

Original Article

Sperm characteristics and ultrastructure of testes of rats after long-term treatment with the methanol subfraction of *Carica papaya* seeds

Boomi Manivannan, Ruchi Mittal, Shipra Goyal, Abdul S. Ansari, Nirmal K. Lohiya

Department of Zoology, Centre for Advanced Studies, University of Rajasthan, Jaipur 302004, India

Abstract

The contraceptive efficacy of *Carica papaya* seeds after short-term evaluation has been well established. We have examined the safety and mechanism of contraception in rats after long-term treatment with the methanol subfraction (MSF) of *C. papaya* seeds. The test substance was administered orally to the male albino rats ($n = 40$) at 50 mg per kg body weight each day for 360 days. Control animals ($n = 40$) received olive oil as a vehicle. Recovery was assessed up to 120 days after treatment withdrawal. Sperm parameters, serum testosterone levels, fertility, histology and ultrastructure of the testis, haematology and serum clinical chemistry were evaluated to establish the safety and efficacy of the test substance. Safety of long-term treatment was evidenced by unaltered health status, organ weight, haematology and clinical chemistry, and by an increase in body weight. The mechanism of contraception was shown by reduction in nuclear and cytoplasmic volume, normal nuclear characteristics and vacuolization in the cytoplasmic organelles of the Sertoli cells, as well as nuclear degeneration in spermatocytes and spermatids indicating disturbed spermatogenesis. Leydig cells were normal. Initial effects were observed in Sertoli cells at 60 days of treatment. Spermatocytes and spermatids were affected after 120–240 days of treatment. A significant decline in sperm count and viability, total inhibition of sperm motility, increased numbers of sperm abnormalities, normal serum testosterone levels and 100% sterility were evident after 60 days of treatment. All the altered parameters, including percent fertility, were restored to control level 120 days after treatment withdrawal. It is concluded that the MSF is safe for long-term treatment and the mechanism of contraception is shown by its effect on spermatid differentiation in the testis, possibly mediated by the Sertoli cell factors.

Asian Journal of Andrology (2009) 11: 583–599. doi: 10.1038/aja.2009.25; published online 3 August 2009.

Keywords: *Carica papaya* seeds, male contraception, spermatozoa, testis, ultrastructure

1 Introduction

It has been well established that the Sertoli cells of the testis play a key role in spermatogenesis, particularly in germ cell differentiation and transformation of spermatids into mature spermatozoa [1]. Several studies have attempted to relate experimentally induced morphological changes in Sertoli cells and Leydig

Correspondence to: Prof. Nirmal K. Lohiya, Department of Zoology, Centre for Advanced Studies, University of Rajasthan, Jaipur 302004, India.
Fax: +91-0141-270-1809
E-mail: lohiyank@hotmail.com; lohiyank@rediffmail.com
Received: 1 November 2008 Revised: 26 February 2009
Accepted: 1 April 2009 Published online: 3 August 2009

cells to the regulation of spermatogenesis [2–4]. It has also been reported that many antifertility compounds produce testicular dysfunction through the premature release of germ cells, induced by disruption of Sertoli cells [5–9]. This sloughing of germ cells causes occlusion of the efferent ductules and long-term testicular damage, including epithelial cell necrosis and inflammatory responses [10].

The seeds of *Carica. papaya* have been well recognized to possess compounds that putatively control fertility. A preclinical, short-term evaluation through a series of experiments using various extraction procedures of the seeds of *C. papaya* revealed spermatogenic arrest, leading to azoospermia or total inhibition of sperm motility in rats, rabbits and langur monkeys [6, 11–17]. The methanol subfraction (MSF) of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds has been identified as a putative candidate for male contraception [12]. However, the evidence was drawn from short-term evaluation. To establish *C. papaya* seed extract as a potent male contraceptive, evaluation of long-term treatment with the product and reversal of its effects is necessary. The mechanism of action after long-term treatment and the safety of long-term treatment are two other areas to be evaluated. It has been speculated that Sertoli cell factors are the primary contributors to azoospermia, mediated through the testis, and asthenozoospermia, mediated through the peritubular cells in the epididymis [13]. Yet another speculation is that the direct cytotoxic effect of the test substance on the germ cells leads to sterility [13, 14]. Further insight into the subcellular characteristics of the testis is expected to provide better evidence for identifying the mode of action of the drug in the target tissue. Therefore, this investigation aimed to identify the reversibility and safety of long-term treatment with *C. papaya* seeds, and their effects on body weight, organ weight, sperm parameters, haematology, clinical chemistry and fertility in albino rats, with particular emphasis on the mechanism of action through cellular and subcellular characteristics of the testis.

2 Materials and methods

2.1 Test material

Fresh seeds of *C. papaya* L. (Caricaceae), of the honeydew variety, were procured commercially, authenticated in the Department of Botany, University of Rajasthan, Jaipur, India (Voucher No. RUBL 16 590),

shade dried, powdered and refluxed with chloroform for 36 h at 58–60°C. The resultant chloroform extract was subjected to silica gel column chromatography (60–120 mesh) and eluted with benzene. The benzene chromatographic fraction of the chloroform extract was concentrated under reduced pressure and subfractionated with methanol. The final product, known as the MSF, was concentrated under reduced pressure and used in this investigation.

2.2 Animals

Adult Wistar albino rats, approximately three months old and weighing 180–200 g, were used in this investigation. The animals were maintained in individual polypropylene cages in the Departmental Animal House Facility with a 12:12-h light:dark schedule. The temperature in the animal house during the study period was maintained at $23 \pm 2^\circ\text{C}$, and the relative humidity ranged between 32% and 70%. The feeding schedule consisted of two rat pellet meals a day, and water was provided *ad libitum*. Daily intake of food and water were quantified precisely. The animals were maintained under veterinary supervision in accordance with the Guidelines for Care and Use of Animals in Scientific Research [18].

2.3 Experimental design

The experiments consisted of the following two phases.

2.3.1 Treatment phase

The animals were divided into two groups, as follows:

Group I (40 animals) — The animals that served as control were treated orally with olive oil at 50 mg per kg body weight each day for 360 days.

Group II (40 animals) — The animals were treated orally with the MSF of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds, at a standardized dose regimen of 50 mg per kg body weight each day for 360 days.

2.3.2 Recovery phase

After the completion of the treatment schedule (360 days), all surviving animals were withdrawn from treatment. Recovery was assessed through sperm parameters and histopathology of the testis for a 120-day observation period or until the altered parameters regained normalcy.

2.4 Parameters

As described below, five animals each from the control and treatment groups were periodically evaluated for various parameters every 60 days during the 360-day treatment period and the 120-day recovery period. Histology and ultrastructure of the testis were evaluated every 120 days during the treatment and recovery periods. One additional histological evaluation of the testis was carried out after 60 days of treatment to evaluate initial effects after completion of one spermatogenetic cycle.

2.4.1 Body and organ weight

Body weight of the animals and the weight of testes, epididymis, seminal vesicles and ventral prostates, excised free of adhering tissues, were evaluated periodically every 60 days during the treatment and recovery periods.

2.4.2 Libido and fertility tests

Libido and fertility tests were carried out every 60 days during the treatment and recovery periods before necropsy. The control and treated animals were exposed to proven fertile female rats at ratios of 1:2 to assess libido, mounting behaviour and fertility status. Success of mating was confirmed by the appearance of a vaginal plug or spermatozoa in the vaginal smear over the subsequent days. The females, if pregnant, were allowed to complete the term, and the fetal mortality, morbidity and teratogenicity, if any, were recorded.

2.4.3 Sperm analysis

The cauda epididymis, wherein the spermatozoa mature and are stored, was chipped in 1 mL of normal physiological saline, and the clear fluid was used for the analysis of sperm concentration, motility, viability and abnormality, as per the procedures described in the WHO Laboratory Manual [19].

The sperm concentration was calculated with the haemocytometric method, using Neubauer's haemocytometer at a 1:19 dilution. The percent motility (rapid linear progressive, slow linear progressive, vibratory and non-motile); percent viability, assessed with the eosin-nigrosin staining method; and percent normal/abnormal spermatozoa, determined with the Papanicolaou staining procedure, were assessed under a phase-contrast microscope (Optiphat, Nikon, Japan).

2.4.4 Biochemical investigations

Androgen-sensitive biochemical markers, namely,

cholesterol [20], glycogen [21] and lactate dehydrogenase (LDH) [22] for the testis; sialic acid [23], *L*-carnitine [24] and neutral α -glucosidase for the epididymis; fructose for the seminal vesicle and acid phosphatase (ACP) for the ventral prostate [25] were estimated quantitatively using homogenates of the respective tissues.

2.4.5 Histology and ultrastructure of the testis

The testis was used for histological and ultrastructural studies at different stages of treatment. After recovery, only histology was carried out.

For histology, tissue was fixed in Bouin's fixative, dehydrated in various grades of ethanol, cleared in benzene, infiltrated and embedded in paraffin wax. Sections, 6 μ m thick, were stained with haematoxylin and eosin.

For ultrastructural studies, tissue, which was cut in to small pieces (1 mm) was fixed immediately in 2.5% glutaraldehyde for 6–24 h, washed thrice in phosphate buffer (0.2 mol L⁻¹; pH 7.4), post-fixed in 1% OsO₄ for 4–6 h, washed in phosphate buffer, dehydrated in acetone, infiltrated and embedded in low-viscosity spur media and polymerized at 60°C for 48 h. Semi-thin sections (1 μ m) were stained with toluidine blue, and ultra thin sections were double stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (Philips CM-10, Eindhoven, The Netherlands).

2.4.6 Safety evaluation

Blood samples were collected by cardiac puncture and were used for routine haematology and serum clinical chemistry.

2.4.6.1 Haematology

Total red blood corpuscles (RBC), white blood corpuscles (WBC), haemoglobin (Hb), packed cell volume (PCV) and standard haematological indices—mean corpuscular volume (MCV), mean corpuscular Hb (MCH) and mean corpuscular Hb concentration (MCHC)—were measured as described earlier [25].

2.4.6.2 Clinical chemistry

Serum glutamate oxalate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), creatinine phosphokinase (CPK), alkaline phosphatase (ALP), LDH, creatinine, cholesterol, glucose, bilirubin, urea, triglycerides (TGL) and high-density lipoprotein (HDL) levels were estimated in a semi-autoanalyser (ERBA;

Smart Lab, Mumbai, India), using the appropriate reagent kits (Transasia Biomedicals Ltd., Mumbai, India). Serum testosterone levels were estimated using ELISA kits (Biochem Immuno Systems, Italy).

