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Dose-dependent Response to an Intratesticular Injection of Calcium Chloride for Induction of Chemosterilization in Adult Albino Rats

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ABSTRACT

This study concerned the minimum and optimum effective doses of calcium chloride needed for induction of chemosterilization in male albino rats, 30 days after a single intratesticular injection of calcium chloride ($CaCl_2.2H_2O$) solution at 2.5, 5, 10 or 20 mg per 100 g body weight per testis. There was a significant diminution in the relative wet weight of the sex organs ($p<0.01$), epididymal sperm count ($p<0.001$), plasma concentration of testosterone ($p<0.01$), testicular activities of Δ^5,3β-hydroxy steroid dehydrogenase (Δ^5,3β-HSD), 17β-hydroxy steroid dehydrogenase (17β-HSD) ($p<0.01$), glutathione *S*-transferase (GST) ($p<0.01$), superoxide dismutase (SOD) ($p<0.01$), and peroxidase ($p<0.01$), significant elevations in testicular content of malondialdehyde (MDA) and conjugated dienes ($p<0.01$), along with derangement of seminiferous tubular architecture and degeneration of the Leydig cells in the testis and elevations in the concentrations in the plasma of LH and FSH ($p<0.01$), commencing at a dose of 5 mg, with the greatest effects at a dose of 20 mg. No significant alterations in these factors occurred at the dose of 2.5 mg in comparison to the control that received only the vehicle. There was no significant alteration in the plasma concentrations of prolactin ($p>0.05$), corticosterone ($p>0.05$) or fasting blood glucose or in the rectal temperature ($p>0.05$) at any of the doses relative to the control group, suggesting that this chemosterilizing procedure did not exert any chronic stress on the experimental animals. From these observations, it may be suggested that 5 mg should be considered as the minimum dose, and 10 mg or 20 mg as the optimum dose, whereas 2.5 mg was ineffective for induction of chemosterilization. There would seem to be little point in using more than 20 mg of calcium chloride for this purpose. Intratesticular injection of calcium chloride at an effective dose may be considered as an alternative to surgical castration.

Keywords: chemical castration, epididymis, enzymes, gonadotrophins, histology, rat, spermatozoa, testis, testosterone

Abbreviations: BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; EDTA, ethylenediaminetetraacetic acid; FSH, follicle-stimulating hormone; GnRH, gonadotrophin-releasing hormone; GSH, reduced glutathione; GST, glutathione *S*-transferase; LH, luteinizing hormone; MDA, malondialdehyde; NAD, nicotinamide-adenine dinucleotide; NAD(P)H, nicotinamide–adenine dinucleotide phosphate, reduced form; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases; PRL, prolactin; rLH-RP-3, rat luteinizing hormone reference preparation 3; rLH-S-5, rat luteinizing hormone standard 5; rFSH-RP-2, rat follicle-stimulating hormone reference preparation 2; rFSH-S-11, rat follicle-stimulating hormone standard 11; rPRL-RP-3, rat prolactin reference preparation 3; rPRL-S-9, rat prolactin standard 9; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TDB, triethanolamine–diethanolamine buffer; TMB, tetramethylbenzidine

