gupta, 2004a.

Generally a greater proportion of spermatozoa lose their cytoplasmic droplet during its transit from corpus to cauda epididymis. This indicates that spermatozoa maturation takes place between the corpus and cauda epididymis since loss of cytoplasmic droplet is considered as an index of spermatozoa maturation in mammals (Bedford, 1975). Further, the location of cytoplasmic droplet changes from proximal to the distal end of the mid-piece in an increasing percentage of spermatozoa during their epididymal transit (Flechon and Hafez, 1975; Hoffer *et al.*, 1981; Kaur *et al.*, 1990; Sastry and Gupta, 2004b). Concurrent to this in the present study the location of the cytoplasmic droplets changes from the middle piece to principal and to the distal end of the tail, and these changes are dose dependent.

Our results show that Cyclophosphamide which is an inhibitor of spermatogenesis also interferes with the maturation of spermatozoa perhaps due to an alteration in epididymal secretory and absorptive functions and due to decline in the serum level testosterone. Regarding the retention of cytoplasmic droplet, there are no reports when we perused the literature on the effect of Cyclophosphamide on testis therefore this study is the only work describing the inhibition of spermatozoa maturation with low dose and high dose treatment.

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