2.4.7 Statistical analysis

Unpaired *t*-test was employed for statistical comparison. Values are expressed as mean \pm standard error (s.e.), and $P < 0.05$ was considered statistically significant.

3 Results

3.1 Health status

No morbidity or mortality was observed in the control or treated animals. All the animals were healthy throughout the study period.

3.2 Body weight

The mean body weight of the vehicle-treated control animals was 176.09 ± 8.41 g. Although there was a mild increase in body weight during the course of the 360-day treatment period and 120-day recovery period, the change was statistically insignificant. Body weight values ranged between 179.42 ± 0.88 g and 183.96 ± 0.95 g (Table 1).

3.3 Organ weight

The mean weights of the vital organs—brain, thyroid, heart, lungs, liver, spleen, adrenal gland and kidney—and reproductive organs—testis, epididymis, seminal vesicle and ventral prostate—did not show significant dif-

ferences throughout the study period. All values ranged within the control limits (Tables 1 and 2).

3.4 Sperm analysis

3.4.1 Sperm concentration

The mean sperm concentration of the vehicle-treated control animals was 21.43 ± 3.63 million mL^{-1} . Treatment with MSF at a concentration of 50 mg per kg body weight per day led to a significant reduction in sperm concentration over days 120–180 of treatment ($P < 0.05$). On days 240–360 of treatment, the values, although still comparatively low, were not statistically different from those in the controls.

After 120 days of treatment withdrawal, the mean sperm concentration was comparable with that in the control rat (Table 3).

3.4.2 Sperm motility

The mean percentage of motile spermatozoa of the vehicle-treated control animals was 70.00 ± 7.51 . Treatment with MSF at a concentration of 50 mg per kg body weight per day resulted in total inhibition of sperm motility from day 60 through to the end of the 360-day treatment period.

Treatment withdrawal resulted in restoration of sperm motility after 60 days, and levels comparable with those in controls were reached after 120 days of treatment withdrawal (Table 3).

3.4.3 Sperm viability

The mean percentage of viable spermatozoa of

Table 1. The body weight and weight of reproductive organs in rats treated orally with the methanol subfraction of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds at 50 mg per kg body weight (bw) per day (values are mean \pm s.e. of five animals).

| Treatment schedule | Body weight (g) | Testis (mg per 100 g bw) | Epididymis (mg per 100 g bw) | Seminal vesicle (mg per 100 g bw) | Ventral Prostate (mg per 100 g bw) |
|--------------------|-------------------|--------------------------|------------------------------|-----------------------------------|------------------------------------|
| Control | 176.09 ± 8.41 | 596.73 ± 8.16 | 187.24 ± 8.81 | 218.02 ± 8.11 | 118.24 ± 6.89 |
| Treatment period | | | | | |
| 60 days | 179.42 ± 0.88 | 566.12 ± 10.08 | 180.12 ± 2.19 | 196.78 ± 9.57 | 107.14 ± 3.84 |
| 120 days | 181.93 ± 2.33 | 572.93 ± 7.18 | 177.63 ± 3.36 | 199.15 ± 9.65 | 116.92 ± 2.69 |
| 180 days | 182.38 ± 2.11 | 574.80 ± 8.71 | 180.84 ± 1.65 | 195.42 ± 9.00 | 109.25 ± 2.51 |
| 240 days | 179.86 ± 0.33 | 560.05 ± 10.21 | 181.72 ± 2.24 | 199.24 ± 8.63 | 108.41 ± 3.79 |
| 300 days | 182.35 ± 2.45 | 569.72 ± 8.59 | 183.48 ± 1.64 | 198.18 ± 9.92 | 116.23 ± 5.65 |
| 360 days | 182.90 ± 1.95 | 565.43 ± 11.98 | 180.34 ± 2.10 | 196.48 ± 8.94 | 105.34 ± 6.08 |
| Recovery period | | | | | |
| 60 days | 182.67 ± 2.00 | 577.71 ± 9.69 | 182.21 ± 1.35 | 201.62 ± 6.51 | 110.15 ± 1.08 |
| 120 days | 183.86 ± 0.95 | 578.24 ± 9.01 | 185.71 ± 0.88 | 200.74 ± 5.36 | 111.72 ± 1.00 |

Table 2. The weight of vital organs in rats treated orally with the methanol subfraction of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds at 50 mg per kg body weight per day (values are mean \pm s.e. of five animals).

| Treatment schedule | Brain (g) | Thyroid (g) | Heart (g) | Lungs (g) | Liver (g) | Spleen (g) | Adrenal (g) | Kidney (g) |
|--------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control | 1.54 \pm 0.06 | 0.03 \pm 0.02 | 0.64 \pm 0.04 | 1.29 \pm 0.31 | 3.03 \pm 0.24 | 0.45 \pm 0.01 | 0.15 \pm 0.06 | 1.54 \pm 0.04 |
| Treatment period | | | | | | | | |
| 60 days | 1.52 \pm 0.22 | 0.02 \pm 0.02 | 0.63 \pm 0.02 | 1.31 \pm 0.12 | 3.45 \pm 0.31 | 0.47 \pm 0.12 | 0.16 \pm 0.05 | 1.42 \pm 0.10 |
| 120 days | 1.43 \pm 0.12 | 0.01 \pm 0.01 | 0.58 \pm 0.19 | 1.28 \pm 0.31 | 3.52 \pm 0.03 | 0.42 \pm 0.07 | 0.12 \pm 0.04 | 1.37 \pm 0.13 |
| 180 days | 1.45 \pm 0.14 | 0.03 \pm 0.02 | 0.61 \pm 0.04 | 1.27 \pm 0.03 | 3.54 \pm 0.29 | 0.56 \pm 0.04 | 0.14 \pm 0.05 | 1.50 \pm 0.13 |
| 240 days | 1.50 \pm 0.12 | 0.01 \pm 0.01 | 0.54 \pm 0.06 | 1.23 \pm 0.14 | 3.49 \pm 0.24 | 0.55 \pm 0.19 | 0.11 \pm 0.04 | 1.41 \pm 0.06 |
| 300 days | 1.48 \pm 0.04 | 0.02 \pm 0.01 | 0.57 \pm 0.03 | 1.24 \pm 0.06 | 3.54 \pm 0.27 | 0.42 \pm 0.06 | 0.09 \pm 0.02 | 1.40 \pm 0.05 |
| 360 days | 1.52 \pm 0.02 | 0.02 \pm 0.01 | 0.56 \pm 0.12 | 1.21 \pm 0.45 | 3.61 \pm 0.24 | 0.51 \pm 0.04 | 0.11 \pm 0.02 | 1.39 \pm 0.01 |
| Recovery period | | | | | | | | |
| 60 days | 1.49 \pm 0.03 | 0.02 \pm 0.01 | 0.61 \pm 0.10 | 1.25 \pm 0.38 | 3.53 \pm 0.12 | 0.46 \pm 0.24 | 0.07 \pm 0.03 | 1.40 \pm 0.02 |
| 120 days | 1.61 \pm 0.02 | 0.03 \pm 0.01 | 0.59 \pm 0.07 | 1.26 \pm 0.18 | 3.62 \pm 0.48 | 0.51 \pm 0.18 | 0.12 \pm 0.07 | 1.42 \pm 0.05 |

Table 3. Cauda epididymal sperm characteristics of rats treated orally with the methanol subfraction of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds at 50 mg per kg body weight per day (values are mean \pm s.e. of five animals).

| Treatment schedule | Sperm density (million mL ⁻¹) | Sperm motility (%) | Sperm viability (%) | Abnormal sperm (%) |
|--------------------|---|--------------------|---------------------|--------------------|
| Control | 21.43 \pm 3.63 | 69.70 \pm 7.51 | 60.08 \pm 5.81 | 36.14 \pm 6.11 |
| Treatment period | | | | |
| 60 days | 14.12 \pm 0.84 | Nil | 36.19 \pm 1.66** | 47.07 \pm 4.41** |
| 120 days | 13.17 \pm 0.23* | Nil | 40.17 \pm 1.81** | 52.75 \pm 6.72** |
| 180 days | 12.28 \pm 0.16* | Nil | 38.74 \pm 1.71** | 51.08 \pm 5.92** |
| 240 days | 18.15 \pm 0.66 | Nil | 41.12 \pm 1.66** | 52.12 \pm 5.68** |
| 300 days | 15.05 \pm 0.33 | Nil | 42.23 \pm 2.66** | 50.18 \pm 6.00** |
| 360 days | 15.14 \pm 0.41 | Nil | 40.11 \pm 1.63** | 50.24 \pm 5.76** |
| Recovery period | | | | |
| 60 days | 18.01 \pm 2.06 | 51.80 \pm 8.03 | 50.78 \pm 5.11 | 42.17 \pm 4.70 |
| 120 days | 22.04 \pm 2.09 | 68.84 \pm 5.88 | 56.91 \pm 4.82 | 38.82 \pm 4.08 |

* $P < 0.05$, ** $P < 0.01$ compared with control values.

the vehicle-treated control animals was 60.08 \pm 5.81. Treatment with MSF at 50 mg per kg body weight per day caused a significant decrease ($P < 0.01$) in the percentage of viable spermatozoa from day 60 of the study period onwards.

Withdrawal of the treatment resulted in the restoration of sperm viability after 120 days of the recovery period. The mean percentage of viable spermatozoa was similar to the level in controls (Table 3).

3.4.4 Sperm abnormality

The mean percentage of abnormal spermatozoa of the vehicle-treated control animals was 36.14 \pm 6.11. Treatment with MSF at a concentration of 50 mg per kg body weight per day resulted in a significant increase ($P < 0.01$) in the percentage of abnormal spermatozoa from day 60 of

treatment onwards, throughout the treatment period.

After treatment withdrawal, there was a gradual reduction in the percentage of abnormal spermatozoa, and after 120 days of recovery, the mean percentage was comparable with that in the control group (Table 3).

3.5 Biochemical investigations

The mean levels of cholesterol, glycogen and LDH in the testis; sialic acid, *L*-carnitine and neutral α -glucosidase in the epididymis; fructose in the seminal vesicle and ACP in the ventral prostate did not show significant changes throughout the treatment or the recovery period (Table 4).

3.6 Histology of the testis

Seminiferous tubules of the testes of control ani-

mals possessed epithelia containing the Sertoli cells and the germ cells of various stages, covering the complete process of spermatogenesis. The Sertoli cells exhibited typical, irregular nuclei and well-defined cytoplasm, which appeared granular. The spermatogonia, oval in shape, were closely associated with the basal lamina. The spermatocytes showed various degrees of condensation of their nuclei and were closely associated with Sertoli cell cytoplasm. Elongated spermatids were embedded in the cytoplasm of the Sertoli cells. The lumen contained mature spermatozoa, and the interstitium contained distinct Leydig cells (Figure 1).