INTRODUCTION

For many years, veterinarians have used surgical methods of castration for sterilization of male animals. However, such methods are costly and time-consuming and bear the risk of postoperative infections, so castration by open surgery requires postoperative care and management of the animals. To overcome these problems, researchers have attempted to develop a suitable alternative method. Various agents have been used, in many species, for nonsurgical chemical sterilization of male animals, including the injection of steroid hormones such as androgens (Matsumoto, 1988), progestagens (Swerdloff *et al.*, 1992), anti-androgens (Dhar and Setty, 1990) and androgens plus progestagens (Wu and Aitken, 1989). However, these treatments failed to achieve the desired result satisfactorily. Agonists for gonadotrophin-releasing hormone (GnRH) have also been used to induce sterilization (Trembley and Belanger, 1984; Dube *et al.*, 1987), with rather better results. However, as the effects of these agents are variable and impermanent, repeated treatment is often necessary, so the methods are not cost effective or suitable for mass-scale application. Immunization techniques have also been used to induce antibodies against gonadotrophins and GnRH (Gonzalez *et al.*, 1989; Dowsett *et al.*, 1991). However, these authors results indicated that such immunization techniques varied in effectiveness and in the duration of azospermia. Adverse vaccination reactions were also observed as another disadvantage of this method. Various chemical agents, including cadmium chloride (Parizek, 1960; Kar, 1961), ferric chloride and ferrous sulphate (Kar *et al.*, 1965), danazol (Dixit *et al.*, 1973), BCG (Naz and Talwar, 1981), glycerol (Wiebe *et al.*, 1989) and lactic acid (Fordyce *et al.*, 1989), have been used by intratesticular injection for the same purpose. All these agents caused some pain and pyrexia or even severe inflammation (orchitis) after intratesticular injection. Some agents (e.g. cadmium chloride, glycerol, lactic acid) caused selective destruction of the testicular parenchyma (Parizek, 1960; Immegart and Threlfall, 2000) with irreversible testicular tissue damage (Heath and Arowolo, 1987). In some cases, the interstitial portion regenerated after an initial phase of testicular atrophy (Gunn and Gould, 1970) and this lead to secondary male behaviour, which caused management problems with the animals (Fordyce *et al.*, 1989). All of these treatments failed to achieve the desired results satisfactorily. Owing to the above complications following the use of these chemicals, an effective chemosterilizing agent has yet to be established. Recent attempts have been made to induce sterilization by intratesticular injection of calcium chloride in male adult stray dogs (Samanta, 1998) and bulls (Mitra and Samanta, 2000), but the results with this chemical agent remain to be defined. Hence, the present experiment was designed to explore the efficacy of calcium chloride for inducing chemosterilization in rats and to further delineate its possible uses for induction of male sterilization.

MATERIALS AND METHODS

Animals

Sixty adult albino rats of the Wistar strain, weighing 130 ± 10 g, were housed four per cage at an ambient temperature of $22 \pm 2^{\circ}$C and in a photoperiod controlled room (light:dark: 12 h:12 h), with free access to standard laboratory food and water. The animals were acclimatized to these laboratory conditions for 15 days prior to experimentation. The Principles of Laboratory Animal Care (NIH, 1985) were followed throughout the duration of the experiment. The experimental protocol also met the National Guidelines on the Proper Care and Use of Animals in Laboratory Research (Indian Science Academy, New Delhi, India) and was approved by the Animals Ethics Committee of the Institute. The body weight of each animal was recorded on the starting day of the experiment. The daily food intake of the animals in each cage was measured by weighing the food given and the residue on the following day.

Drug treatment and recording of rectal temperature

To evaluate the effective dose of calcium chloride for induction of chemosterilization, 48 animals were divided into four equal groups and treated with sterile analytical-grade calcium chloride ($CaCl_2.2H_2O$; Merck, Mumbai, India). The twelve animals in each group were given single bilateral intratesticular injections of 2.5, 5, 10 or 20 mg of calcium chloride in 0.1 ml normal saline per 100 g body weight into each testis. The remaining 12 animals received only 0.1 ml of sterile normal saline per 100 g body weight into each testis and were considered as vehicle-treated controls. The intratesticular injections were performed carefully under light ether anaesthetic with single-use insulin syringes. The needle was directed from the caudoventral aspect of each testis, approximately 5 mm from the epididymal tail, towards the dorsocranial aspect of the testis. The solution was carefully deposited along the entire route from the proximal to the distal end by linear infiltration while withdrawing the needle. All necessary care was taken to prevent seepage of the solution from the injection site. Nothing more was done immediately after the injection of the calcium chloride solution. At three-day intervals thereafter, the rectal temperature of each rat was recorded and this was also noted just before the rats were killed. No further injections were performed on any of the animals throughout the rest of the experiment. All the animals were killed 30 days after the injection of calcium chloride or normal saline. Blood was collected from the tip of the tail for the measurement of fasting blood glucose from all the groups of animals, as this is an indicator of the stress response (Cohen *et al.*, 1990). The rats were finally placed under light ether anaesthesia for collection of blood samples from the dorsal aorta using a heparinized syringe (23-gauge needle) and for recording the final body weight. Plasma samples were separated by centrifugation, frozen and stored at -20°C until all the samples had been collected and were then used for the determination of the LH, FSH, testosterone, corticosterone and prolactin concentrations. The

rats were killed by decapitation and the relevant organs were dissected out. The testis, epididymis, prostate and seminal vesicle were collected and their wet weights were recorded. The right testis from each animal was used for the histopathological study, while the other testis was used for biochemical assays of the key androgenic enzyme activities and antioxidant enzyme activities and for measurement of products of free radicals, malondialdehyde (MDA) and conjugated dienes.