The testes of animals treated with MSF for 60 and 120 days displayed round seminiferous tubules with thick basal lamina. The Sertoli cells and germ cells were normal. The Sertoli cells showed vacuolization, and the spermatogonia, spermatocytes and spermatids, round as well as elongated, appeared normal, with normal nuclear and cytoplasmic characteristics. However, a few of the spermatocytes of the animals treated for 120 days showed signs of nuclear pyknosis or degeneration. The cytoplasm of all germ cells appeared granular. The lumen contained spermatozoa, and the Leydig cells were normal (Figures 2 and 3).

After 240 days of MSF treatment, disruption of spermatogenesis was evident. The basal lamina of the seminiferous tubules was comparatively thin. Vacuolization was evident in the cytoplasm of the Sertoli cells, spermatocytes and spermatids. The nuclei of the spermatocytes and spermatids showed pyknosis. However,

the spermatogonia and Leydig cells appeared normal. The lumen contained spermatozoa (Figure 4).

After 360 days of MSF treatment, severe disruption of spermatogenesis was evident, although the spermatogonia remained unaffected. The seminiferous tubules were round and occasionally showed irregular configurations. The basal lamina appeared thin. Degeneration of the epithelium was common in the majority of the seminiferous tubules. The Sertoli cells showed vacuolization, and the spermatocytes and spermatids showed nuclear pyknosis and cytoplasmic vacuolization. Disruption in the plasma membrane and extrusion of cytoplasmic contents were evident in the majority of the spermatocytes and spermatids. The effects were more severe in spermatid differentiation. The lumen contained germ cell debris and fewer spermatozoa, but the Leydig cells appeared normal (Figure 5).

After 120 days of recovery, restoration of proper spermatogenesis was evident. The seminiferous epithelium was comparable with that of control animals. The Sertoli cells and germ cells showed closer associations, with normal nuclear and cytoplasmic characteristics. The lumen contained packed spermatozoa, and the Leydig cells appeared normal (Figure 6).

3.7 Ultrastructure of the testis

Ultrastructural analysis of the testes of control animals showed that the Sertoli cells and germ cells had cellular characteristics typical of those seen in active spermatogenesis. The Sertoli cells of control animals

Table 4. Reproductive tissue biochemical parameters of rats treated orally with the methanol subfraction of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds at 50 mg per kg body weight per day (values are mean \pm s.e. of five animals).

| Treatment Schedule | Testis | | | | Epididymis | | Seminal vesicle | Ventral prostate |
|--------------------|-----------------------------------|--------------------------------|---------------------------|-----------------------------------|-----------------------------------|---|--------------------------------|---------------------------|
| | Cholesterol (mg g ⁻¹) | Glycogen (mg g ⁻¹) | LDH (U mg ⁻¹) | Sialic acid (mg g ⁻¹) | L-carnitine (nmol per mg protein) | α -Glucosidase (mg PNP per mg protein) | Fructose (mg g ⁻¹) | ACP (BU g ⁻¹) |
| Control | 4.26 \pm 0.20 | 2.10 \pm 0.22 | 25.25 \pm 2.06 | 4.80 \pm 1.25 | 1.73 \pm 0.08 | 101.31 \pm 3.98 | 5.98 \pm 2.02 | 2.03 \pm 1.13 |
| Treatment period | | | | | | | | |
| 60 days | 4.31 \pm 0.21 | 2.19 \pm 0.13 | 28.64 \pm 1.00 | 5.76 \pm 0.16 | 1.65 \pm 0.13 | 98.53 \pm 5.36 | 4.76 \pm 2.29 | 2.99 \pm 0.23 |
| 120 days | 4.58 \pm 0.28 | 2.06 \pm 0.22 | 24.12 \pm 1.13 | 4.72 \pm 1.35 | 1.58 \pm 0.25 | 96.90 \pm 7.31 | 5.76 \pm 0.26 | 2.58 \pm 0.21 |
| 180 days | 4.49 \pm 0.31 | 2.16 \pm 0.25 | 23.23 \pm 1.08 | 5.21 \pm 1.08 | 1.61 \pm 0.31 | 99.40 \pm 3.15 | 6.78 \pm 0.23 | 2.78 \pm 0.09 |
| 240 days | 4.51 \pm 0.11 | 2.08 \pm 0.11 | 27.16 \pm 1.45 | 5.10 \pm 1.18 | 1.60 \pm 0.35 | 100.11 \pm 1.38 | 6.18 \pm 0.28 | 3.20 \pm 0.16 |
| 300 days | 4.39 \pm 0.18 | 2.17 \pm 0.21 | 26.09 \pm 1.56 | 4.52 \pm 1.21 | 1.71 \pm 0.11 | 98.92 \pm 2.35 | 4.98 \pm 0.28 | 3.16 \pm 0.15 |
| 360 days | 4.45 \pm 0.21 | 2.08 \pm 0.19 | 28.03 \pm 1.13 | 4.98 \pm 1.11 | 1.58 \pm 0.99 | 100.61 \pm 1.00 | 6.85 \pm 0.18 | 2.48 \pm 0.14 |
| Recovery period | | | | | | | | |
| 60 days | 4.29 \pm 0.21 | 2.19 \pm 0.15 | 27.65 \pm 1.76 | 4.77 \pm 0.08 | 1.63 \pm 0.75 | 101.34 \pm 0.85 | 5.80 \pm 0.23 | 2.80 \pm 0.08 |
| 120 days | 4.36 \pm 0.11 | 2.10 \pm 0.08 | 31.08 \pm 0.09 | 4.98 \pm 0.16 | 1.68 \pm 0.81 | 100.81 \pm 0.91 | 5.88 \pm 0.18 | 2.50 \pm 0.01 |

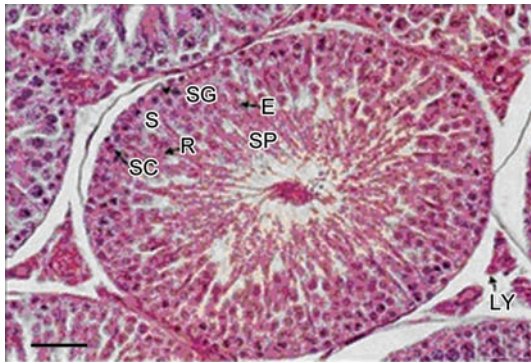


Figure 1. Histology of the testis of a control animal. The seminiferous tubules contain Sertoli cells and germ cells of various stages, covering the entire process of spermatogenesis. The lumen contains mature spermatozoa. The interstitium contains distinct Leydig cells. Scale bar = 100 μ m. S, Sertoli cells; SG, spermatogonia; SC, spermatocytes; R, round spermatid; E, elongated spermatid; SP, spermatozoa; LY, Leydig cells.

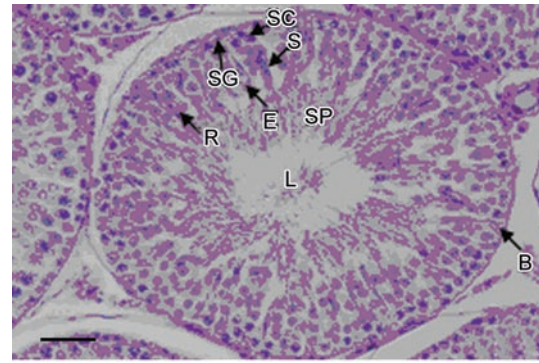


Figure 2. Histology of the testis of rats treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 60 days. Vacuolization in Sertoli cells is evident. Spermatogonia, spermatocytes and spermatids are unaffected, showing normal nuclear and cytoplasmic characteristics. The lumen contains spermatozoa. The Leydig cells are normal. Scale bar = 100 μ m. S, Sertoli cells; SG, spermatogonia; SC, spermatocytes; E, elongated spermatid; B, basal lamina; LY, Leydig cells; R, round spermatid; L, lumen.

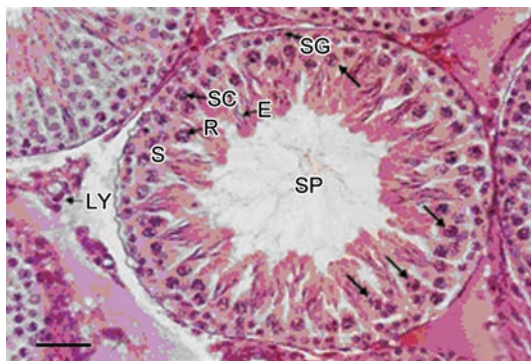


Figure 3. Histology of the testis of rats treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 120 days. Sertoli cells show vacuolization, whereas the spermatogonia, spermatocytes and spermatids (round as well as elongated) appear normal, with normal nuclear and cytoplasmic characteristics. A few of the spermatocytes show nuclear pyknosis (arrows). The cytoplasm of all germ cells appears granular. The lumen contains spermatozoa. The Leydig cells are normal. Scale bar = 100 μ m. S, Sertoli cells; SG, spermatogonia; SC, spermatocytes; R, round spermatid; E, elongated spermatid; SP, spermatozoa; LY, Leydig cells.