Assay of the concentrations of LH and FSH in the plasma

The LH and FSH contents in the plasma were measured by double antibody radio-immunoassay (RIA) (Chowdhury *et al.*, 1983) with reagents supplied by the Rat Pituitary Distribution Programme and NIDDK (Bethesda, MD, USA). Highly purified rat LH (rLH-I-4) and rat FSH (rFSH-I-8) were iodinated with 1 mCi ^{125}I (Bhaba Atomic Research Centre, Mumbai, India) and freshly prepared chloramine T (Sigma, St Louis, MO, USA) (Greenwood *et al.*, 1963). NIDDK-rLH-RP-3 was used as a standard and NIDDK anti-rLH-S-5 was used for the LH assay. The limit of detection of rLH was 0.05 ng at 80%. For the FSH assay, NIDDK-rFSH-RP-2 was used as a standard and NIDDK anti-rFSH-S-11 was used for the assay. The limit of detection was 0.04 ng at 98%. All the samples were assayed on the same day to avoid inter-assay variation. Intra-assay variation was 3.5%.

Assay of the concentration of testosterone in the plasma

The plasma concentration of testosterone was measured by an immunoenzymatic method using an ELISA reader (Merck, Tokyo, Japan) according to the standard protocol given by National Institute of Health and Family Welfare (NIHFW, 2001). Horseradish peroxidase-labelled antigen (IBL, Hamburg, Germany) was used to compete with unlabelled antigen for binding with a limited number of antibody sites on the micro plates (solid phase). After incubation for 1 h, separation of bound and free enzyme-labelled antigen was performed by washing. The substrate of the enzyme, H_2O_2 and the chromogen (TMB, 100 µl, IBL) was added and the enzymic reaction was stopped after 15 min by the addition of 100 µl of a stop solution supplied by IBL. The testosterone concentration in the sample was calculated on the basis of five standards supplied by IBL. The absorbance of the standards and samples was monitored against the blank at 450 nm. The cross-reaction of the testosterone antibody with dehydro-testosterone was 10% and the intra-run precision had a coefficient of variation of 6.2%. All the samples were run at one time, so there was no inter-assay precision coefficient.

Assay of the concentration of prolactin in the plasma

The concentrations of prolactin (PRL) in the plasma were measured by radio-immunoassay according to the procedure of Jacobs (1979), using reagents supplied by

the Rat Pituitary Distribution Programme and NIDDK. Carrier-free ^{125}I for hormone iodination was obtained from Bhaba Atomic Research Centre. Pure rat prolactin (rPRL-I-6) was iodinated with 1 mCi ^{125}I using chloramine-T (Sigma) (Greenwood *et al.*, 1963). NIDDK-rPRL-RP-3 was used as a standard and NIDDK anti-rPRL-S-9 was used for the assay. Goat anti-rabbit γ-globulin was used as the second antibody (Indo-Medicine, Friendswood, TX, USA). The intra-assay variation was 6% and all the samples were assayed on the same day to avoid inter-assay variation.

Estimation of the concentrations in the plasma of corticosterone and fasting blood glucose

The concentration of corticosterone in the plasma was measured spectroflurimetrically (F-3010, Hitachi, Tokyo, Japan) according to the method of Glic and colleagues (1964), as modified by Silber (1966). The fluorescence was measured at 463 nm (excitation) and 518 nm (emission) by setting the instrument at a spectroflurimetric reading of 80 with a standard solution of 1.6 μg/ml corticosterone (Sigma). A minimum of 1.6 μg of corticosterone/100 ml serum can be measured by this method. The concentration of corticosterone was expressed as μg/100 ml of plasma. Fasting blood glucose concentration was measured using a single touch glucometer (Blood Life Scan, Johnson & Johnson, Milpitas, CA, USA) and the concentration was expressed as mg/100 ml.