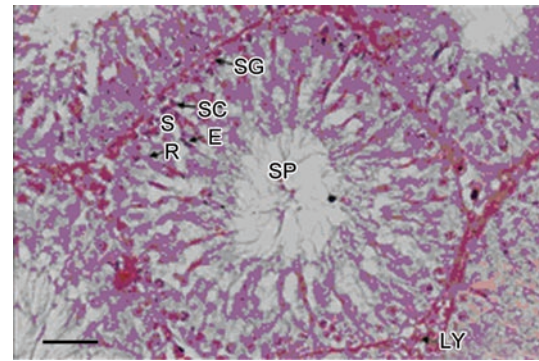


Figure 4. Histology of the testis of rats treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 240 days. Disruption of spermatogenesis is evident. Vacuolization is evident in the cytoplasm of Sertoli cells, spermatocytes and spermatids. The nuclei of spermatocytes and spermatids show pyknosis. Spermatogonia and Leydig cells appear normal. The lumen contains spermatozoa. Scale bar = 100 μ m. S, Sertoli cells; SG, spermatogonia; SC, spermatocytes; R, round spermatid; E, elongated spermatid; SP, spermatozoa; LY, Leydig cells.

showed distinct nuclear and cytoplasmic characteristics consistent with an active secretory state. The nuclei were irregular, with patchy chromatin material, deep indentations and well-defined nucleoli. Adjacent to the nuclei, the cytoplasm was characterized by cisternae

of rough endoplasmic reticulum, in close association with lipid droplets. Mitochondria were well defined and scattered throughout the cytoplasm. Golgi bodies were also well defined, and free ribosomes and glycogen granules were dispersed throughout the cytoplasm. The plasma membranes were conspicuous

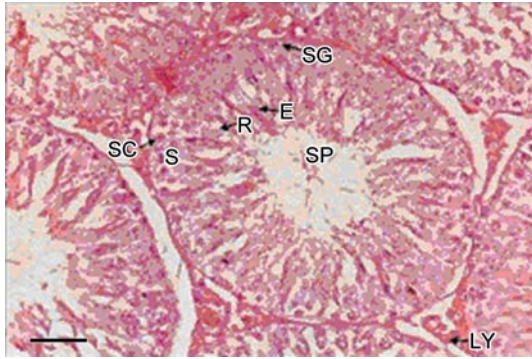


Figure 5. Histology of the testis of rats treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 360 days. Degeneration of the epithelium is common in the majority of the seminiferous tubules. Sertoli cells show vacuolization, and the spermatocytes and spermatids show nuclear pyknosis and cytoplasmic vacuolization. The lumen contains germ cell debris and fewer spermatozoa. Leydig cells appear normal. Scale bar = 100 μm . S, Sertoli cells; SG, spermatogonia; SC, spermatocytes; R, round spermatid; E, elongated spermatid; SP, spermatozoa; LY, Leydig cells.

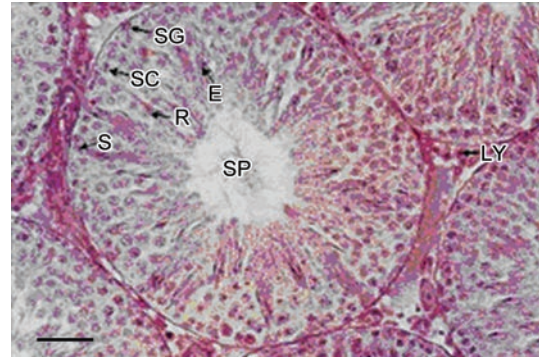


Figure 6. Histology of the testis of rats 120 days after treatment withdrawal. Restoration of spermatogenesis is evident. The seminiferous epithelium is comparable with that of a control animal. The Sertoli cells and germ cells show closer association to each other, with normal nuclear and cytoplasmic characteristics. The lumen contains packed spermatozoa, and the Leydig cells appear normal. Scale bar = 100 μm . S, Sertoli cells; SG, spermatogonia; SC, spermatocytes; R, round spermatid; E, elongated spermatid; SP, spermatozoa; LY, Leydig cells.

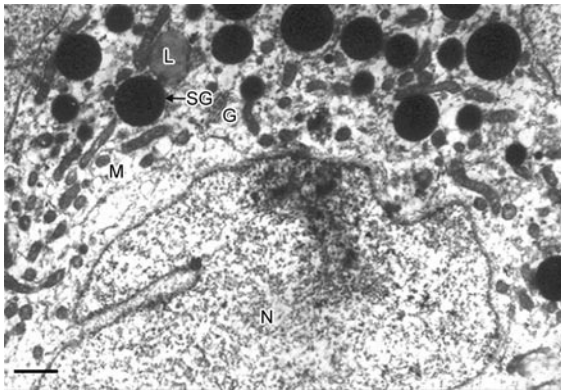


Figure 7. Ultrastructure of the testis of control animals showing the Sertoli cells. The nucleus appears irregular, with indentations. The cytoplasmic organelles indicate an active secretory state. The mitochondria, lipid droplets and rough endoplasmic reticulum are prominent and scattered throughout the cytoplasm. Secretory granules are abundant. Scale bar = 1.79 μm . G, Golgi bodies; L, lipid droplets; M, mitochondria; N, nucleus; SG, secretory granules.

and tortuous, showing close associations with adjacent spermatocytes and spermatids. Secretory granules were abundant (Figures 7 and 8). The spermatocytes, both primary and secondary, displayed round configurations with prominent nuclei. The nuclei contained distinct chromatin networks in the process of condensation and

well-defined nuclear membranes. The cytoplasm of the primary spermatocytes appeared granular, characterized by dispersed mitochondria and loose networks of rough endoplasmic reticulum. Cytoplasmic organelles of secondary spermatocytes were relatively sparse (Figures 9 and 10). Round spermatids were characterized by well-defined nuclei with distinct nuclear membranes and chromatin networks, and their cytoplasm was occupied predominantly with mitochondria. Formation of acrosome aggregations, marked by Golgi vesicles, appeared normal (Figure 11). The Leydig cells showed distinct nuclear characteristics. The cytoplasm showed prominent lipid bodies, well-defined vesicles of smooth endoplasmic reticulum and mitochondria surrounding the nucleus (Figure 12).

After 120 days of MSF treatment, the Sertoli cells showed evidence of severe vacuolization in the cytoplasm. However, secretory granules, prosecretory granules, mitochondria and Golgi bodies were scattered throughout the cytoplasm. The nuclei appeared normal with indentations and patchy chromatin material. Association of the germ cells with the cytoplasm of the Sertoli cells appeared normal (Figure 13). The spermatocytes appeared normal, containing distinct nuclei with patchy chromatin material and normal cytoplasm. Cytoplasmic organelles, however, were poorly defined. The plasma membranes of the spermatocytes were

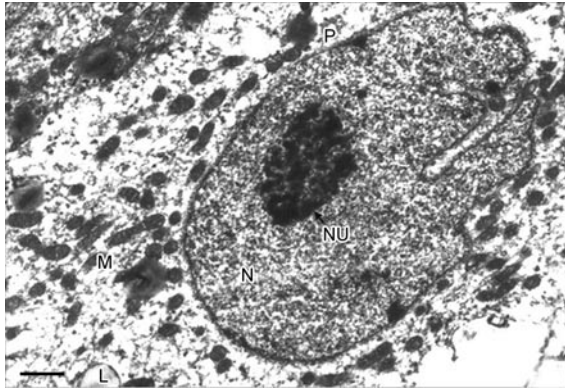


Figure 8. Ultrastructure of the testis of control animals showing the Sertoli cell cytoplasm. The nucleus shows indentation and a prominent nucleolus. The mitochondria are well defined. Prosecretory granules, rosettes of glycogen granules and free ribosomes are scattered throughout the cytoplasm. Scale bar = 2 μm . L, lipid droplets; M, mitochondria; N, nucleus; NU, nucleolus; P, prosecretory granules.

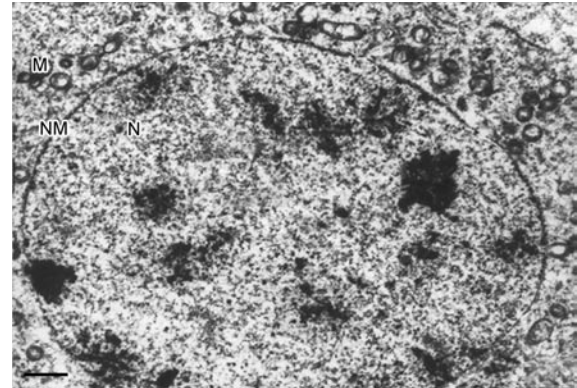


Figure 10. Ultrastructure of the testis of control animals, showing a secondary spermatocyte. The nucleus contains condensed chromatin material and a distinct nuclear membrane. Mitochondria are scattered throughout the cytoplasm. Scale bar = 2.17 μm . M, mitochondria; N, nucleus; NM, nuclear membrane.

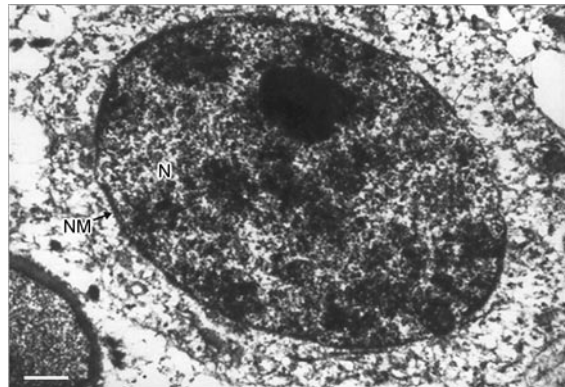


Figure 9. Ultrastructure of the testis of control animals showing the primary spermatocytes. The cytoplasm appears granular. The nucleus contains distinct chromatin networks, with well-defined nucleoli and prominent nuclear membranes. Scale bar = 2.17 μm . N, nucleus; NM, nuclear membrane.

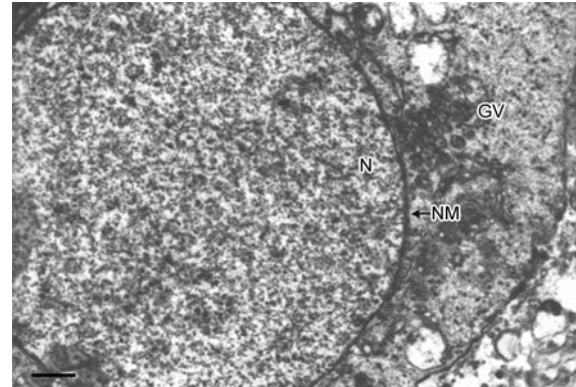


Figure 11. Ultrastructure of the testis of control animals showing a round spermatid. The nucleus is round, with a prominent nuclear membrane. Mitochondria occupy the periphery of the cytoplasm. Golgi vesicles are well defined. Scale bar = 2.78 μm . GV, Golgi vesicles; N, nucleus; NM, nuclear membrane.

distinct and showed close associations with Sertoli cell cytoplasm (Figure 14). Round spermatids showed well-defined nuclear and cytoplasmic characteristics. The nuclei appeared round, with well-defined nuclear membranes and chromatin structures. The cytoplasm showed scattered mitochondria and cisternae of rough endoplasmic reticulum. Acrosome vesicles, evidenced by the accumulation of Golgi vesicles, were also well defined (Figure 15).