Assay of key androgenic enzyme activities in the testes

The testicular Δ^5,3β-HSD activity was measured biochemically (Talalay, 1962). The testicular tissue of each animal was homogenized in 20% spectroscopic-grade glycerol (BDH, Mumbai, India) containing 5 mmol/L potassium phosphate (Loba, Mumbai, India) and 1 mmol/L EDTA (Organon, Calcutta, India), at a tissue concentration of 100 mg/ml homogenizing mixture, and centrifuged at 10 000*g* for 30 min at 4°C. The enzymatic activity was measured using the tissue supernatant in the presence of the necessary reagents in a spectrophotometer (U-2001, Hitachi, Japan) cuvette at 340 nm against a blank. One unit of enzyme activity is the amount causing a change in absorbance of 0.001/min at 340 nm.

The activity of testicular 17β-HSD was measured biochemically (Jarabak *et al.*, 1962) using the same supernatant as was prepared for the assay of Δ^5,3β-HSD (above). The supernatant (1 ml) was used along with the necessary reagents for the measurement of the enzyme activity in a spectrophotometer cuvette at 340 nm against a blank. One unit of enzyme activity is equivalent to a change in absorbance of 0.001/min at 340 nm.

Assay of peroxidase (PX) activity in the testes

The peroxidase activity in the testicles was determined by the modified procedure described by Sadasivam and Manickam (1996). The testicular tissue was homogenized in sodium phosphate-buffered saline (0.1 mol/L, pH 7.4) to give a tissue concentration of 100 mg/ml. The homogenate was centrifuged at 1000*g* for 15 min at 5°C. In a spectrophotometer cuvette, 0.1 ml of the supernatant was mixed with 3 ml of phosphate buffer (pH 7.4) at 25°C, 0.05 ml of 20 mmol/L guaiacol (Glaxo, Mumbai, India) solution and 0.3 ml of 12.3 mmol/L H_2O_2 solution (0.042%). The cuvette was placed in a spectrophotometer (Hitachi) and left until the absorbance had increased by 0.05. The time in min (Δ^t) required for the absorbance to increase by 0.1 was then noted. Estimation of the protein in the samples was made by a standard method (Lowry *et al.*, 1951).

Assay of superoxide dismutase (SOD) and glutathione S-*transferase (GST) activities in the testes*

Testicular tissue was homogenized in chilled 100 mmol/L Tris HCl buffer containing 0.16 mol/L KCl (pH 7.4) to give a tissue concentration of 10% (w/v) and centrifuged at 10 000*g* for 20 min at 4°C. The SOD activity in the supernatant was measured according to a standard protocol (Paoletti and Macoli, 1990). The reaction mixture was prepared by mixing 0.8 ml of TDB (Merck), 40 ml of 7.5 mmol/L NAD(P)H (Sigma), 25 ml of (100 mmol/L-50 mmol/L) EDTA-$MnCl_2$ and 0.1 ml of the supernatant in Tris HCl buffer (pH 7.4) containing 0.16 mol/L KCl. The activity of SOD in this mixture was monitored using a UV spectrophotometer (Hitachi) from the rate of oxidation of NAD(P)H. Glutathione *S*-transferase activity was also measured spectrophotometrically (Habig *et al.*, 1974) using CDNB (1-chloro-2,4-dinitrobenzene, Sigma) as substrate. The assay mixture (3 ml) contained 0.1 ml of 1 mmol/L CDNB in ethanol, 0.1 ml of 1 mol/L GSH (SRL, Mumbai, India), 2.7 ml of 100 mmol/L potassium phosphate buffer (pH 6.5) and 0.1 ml of the supernatant of the tissue homogenate. The formation of the adduct of CDNB, *S*-2,4-dinitrophenylglutathione, was monitored by measuring the net increase in absorbance at 340 nm against the blank. The enzyme activity was calculated using the extinction coefficient 9.6 mol^{-1} cm^{-1} and expressed as nmol of product formed per min per mg of protein.

Estimation of lipid peroxidation from the concentrations of malondialdehyde (MDA) and conjugated dienes

The testicular tissue was homogenized (10% w/v) in ice-cold phosphate buffer (0.1 mol/L, pH 7.4) and the homogenate was centrifuged at 15 000*g* in 4°C for 3 min. The supernatant was used for the estimation of MDA and conjugated dienes. MDA , also known as TBARS, the product formed being due to the peroxidation of lipids, was determined by the reaction of thiobarbituric acid (Merck, Darmstadt, Germany) with

malondialdehyde by the method of Ohkawa and colleagues (1979). The amount of MDA formed was measured by taking the absorbance at 530 nm (extinction coefficient, $\varepsilon = 1.56 \times 10^5$ mol^{-1} cm^{-1}) using a UV spectrophotometer (Hitachi). Conjugated dienes were determined by a standard method (Slater, 1984). The lipids were extracted with chloroform–methanol (2:1), followed by centrifugation at 1000*g* for 5 min. The chloroform layer was evaporated to dryness under a stream of nitrogen. The lipid residue was dissolved in 1.5 ml of cyclohexane and the absorbance at 233 nm measured the amount of hydroperoxide formed.

Histopathological studies on the testes

The right testis from each animal was fixed in Bouin's fixative and embedded in paraffin wax. A section 5 μm thick was cut from the middle portion of each testis, stained with hematoxylin–eosin and examined under light microscopy at 400× magnification. The structures of the seminiferous tubules and interstitial spaces in the testis were examined.

Epididymal sperm count

Spermatozoa were collected from an equal length of the caudae of the excised epididymis of each rat by flushing it with the same volume (10 ml) of a suspension medium containing 140 mmol/L NaCl, 0.3 mmol/L KCl, 0.8 mmol/L Na_2HPO_4, 0.2 mmol/L KH_2PO_4 and 1.5 mmol/L D-glucose (pH adjusted to 7.3 by adding 0.1 mol/L NaOH) (Merck, Mumbai, India). The collected sample was centrifuged at 100*g* for 2 min and the precipitate was resuspended in 10 ml of fresh medium. The numbers of spermatozoa in a 100 μl suspension were counted in four chambers of the leukocyte counting chamber on a haemocytometer slide (Alvarez and Story, 1984) under a phase-contrast microscope (Axiolab, Carlzeiss, Oberkochem, Germany). The sperm count was expressed as the number of sperm per ml of suspension.

Statistical analysis

One-way ANOVA followed by a multiple two-tailed *t*-test with Bonferroni modification was used for statistical analysis of the data (Das, 1998). Differences were considered significant when $p < 0.05$.

RESULTS

All the animals tolerated the intratesticular injections of calcium chloride and did not appear to suffer from any agitation, fever or marked inflammatory swelling of the testis. There was no apparent discomfort exhibited by any of the rats, either on

recovery from ether anaesthesia after the injection or at any point during the course of the experiment. There was no apparent change in food consumption among any of the groups of animals throughout the experimental period. All the rats injected with calcium chloride survived the study in good, healthy condition. There was no significant alteration in body weight in any of the treated groups in comparison to the controls. The rectal temperature of the rats treated with calcium chloride at doses of 10 mg or 20 mg was elevated ($p < 0.05$) by the third day after injection relative to the controls but, from the sixth day after injection, there was no significant difference in rectal temperature compared to the controls. The rectal temperature in the rats given either the 2.5 mg or 5 mg dose did not show any significant variation relative to controls throughout the experimental schedule (Figure 1).