After 240 days of MSF treatment, vacuolization of

the Sertoli cells was comparable with that of the Sertoli cells after 120 days of MSF treatment. However, the nuclei showed deep indentations, with patchy chromatin material, and there were fewer cytoplasmic organelles. Mitochondria were few and vacuolated. The cytoplasm of the associated germ cells was poorly defined (Figures 16 and 17). The nuclei of spermatocytes and round spermatids showed evidence of pyknosis, characterized by poorly defined chromatin structures and nuclear membranes. Mitochondria adjacent to the nuclei were

vacuolated. Other cytoplasmic organelles in the spermatocytes and spermatids were sparse (Figures 18 and 19). Elongated spermatids embedded in the Sertoli cell cytoplasm were characterized by poorly defined membranes and cytoplasmic vacuolization. Mitochondria present in the cytoplasm also showed vacuolization

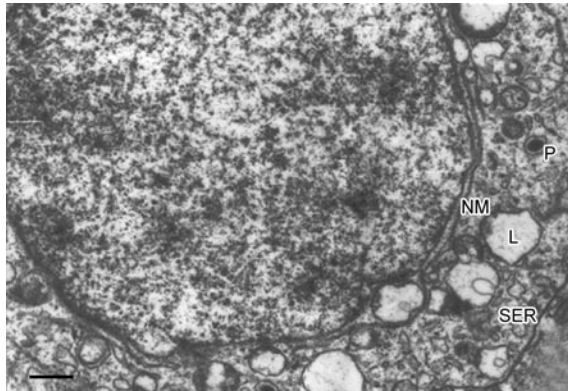


Figure 12. Ultrastructure of the testis of a control animal, showing a Leydig cell. The cell is characterized by a round nucleus, containing a prominent nuclear membrane. The cytoplasm shows vesicles of smooth endoplasmic reticulum, prosecretory granules, prominent mitochondria and lipid bodies. Scale bar = 2.78 μm . L, lipid bodies; NM, nuclear membrane; P, prosecretory granules; SER, smooth endoplasmic reticulum.

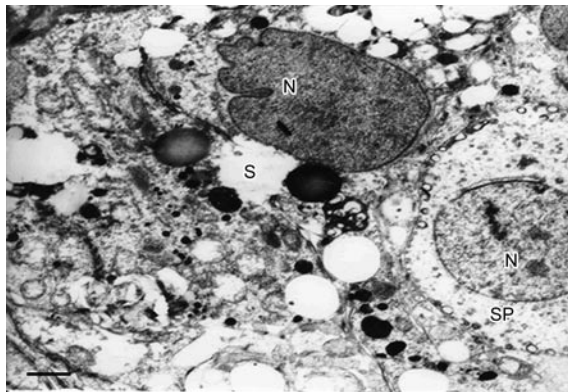


Figure 13. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 120 days showing the Sertoli cells. Vacuolization in the cytoplasm is evident. Secretory granules, prosecretory granules, mitochondria and Golgi bodies are scattered in the cytoplasm. The nucleus appears normal, with indentations and patchy chromatin material. Association of germ cells with the cytoplasm of the Sertoli cells appears normal. Scale bar = 2.38 μm . S, Sertoli cell; SP, spermatid; N, nucleus.

(Figure 20).

After 360 days of MSF treatment, the ultrastructural characteristics of the Sertoli cells and germ cells of the testis were indicative of disturbed spermatogenesis. The nuclear and cytoplasmic volumes of the Sertoli cells were reduced. However, the nuclear features

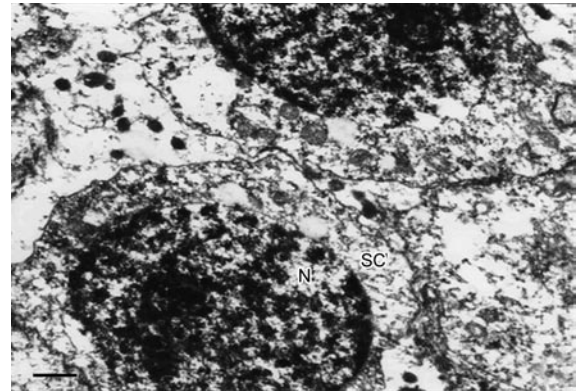


Figure 14. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 120 days, showing the spermatocytes, with distinct nucleus, patchy chromatin material and normal cytoplasm. Cytoplasmic organelles, however, are poorly defined. The plasma membranes of the spermatocytes are distinct and show closer association with the Sertoli cell cytoplasm. Scale bar = 2.78 μm . SC, spermatocytes; N, nucleus.

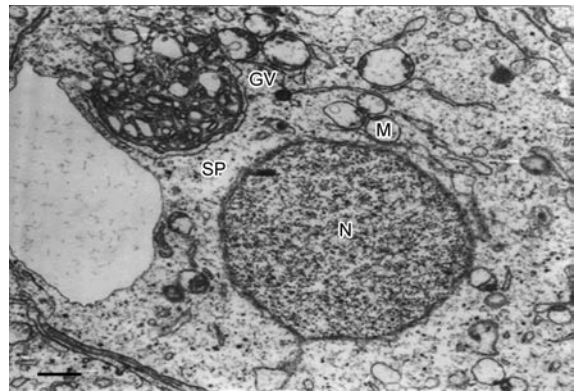


Figure 15. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 120 days, showing the spermatids. The nucleus appears round, with well-defined nuclear membranes and chromatin structures. The cytoplasm shows scattered mitochondria and cisternae of rough endoplasmic reticulum. The acrosome vesicles are evidenced by accumulation of Golgi vesicles. Scale bar = 2.78 μm . SP, spermatids; N, nucleus; M, mitochondria; GV, Golgi vesicles.

appeared normal. The cytoplasm showed vacuolization, and the cytoplasmic organelles were relatively sparse. Although a few mitochondria were found scattered around the cytoplasm, they showed vacuolization (Figure 21). Nuclear degeneration was evident in the spermatocytes and spermatids, and the cytoplasmic organelles in these cells were sparse. Although present in the peripheral cytoplasm of the spermatids, few mitochondria displayed vacuolization. Membrane damage in the acrosomal cap was also evident. Leydig cells

appeared normal (Figures 22–24).

3.8 Haematology

Total RBC, WBC, Hb, PCV, MCV, MCH and MCHC levels did not show appreciable changes after either 360 days of treatment with MSF at a concentration of 50 mg per kg body weight per day or after 120 days of recovery (Table 5).

3.9 Clinical chemistry

The mean levels of serum SGPT, SGOT, CPK, ALP,

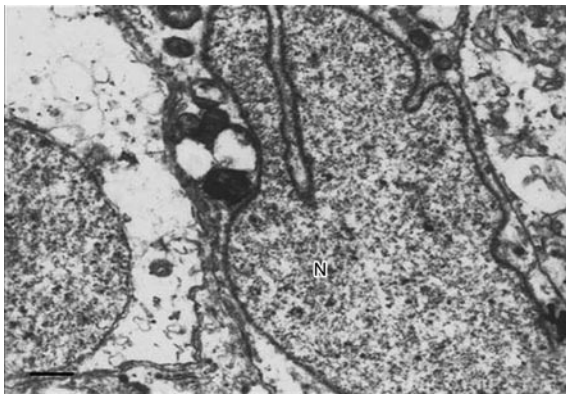


Figure 16. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 240 days, showing the Sertoli cells. The nuclei show indentations and patchy chromatin material. Mitochondria show vacuolization. The cytoplasm of the associated germ cells was poorly defined. Scale bar = 2.78 μm . N, nucleus.

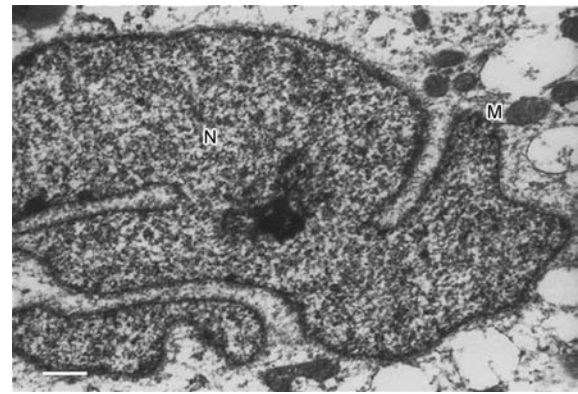


Figure 17. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 240 days, showing the Sertoli cells. The nucleus shows deep indentations, with patchy chromatin material, and the cytoplasmic organelles are fewer in number. Mitochondria are sparse and vacuolated. Scale bar = 2.38 μm . N, nucleus; M, mitochondria.

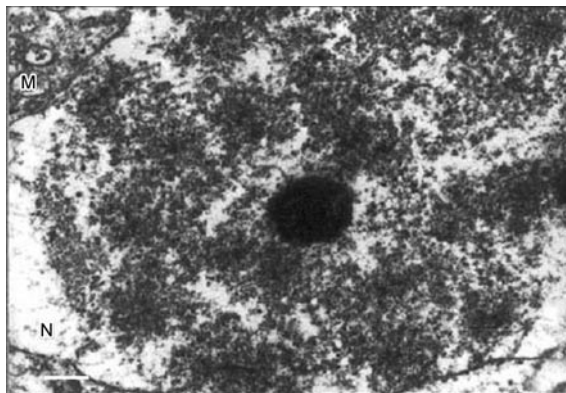


Figure 18. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 240 days, showing the spermatocytes. The nucleus shows signs of pyknosis. Mitochondria show vacuolization. Scale bar = 2.38 μm . N, Nucleus; M, mitochondria.

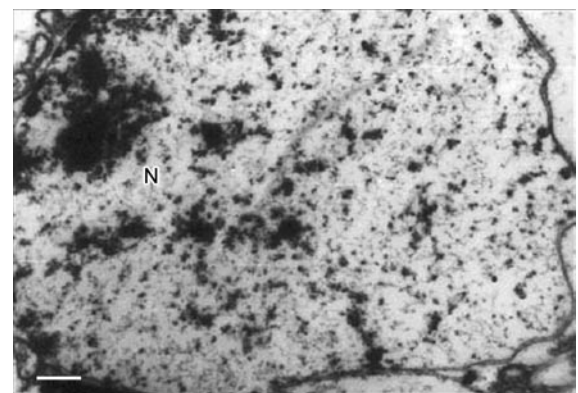


Figure 19. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 240 days, showing a round spermatid. The nucleus shows signs of pyknosis. Scale bar = 2.38 μm . N, nucleus.

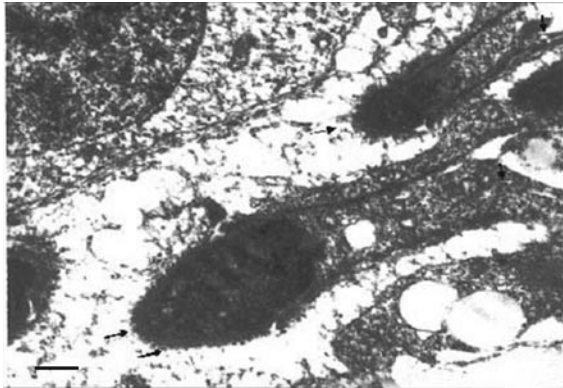


Figure 20. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 240 days, showing the elongated spermatids. The nuclear membranes are poorly defined (arrows). Mitochondria show vacuolization. Other cytoplasmic organelles are sparse. Scale bar = 2.78 μ m.

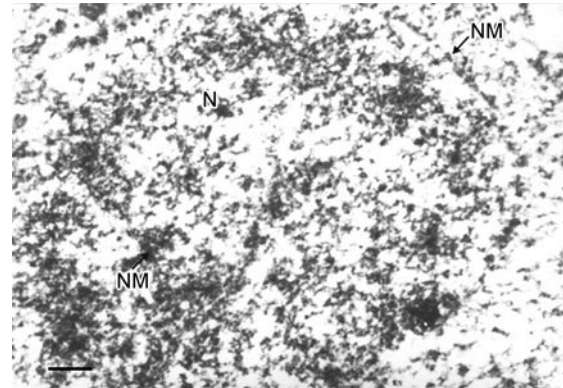


Figure 22. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 360 days, showing the spermatocytes. The cell is severely affected. The nuclear membranes and nuclear contents are poorly defined. Cytoplasmic organelles are sparse. Scale bar = 2.38 μ m. N, nucleus; NM, nuclear membrane.

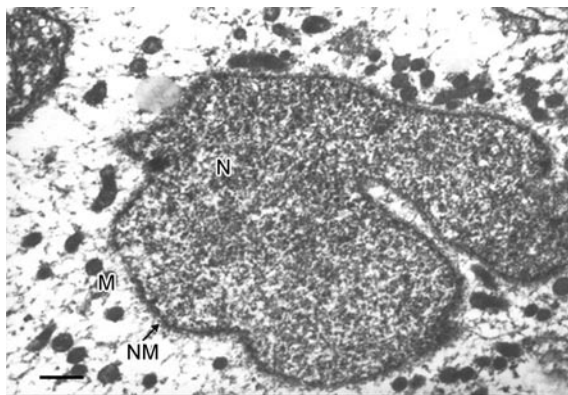


Figure 21. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 360 days, showing the Sertoli cells. The nucleus is irregular, with deep indentations. Cytoplasmic organelles are relatively sparse. However, well-defined mitochondria are scattered throughout the cytoplasm. Scale bar = 2.17 μ m. M, mitochondria; N, nucleus; NM, nuclear membrane.

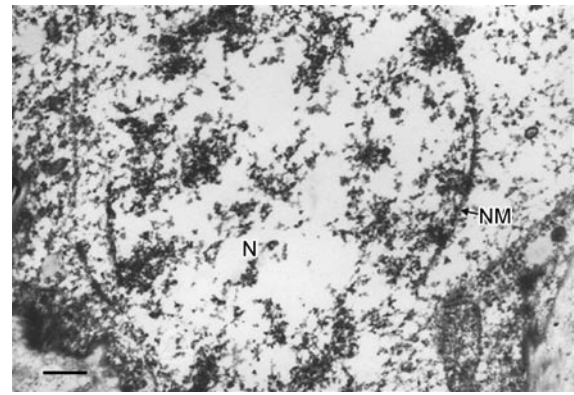


Figure 23. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 360 days, showing the round spermatids. The nucleus shows signs of pyknosis. The cytoplasmic organelles are sparse. Scale bar = 2.78 μ m. N, nucleus; NM, nuclear membrane.

LDH, creatinine, cholesterol, glucose, bilirubin, urea, TGL and HDL did not show significant changes (Table 6).

3.10 Hormone assay

The mean serum testosterone level showed fluctuations in the level of testosterone within the control range during treatment with MSF at 50 mg per kg body weight per day for 360 days and after withdrawal of treatment for 120 days (Table 7).

3.11 Fertility test

Fertility tests, conducted every 60 days, showed normal viable offspring in the vehicle-treated control animals (5.0 ± 0.6 , mean \pm s.e.). In the MSF-treated animals, fertility tests showed no fertility throughout the treatment period. Normal, viable offspring (free of mortality, morbidity or visible teratogenic symptoms) were produced after 120 days of treatment withdrawal in the treated animals (Table 8). The libido of the treat-

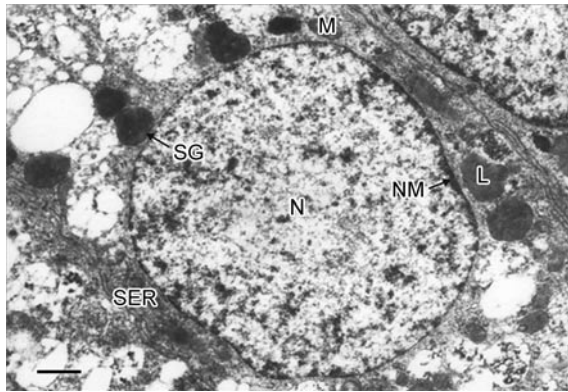


Figure 24. Ultrastructure of the testis of rat treated with methanol subfraction (MSF) at 50 mg per kg body weight per day for 360 days, showing the Leydig cells. The cell appears normal. Lipid bodies, secretory granules, mitochondria and stacks of smooth endoplasmic reticulum are prominent. Nucleus and nuclear membrane appear normal. Scale bar = 2.78 μm . L, lipid bodies; M, mitochondria; N, nucleus; NM, nuclear membrane; SER, smooth endoplasmic reticulum; SG, secretory granule.

ed animals, as evidenced by mounting behaviour (visual observation), remained unaffected throughout the study period.

4 Discussion

Many of the antifertility compounds identified in plants to date perturb spermatogenesis by causing tes-

ticular dysfunction. The roots of *Aristolochia indica* and *Plumbago zeylanica*; the leaves of *Azadirachta indica*, *Catharanthus roseus*, *Vinca rosea* and *Ocimum sanctum*; the flowers of *Hibiscus rosa-sinensis* and *Malvaviscus conzatii*; the seeds of *C. papaya* and *Vitex negundo*; and the fruit of *Momordica charantia* have been identified as candidates for male fertility regulation. Reduction in weight of the testis, epididymis, seminal vesicle and ventral prostate; suppression of spermatogenesis; degeneration of epididymal epithelium; and regression or absence of secretory activity of the seminal vesicle and ventral prostate are some of the common outcomes of the use of these plants, as well as several hundred plants undergoing preliminary screening for antifertility activity [27–29].

In our earlier, preliminary investigations, aqueous, methanol, ethanol, ethyl acetate and chloroform extracts of *C. papaya* seeds were shown to possess significant antifertility effects [11, 12, 28, 30]. However, despite species variation (that is, sperm motility inhibition in rats, and azoospermia in rabbits and langur monkeys [15–17]), it has been observed that the chloroform extract, the benzene chromatographic fraction of the chloroform extract and its methanol and ethyl acetate subfractions exerted 100% reversible contraceptive efficacy without side effects.

The results of this investigation show that long-term treatment with MSF for a period up to one year is safe and effective in completely inhibiting sperm motil-

Table 5. Haematological parameters of rats treated orally with the methanol subfraction of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds at 50 mg per kg body weight per day (values are mean \pm s.e. of 5 animals).

| Treatment schedule | RBC (10^6 mm^{-3}) | WBC (10^3 mm^{-3}) | Hb (g per 100 mL) | PCV (%) | MCV (μ^3) | MCH (pg) | MCHC (%) |
|--------------------|--------------------------------|--------------------------------|-------------------|------------------|------------------|------------------|------------------|
| Control | 6.85 \pm 2.21 | 8.53 \pm 1.76 | 15.54 \pm 1.26 | 45.82 \pm 2.51 | 66.30 \pm 2.08 | 22.90 \pm 2.41 | 34.34 \pm 1.32 |
| Treatment period | | | | | | | |
| 60 days | 6.63 \pm 1.01 | 8.43 \pm 0.94 | 15.10 \pm 1.33 | 44.80 \pm 1.40 | 67.10 \pm 1.69 | 22.81 \pm 1.51 | 33.90 \pm 1.92 |
| 120 days | 6.61 \pm 2.01 | 8.42 \pm 1.04 | 14.82 \pm 1.92 | 43.64 \pm 1.60 | 67.51 \pm 1.17 | 22.43 \pm 1.62 | 33.18 \pm 1.67 |
| 180 days | 6.68 \pm 1.00 | 8.43 \pm 1.01 | 15.31 \pm 1.00 | 45.00 \pm 1.13 | 66.12 \pm 1.00 | 23.10 \pm 1.08 | 33.43 \pm 1.85 |
| 240 days | 6.73 \pm 1.03 | 8.39 \pm 1.05 | 14.10 \pm 1.16 | 44.61 \pm 1.44 | 66.34 \pm 1.80 | 22.42 \pm 1.32 | 34.00 \pm 1.23 |
| 300 days | 6.76 \pm 1.04 | 8.46 \pm 0.91 | 15.91 \pm 1.00 | 43.90 \pm 1.16 | 65.44 \pm 1.93 | 22.12 \pm 1.16 | 33.83 \pm 1.10 |
| 360 days | 6.80 \pm 0.29 | 8.36 \pm 1.13 | 16.24 \pm 0.98 | 45.12 \pm 1.00 | 65.90 \pm 1.04 | 22.48 \pm 1.11 | 33.54 \pm 1.13 |
| Recovery period | | | | | | | |
| 60 days | 6.73 \pm 1.01 | 8.46 \pm 1.16 | 15.20 \pm 1.01 | 45.24 \pm 0.01 | 66.31 \pm 0.91 | 23.52 \pm 0.98 | 34.10 \pm 1.00 |
| 120 days | 6.80 \pm 0.39 | 8.41 \pm 0.39 | 16.62 \pm 1.02 | 45.42 \pm 0.08 | 66.92 \pm 0.75 | 22.80 \pm 0.59 | 34.62 \pm 0.71 |

Abbreviations: RBC, red blood corpuscles; WBC, white blood corpuscles; Hb, haemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular Hb; MCHC, mean corpuscular Hb concentration

Table 6. Serum clinical biochemistry of rats treated orally with methanol subfraction of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds at 50 mg per kg body weight per day (values are mean \pm s.e. of five animals).