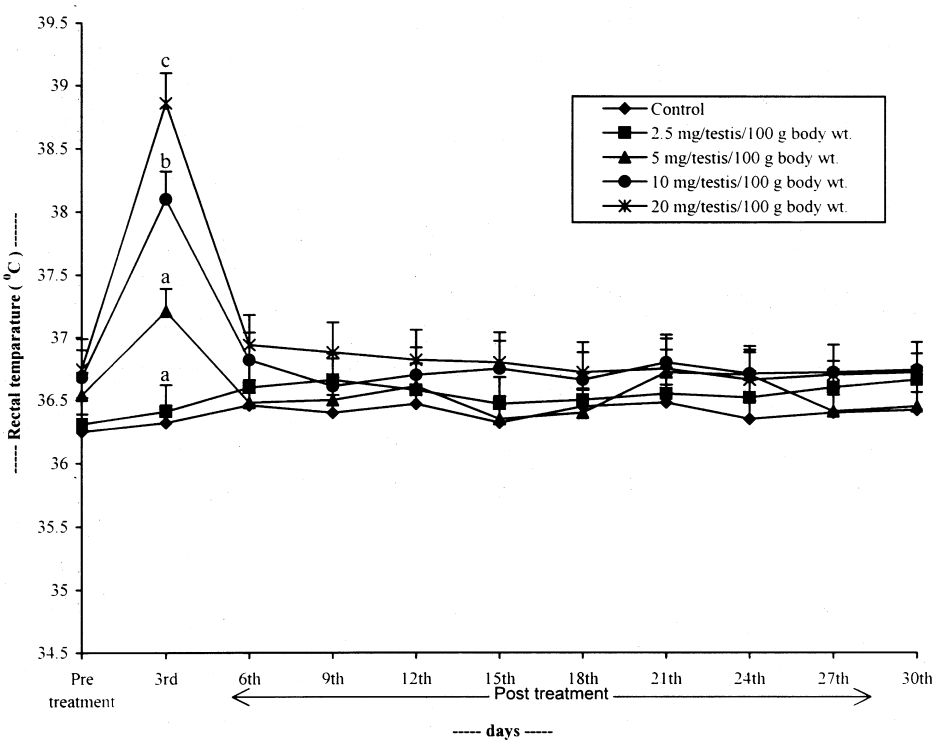

Figure 1. Graphical representation of the effect of an intratesticular injection of calcium chloride on rectal temperature (°C) in albino rats. Data are mean ± SE; ANOVA followed by multiple two-tailed *t*-test ($^{a}p > 0.05$, $^{b,c}p < 0.05$, compared to the respective control)

Changes in somatic indices for the sex organs

Intratesticular injection of calcium chloride at doses of 5 mg, 10 mg or 20 mg led to a significant graded diminution ($p < 0.01$) in the testicular-somatic, epididymal-somatic, prostato-somatic and seminal vesiculo-somatic indices relative to the controls. In the rats that received 2.5 mg calcium chloride, none of these parameters differed from the controls (Table I).

Effect on plasma LH and FSH concentrations

Intratesticular injections of 5 mg, 10 mg or 20 mg of calcium chloride led to a significant graded elevation ($p < 0.01$) in the concentrations in the plasma of LH and FSH in comparison to the animals treated with the vehicle. The level of elevation in these hormone concentrations was greatest at the dose of 20 mg of calcium chloride. The plasma concentration of LH and FSH in rats treated with 2.5 mg of calcium chloride did not show any significant change in comparison to the controls (Figure 2).

Effects on plasma testosterone concentration

The concentration of testosterone in the plasma was significantly reduced in a graded manner ($p < 0.01$) at doses of 5 mg, 10 mg or 20 mg of calcium chloride in comparison to the controls treated with the vehicle, as well as between the treated groups. Calcium chloride treatment at the dose of 2.5 mg did not exhibit any remarkable change in the concentration of this hormone in comparison to the controls (Figure 3).

Effects on the concentrations in the plasma of prolactin, corticosterone and fasting blood glucose

No significant ($p > 0.05$) alterations in the plasma concentrations of prolactin, corticosterone or fasting blood glucose were observed in any of the treated groups with respect to the controls treated with the vehicle or between the treated groups (Table II, Figure 3).

Effects on testicular Δ^5,3β-HSD and 17β-HSD activities

A graded inhibitory response ($p < 0.01$) from the calcium chloride treatment was noted on the testicular Δ^5,3β-HSD and 17β-HSD activities. There was no significant change in the activities of testicular Δ^5,3β-HSD or 17β-HSD after calcium chloride treatment at the dose of 2.5 mg in comparison to the controls. The level of inhibition in the activities of these enzymes was greatest at the dose of 20 mg of calcium chloride (Figure 4).

TABLE I
Effect of intratesticular injection of calcium chloride on sex organ somatic indices and epididymal sperm count in albino rats

Condition	Testicular-somatic index (mg/100 g bw)	Prostato-somatic index (mg/100 g bw)	Seminal vesiculo-somatic index (mg/100 g bw)	Epididymal-somatic index (mg/100 g bw)	Epididymal sperm count (no./ml)
Control	1380 ± 35.45^{a}	220 ± 15.20^{a}	540 ± 25.80^{a}	380 ± 15.26^{a}	12410 ± 460.40^{a}
2.5 mg/testis/100 g body wt	1294 ± 30.50^{a}	210 ± 12.50^{a}	528 ± 22.10^{a}	365 ± 13.26^{a}	11600 ± 410.20^{a}
5 mg/testis/100 g body wt	710 ± 24.16^{b}	150 ± 8.32^{b}	310 ± 20.50^{b}	242 ± 10.55^{b}	2120 ± 120.50^{d}
10 mg/testis/100 g body wt	402 ± 18.85^{c}	104 ± 6.15^{c}	220 ± 16.55^{c}	195 ± 8.56^{c}	740 ± 80.30^{d}
20 mg/testis/100 g body wt	116 ± 10.25^{d}	48 ± 4.18^{d}	42 ± 8.10^{d}	78 ± 8.10^{d}	310 ± 45.20^{d}

Data are mean $\pm$ SE (n = 12)

ANOVA followed by multiple two-tailed t-test. The different superscripts (a, b, c, d) in each column indicate the probability that the figure differs significantly $^{a}p > 0.05$; $^{b,c}p < 0.01$; $^{d}p < 0.001$ compared to the respective control

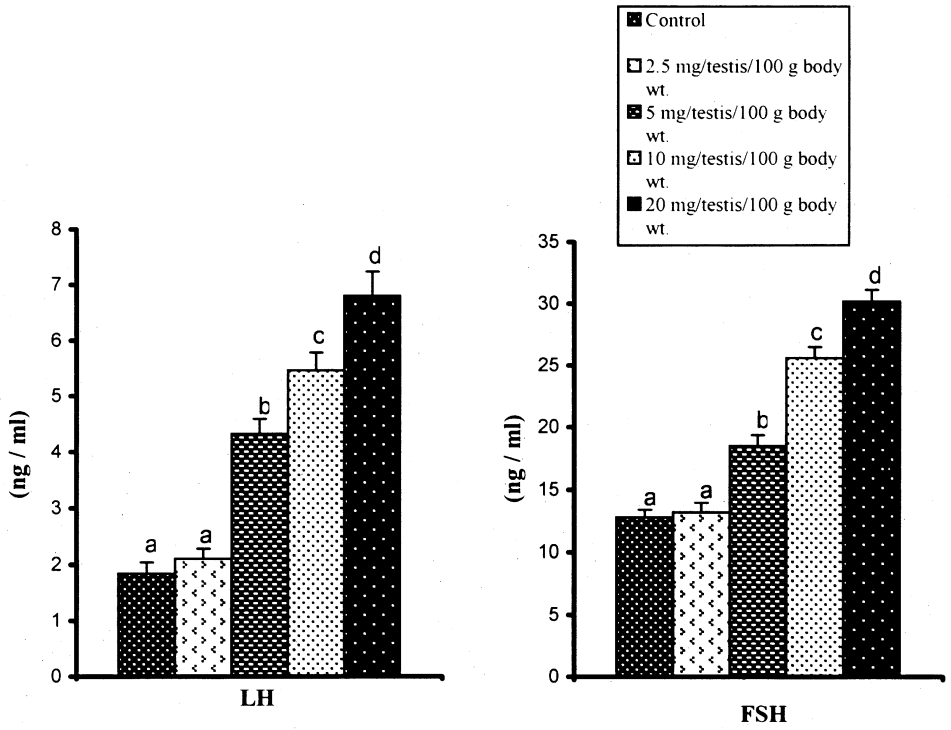

Figure 2. Graphical representation of the effect of an intratesticular injection of calcium chloride on the plasma concentration of LH and FSH in albino rats. Data are mean ± SE, n = 12; ANOVA followed by a multiple two-tailed t-test. Figures with different superscripts (a, b, c, d) on each bar indicate the probability that they differ significantly ($^{b,c}p < 0.05$, $^{d}p < 0.001$, from the respective control)

Effects on testicular peroxidase, superoxide dismutase (SOD) and glutathione S-transferase (GST) activities

There was no significant change in the activities of testicular peroxidase, SOD or GST after calcium chloride treatment at the dose of 2.5 mg in comparison to the controls treated with the vehicle. In contrast, the activities of these antioxidant enzymes were significantly diminished ($p < 0.01$) following treatment with 5 mg, 10 mg or, even more markedly, 20 mg of calcium chloride (Table II, Figure 5).