| Treatment schedule | SGPT (IU L ⁻¹) | SGOT (IU L ⁻¹) | CPK (IU L ⁻¹) | ALP (IU L ⁻¹) | LDH (U mL ⁻¹) | Creatinine (mg per 100 mL) | Cholesterol (mg per 100 mL) | Glucose (mg per 100 mL) | Bilirubin (mg per 100 mL) | Urea (mg per 100 mL) | Triglycerides (mg per 100 mL) | HDL-Cholesterol (mg per 100 mL) |
|--------------------|----------------------------|----------------------------|---------------------------|---------------------------|---------------------------|----------------------------|-----------------------------|-------------------------|---------------------------|----------------------|-------------------------------|---------------------------------|
| Control | 2.03 \pm 0.53 | 2.02 \pm 0.81 | 4.81 \pm 0.97 | 10.60 \pm 1.15 | 24.08 \pm 1.88 | 2.05 \pm 0.94 | 100.82 \pm 2.02 | 87.82 \pm 1.64 | 0.63 \pm 0.06 | 23.12 \pm 2.73 | 75.15 \pm 1.03 | 48.90 \pm 0.68 |
| Treatment period | | | | | | | | | | | | |
| 60 days | 2.14 \pm 0.03 | 1.95 \pm 0.16 | 4.72 \pm 0.76 | 9.82 \pm 0.76 | 21.25 \pm 2.86 | 2.08 \pm 0.08 | 101.80 \pm 1.37 | 86.17 \pm 1.57 | 0.52 \pm 0.02 | 25.05 \pm 1.03 | 73.86 \pm 1.09 | 41.84 \pm 2.93 |
| 120 days | 1.91 \pm 0.08 | 2.13 \pm 0.85 | 4.67 \pm 0.56 | 10.45 \pm 0.83 | 24.12 \pm 0.54 | 2.19 \pm 0.93 | 102.12 \pm 1.31 | 83.93 \pm 1.98 | 0.67 \pm 0.03 | 21.91 \pm 3.56 | 70.83 \pm 2.95 | 40.72 \pm 1.95 |
| 180 days | 2.00 \pm 0.05 | 2.02 \pm 0.49 | 4.81 \pm 0.30 | 9.63 \pm 0.15 | 23.67 \pm 1.00 | 2.01 \pm 0.08 | 103.05 \pm 1.44 | 85.74 \pm 2.00 | 0.59 \pm 0.05 | 24.82 \pm 0.71 | 73.81 \pm 2.01 | 38.51 \pm 4.08 |
| 240 days | 1.95 \pm 0.06 | 1.85 \pm 0.10 | 4.73 \pm 0.84 | 10.35 \pm 0.96 | 21.64 \pm 1.66 | 1.92 \pm 0.56 | 102.53 \pm 1.28 | 86.71 \pm 1.66 | 0.64 \pm 0.03 | 23.08 \pm 1.36 | 74.60 \pm 1.96 | 47.62 \pm 0.85 |
| 300 days | 1.95 \pm 0.03 | 1.92 \pm 0.89 | 4.58 \pm 0.94 | 11.00 \pm 0.77 | 22.79 \pm 0.98 | 1.90 \pm 0.84 | 102.42 \pm 1.33 | 87.50 \pm 1.00 | 0.56 \pm 0.02 | 22.82 \pm 2.01 | 73.01 \pm 2.01 | 46.82 \pm 2.36 |
| 360 days | 2.15 \pm 0.08 | 2.01 \pm 0.50 | 4.63 \pm 0.88 | 10.63 \pm 0.20 | 23.10 \pm 0.85 | 2.05 \pm 0.06 | 101.94 \pm 1.65 | 86.09 \pm 2.10 | 0.59 \pm 0.02 | 24.10 \pm 1.06 | 75.14 \pm 1.98 | 45.85 \pm 3.85 |
| Recovery period | | | | | | | | | | | | |
| 60 days | 2.00 \pm 0.98 | 1.90 \pm 0.88 | 4.79 \pm 0.51 | 11.60 \pm 0.21 | 23.00 \pm 1.01 | 1.90 \pm 0.63 | 102.83 \pm 1.84 | 88.05 \pm 1.05 | 0.68 \pm 0.03 | 22.35 \pm 0.85 | 74.80 \pm 0.85 | 47.91 \pm 1.96 |
| 120 days | 2.41 \pm 0.10 | 1.92 \pm 0.98 | 4.84 \pm 0.99 | 10.98 \pm 0.99 | 24.12 \pm 0.09 | 2.12 \pm 0.38 | 102.12 \pm 1.02 | 86.91 \pm 1.58 | 0.53 \pm 0.02 | 23.08 \pm 0.85 | 74.62 \pm 2.61 | 47.12 \pm 2.08 |

Table 7. Serum levels of testosterone in rats treated orally with the methanol subfraction of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds at 50 mg per kg body weight per day (values are mean \pm s.e. of five animals)

| Treatment schedule | Testosterone (ng mL ⁻¹) |
|--------------------|-------------------------------------|
| Control | 3.91 \pm 0.41 |
| Treatment period | |
| 60 days | 3.26 \pm 0.90 |
| 120 days | 3.88 \pm 0.77 |
| 180 days | 3.16 \pm 0.15 |
| 240 days | 2.75 \pm 0.71 |
| 300 days | 3.72 \pm 0.31 |
| 360 days | 2.91 \pm 0.44 |
| Recovery period | |
| 60 days | 2.76 \pm 0.94 |
| 120 days | 3.13 \pm 0.45 |

ity in rats. This inhibition coincides with a gradual and significant decline in cauda epididymal sperm density, percentage of viable spermatozoa and a significant increase in sperm anomalies, degenerated germinal epithelium, vacuolization in Sertoli cells and proliferating germ cells. Furthermore, disturbances in spermatid differentiation of the testis, normal serum testosterone levels and sterility indicate significant contraceptive efficacy. The onset of contraceptive efficacy is evident within 60 days of treatment, as confirmed by fertility tests.

It is likely that any contraceptive agent that affects sperm motility would influence spermatozoa indirectly through disruption of epididymal epithelial cell function or act directly on the spermatozoa by affecting their enzymes [31]. In this investigation, although the increase in abnormal sperm count and total inhibition of sperm motility suggest that MSF's target is within the internal milieu of the epididymis, the lack of significant alterations in the epididymal epithelium (unpublished observations) and the unaltered level of epididymal markers (sialic acid, *L*-carnitine and neutral α -glucosidase) in the cauda epididymis indicate that it is less likely that MSF affects the sperm motility by altering the epididymis itself.

In normal testicular function, both Sertoli cells and Leydig cells are central to the regulation of fertility and reproductive health. These cells are susceptible targets in any of the experimentally induced changes in testis, particularly in male contraceptive strategies [1, 3]. Spermatogenesis is a complex process that requires a specific microenvironment generated by somatic cells to

Table 8. Reproductive performance of the male rats treated orally with the methanol subfraction of the benzene chromatographic fraction of the chloroform extract of *C. papaya* seeds at 50 mg per kg body weight per day (values are mean \pm s.e. of five animals).

| Treatment schedule | No. of males/females | No. of females delivered | No. of litters | % Fertility |
|--------------------|----------------------|--------------------------|----------------|-------------|
| Control | 5/10 | 10 | 52 | 100 |
| Treatment period | | | | |
| 60 days | 5/10 | 0 | 0 | 0 |
| 120 days | 5/10 | 0 | 0 | 0 |
| 180 days | 5/10 | 0 | 0 | 0 |
| 240 days | 5/10 | 0 | 0 | 0 |
| 300 days | 5/10 | 0 | 0 | 0 |
| 360 days | 5/10 | 0 | 0 | 0 |
| Recovery period | | | | |
| 60 days | 5/10 | 6 | 37 | 60 |
| 120 days | 5/10 | 10 | 49 | 100 |

provide support and nurturing for the developing germ cells throughout their proliferation and differentiation [3]. Somatic cells form a blood–testis barrier through tight junctions between adjacent Sertoli cells, which creates a unique environment for germ cell development and also protects the differentiated haploid germ cells from recognition by the immune system. In addition, they phagocytose the residual cytoplasm from mature sperm cells and may absorb interstitial fluid, which is secreted into the lumen of the seminiferous tubule to aid sperm transport to the rete testis and epididymis [3].

It is well known that the Sertoli cells are susceptible to testicular damage. The presence of agranular endoplasmic reticulum and stacks of granular endoplasmic reticulum, numerous mitochondria and multiple Golgi bodies in the basal part of the cytoplasm are characteristics of Sertoli cells, which are metabolically active, producing protein and steroids [1]. Proteins necessary for the differentiation of germ cells are secreted at their highest rates in the testis during spermatid elongation and spermiation. Physiologically, Sertoli cells are responsible for protein secretion, which aids in specific steps of germ cell maturation [3]. These proteins, in addition to being synthesized by Sertoli cells, are captured at the basal pole of the Sertoli cells, transported upwards to their adluminal parts and transferred to the germ cells [3]. Sertoli cells in a regressed testis do not contain conspicuous stacks of granular endoplasmic reticulum, which is consistent with the reduced protein secretory function of these Sertoli cells [1].

In the case of gossypol-induced spermatogenic arrest in laboratory animals, it has been reported that the

specific targets of gossypol's action on spermatogenesis are the mid and late stages of spermatids and spermatocytes, with accompanying degenerative changes in the Sertoli cells [32]. The subcellular target for gossypol is the mitochondria of the spermatogenic cells, and the spermatozoa derived from the testes of the animals treated with gossypol show remarkable damage in the mitochondrial sheaths. Inhibition of mitochondrial ATP production in isolated hamster spermatids has also been well documented, suggesting that the mitochondria and their energy production are the specific targets of the action of gossypol on spermatogenic cells [32].

The main aim of this investigation was to ascertain the safety and mechanism of action of long-term treatment with MSF—specifically, whether the effect of MSF is a simple cytotoxic effect on the germ cells or is mediated by other factors, such as Sertoli cells, on the basis of the evidence obtained from earlier investigations after short-term treatment with *C. papaya* seed products [7, 11–17]. Such experiments would establish *C. papaya* seeds as a potent male contraceptive. In this investigation, damage to Sertoli cells was evident in the MSF-treated animals. The damage was characterized by cytoplasmic vacuolization and mitochondrial damage, similar to the results observed in gossypol-treated rats [32]. The ultrastructural defects in spermatocytes and spermatids, particularly in those that interfere with the spermiation process, could be because of disturbances in the microenvironment of the Sertoli cells, disturbances that affect the protein synthesis machinery essential for germ cell differentiation. Persistent damage to the Sertoli cells may have resulted

in faulty spermatid differentiation, which ultimately led to teratozoospermia. Treatment-induced vacuolization of the Sertoli cells resulted in changes or decreases in seminiferous tubule fluid secretion, which further resulted in apical sloughing or shedding and germ cell death [4]. Although a reduction in the weight of the testes was observed in this investigation, it was statistically insignificant and possibly attributable to the fact that the changes in the Sertoli cells and germ cells were at a subcellular level. These changes led to cytoplasmic vacuolization and nuclear pyknosis, without loss of germ cells or Sertoli cells and without affecting the process of spermatogenesis, as in gossypol-treated rats [4]. Recent evidence also suggests a role for oxidative stress, caused by many factors, including tissue damage, in defective sperm function [33, 34]. The initial damage in the Sertoli cells, leading to apoptosis of germ cells, could result in generation of reactive oxygen species (ROS), which alter sperm cell membrane integrity, leading to loss of motility [33, 34].

It is concluded that long-term daily treatment with MSF for a period of 360 days results in necrotic changes in the seminiferous tubules of the testis, showing sparse cytoplasmic organelles and vacuolization in Sertoli cells, and nuclear pyknosis in spermatocytes and spermatids. Available data indicate that the effect originates in the Sertoli cells, as evidenced by the morphology of the testis after 60–120 days of treatment, whereas histology and ultrastructural analysis indicated damage only in the Sertoli cells. Initial damage in a few of the spermatocytes at 120 days and severe damage to the spermatocytes and spermatids after 240 days of treatment indicate that the deficient Sertoli cell factors (proteins) might subsequently affect spermatocyte and spermatid maturation. In particular, nuclear decondensation, acrosome formation and mitochondria may be affected, which may in turn result in defective spermatozoon production in the testes, reflected in increased numbers of abnormalities and loss of motility in the cauda epididymal spermatozoa. It has been reported that the morphogenetic factor involved in development of the normal head shape of spermatozoa is the degree of chromatin packing during the process of spermiogenesis. Head abnormalities detected at short time intervals in epididymal sperm imply damage to the nuclei of late elongated spermatids. Persistence of teratozoospermia for a longer duration implies damage to Sertoli cells, which affects germ cells and results in faulty spermatid differentiation [35]. In addition,

the possibility that oxidative stress leads to excessive generation of ROS from apoptotic cells and defective spermatozoa that in turn cause alterations in sperm parameters after 60 days of treatment cannot be ruled out [33, 34]. Normal subcellular characteristics of the Leydig cells, together with the unaltered serum testosterone levels throughout the study period, and unaltered androgenic parameters (weight of accessory reproductive organs, fructose levels of seminal vesicles and ACP levels of ventral prostates), eliminate any possibility of hormone-mediated disruption of spermatogenesis.

Acknowledgment

This study was supported by the Indian Council of Medical Research, New Delhi, India. We are grateful to the Special Assistance Programme (Phase III)/Centre for Advanced Studies (CAS), University Grants Commission, New Delhi and the Head of the Department, for providing infrastructural facilities. Ultrastructural studies were carried out at the EM-SAIF-DST Facility, Department of Anatomy, All India Institute of Medical Sciences (AIIMS), New Delhi, India.

References

1. Pudney J. Fine structural changes in Sertoli and Leydig cells during the reproductive cycle of the ground squirrel, *Citellus lateralis*. *J Reprod Fertil* 1986; 77: 37–49.
2. Gerard H, Gerard A, Ennya A, Felden F, Gueant JL. Spermatogenic cells do internalize Sertoli androgen-binding protein: A transmission electron microscopy, autoradiographic study in the rat. *Endocrinology* 1994; 134: 1515–27.
3. Boekelheide K. Sertoli cell toxicants. In: Russel LD, Griswold MD, editors. *The Sertoli Cell*. Clearwater, Florida: Cache River Press; 1993. p 552–75.
4. Hoffer AP. Effects of gossypol on the seminiferous epithelium in the rat: a light and electron microscope study. *Biol Reprod* 1983; 28: 1007–20.
5. Kasturi M, Ahamed RN, Pathan KM, Manivannan B, Aladakkatti RH. Ultrastructural changes induced by leaves of *Azadirachta indica* (Neem) in the testis of albino rats. *J Basic Clin Physiol Pharmacol* 2002; 13: 311–28.
6. Lohiya NK, Manivannan B, Mishra PK, Pathak N, Sriram S, *et al.* Chloroform extract of *Carica papaya* induces long-term reversible azoospermia in langur monkey. *Asian J Androl* 2002; 4: 17–26.
7. Manivannan B, Mishra PK, Pathak N, Sriram S, Bhande SS, *et al.* Ultrastructural changes in the testis and epididymis of rats following treatment with the benzene chromatographic fraction of the chloroform extract of the seeds of *Carica papaya*. *Phytother Res* 2004; 18: 285–9.
8. Al-Majed AA, Al-Yahya AA, Al-Bekairi AM, Al-Shabanah

- OA, Qureshi S. Reproductive, cytological and biochemical toxicity of Yohimbe in male Swiss albino mice. *Asian J Androl* 2006; 8: 469–76.
9. Ansari AS, Shrivastava S, Goyal S, Lohiya NK. Leading plant products for fertility regulation: a review. In: Sharma RS, Rajanna A, Rajalakshmi M, editors. *Recent Advances and Challenges in Reproductive Health Research*. New Delhi: Indian Council of Medical Research; 2008. p117–34.
 10. Raji Y, Akinsomisoye OS, Salman TM. Antispermato-genic activity of *Morinda lucida* extract in male rats. *Asian J Androl* 2005; 7: 405–10.
 11. Lohiya NK, Goyal RB, Jayaprakash D, Ansari AS, Sharma S. Antifertility effects of aqueous extract of *Carica papaya* seeds in male rats. *Planta Med* 1994; 60: 400–4.
 12. Lohiya NK, Goyal RB, Jayaprakash D, Ansari AS, Srivas-tava S, *et al.* Antifertility effects of *Carica papaya* seeds in male rats. In: Puri CP, Van Look PFA, editors. *Current Concepts in Fertility Regulation and Reproduction*. New Delhi: Wiley Eastern Ltd.; 1994. p177–92.
 13. Lohiya NK, Pathak N, Mishra PK, Manivannan B. Revers-ible contraception with chloroform extract of *Carica pa-paya* Linn. seeds in male rabbits. *Reprod Toxicol* 1999; 13: 59–66.
 14. Lohiya NK, Mishra PK, Pathak N, Manivannan B, Jain SC. Reversible azoospermia by oral administration of the ben-zene chromatographic fraction of the chloroform extract of the seeds of *Carica papaya* in rabbits. *Adv Contracept* 1999; 15: 141–61.
 15. Lohiya NK, Mishra PK, Pathak N, Manivannan B, Bhande SS, *et al.* Efficacy trial on the purified compounds of the seeds of *Carica papaya* for male contraception in albino rat. *Reprod Toxicol* 2005; 20: 135–48.
 16. Lohiya NK, Manivannan B, Garg S. Toxicological investga-tions on the methanol sub-fraction of the seeds of *Carica papaya* as a male contraceptive in albino rats. *Reprod Toxicol* 2006; 22: 461–8.
 17. Lohiya NK, Manivannan B, Goyal S, Ansari AS. Sperm motility inhibitory effect of the benzene chromatographic fraction of the chloroform extract of the seeds of *Carica pa-paya* in langur monkey, *Presbytis entellus entellus*. *Asian J Androl* 2008; 10: 298–306.
 18. INSA. Guidelines for Care and Use of Animals for Scientific Research. New Delhi: Indian National Science Academy; 2000.
 19. World Health Organisation. WHO Laboratory Manual for Examination of the Human Semen and Sperm-Cervical Mu-cus Interaction. New York: Cambridge University Press; 1999.
 20. King EJ, Wooton IDP. *Micro Analysis in Medical Biochem-istry*. London: Livingstone Churchill; 1959. p 42.
 21. Montgomery R. Determination glycogen. *Arch Biochem Biophys* 1957; 67: 378–86.
 22. Bergmeyer HU. *Methods of Enzymatic Analysis*. 2nd edn, New York and London: Academic Press; 1965.
 23. Svennerholm L. Isolation and determination of neuraminic (sialic) acid by resorcinol reaction. In: Glick D, editor. *Methods of Biochemical Analysis*. New Delhi: Interscience; 1960. p 214.
 24. Cooper TG, Yeung CH, Nashan D, Nieschlag E. Epididymal markers in human infertility. *J Androl* 1988; 9: 91–101.
 25. WHO. *Laboratory Manual for Examination of Human Semen and Sperm-Cervical Mucus Interaction*. New York: Cambridge University Press; 1992.
 26. Lynch MJ, Raphael SS, Melor LD, Spare PD, Inwood MJH. *Medical Laboratory Technology and Clinical Pathology*. London: WB Saunders Company; 1969.
 27. Farnsworth NR, Waller DP. Current status of plant products reported to inhibit sperm. *Res Front Fertil Regul* 1982; 2: 1–16.
 28. Lohiya NK, Manivannan B, Mishra PK, Pathak N. Pros-pects of developing a plant based male contraceptive pill. In: Chaudhury SR, Gupta CM, Kamboj VP, editors. *Current Status in Fertility Regulation: Indigenous and Modern Ap-proaches*. Lucknow: Central Drug Research Institute; 2001. p 99–119.
 29. Kamal R, Gupta RS, Lohiya NK. Plants for male fertility regulation. *Phytother Res* 2003; 17: 579–90.
 30. Lohiya NK, Goyal RB, Jayaprakash D, Sharma S, Kumar M, *et al.* Induction of reversible antifertility with a crude ethan-ol extract of *Carica papaya* seeds in albino male rats. *Int J Pharmacog* 1992; 30: 308–20.
 31. Cooper TG, Yeung CH. Approaches to post-testicular con-traception. *Asian J Androl* 1999; 1: 29–36.
 32. Oko R, Hrudka F. Segmental aplasia of the mitochondrial sheath and sequelae induced by gossypol in rat spermatozoa. *Biol Reprod* 1982; 26: 183–95.
 33. Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol* 2008; 59: 2–11.
 34. Tremellen K. Oxidative stress and male infertility - a clini-cal perspective. *Hum Reprod Update* 2008; 14: 243–58.
 35. Huynh PN, Hikim AP, Wang C, Stefanovic K, Lue YH, *et al.* Long-term effects of triptolide on spermatogenesis, epididymal sperm function, and fertility in male rats. *J Androl* 2000; 21: 689–99.