TABLE II
Effect of intratesticular injection of calcium chloride on plasma concentration of prolactin, fasting blood glucose concentration, testicular peroxidase activities and MDA, and conjugated dienes content of testis in albino rats

Condition	Plasma prolactin (μg/L)	Fasting blood glucose (mg/100 ml)	Testicular peroxidase (units/mg protein)	MDA (nmol/mg tissue)	Conjugated dienes (nmol/mg tissue)
Control	52.60 ± 0.44^{a}	82.44 ± 1.12^{a}	2.40 ± 0.16^{a}	20.10 ± 0.34^{a}	142.96 ± 3.10^{a}
2.5 mg/testis/100 g body wt	52.85 ± 0.40^{a}	82.16 ± 0.82^{a}	2.24 ± 0.12^{a}	20.78 ± 0.40^{a}	144.10 ± 4.35^{a}
5 mg/testis/100 g body wt	53.41 ± 0.68^{a}	83.20 ± 1.04^{a}	1.18 ± 0.08^{b}	28.45 ± 0.44^{b}	180.25 ± 4.70^{b}
10 mg/testis/100 g body wt	53.72 ± 0.62^{a}	83.14 ± 1.10^{a}	0.75 ± 0.04^{c}	35.28 ± 0.32^{c}	265.10 ± 6.64^{c}
20 mg/testis/100 g body wt	53.86 ± 0.81^{a}	82.65 ± 0.95^{a}	0.46 ± 0.03^{d}	44.84 ± 0.46^{d}	292.34 ± 8.12^{d}

Data are mean ± SE ($n = 12$)

ANOVA followed by multiple two-tailed t-test. The different superscripts (a, b, c, d) in each column indicate the probability that the figure differs significantly $^{a}p > 0.05$; $^{b,c}p < 0.01$; $^{d}p < 0.001$ compared to the respective control

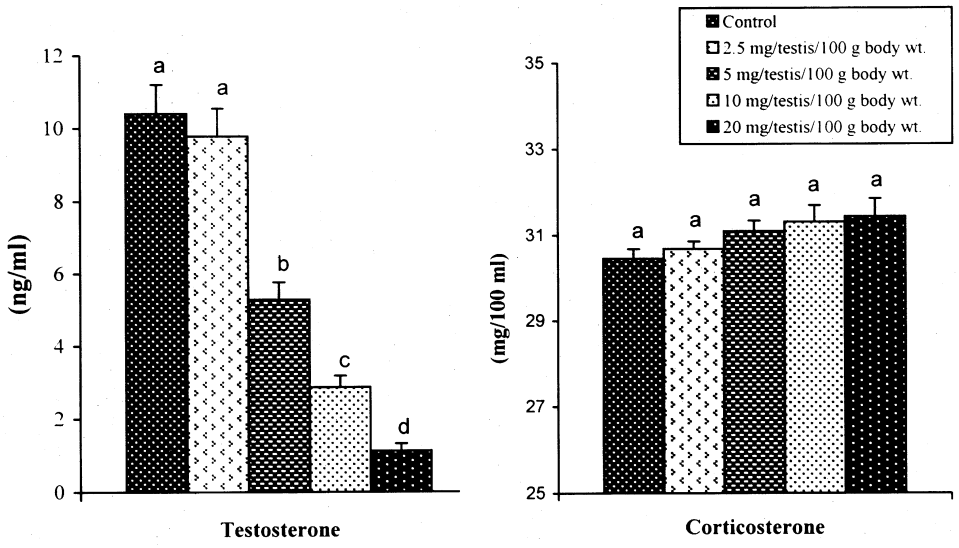

Figure 3. Graphical representation of the effect of an intratesticular injection of calcium chloride on the plasma concentration of testosterone and corticosterone in albino rats. Data are mean ± SE, $n = 12$; ANOVA followed by a multiple two-tailed t-test. Figures with different superscripts (a, b, c, d) on each bar indicate the probability that they differ significantly ($^{a}p > 0.05$, $^{b,c}p < 0.01$, $^{d}p < 0.001$, from the respective control)

Changes in the testicular contents of malondialdehyde (MDA) and conjugated dienes

The testicular contents of malondialdehyde (MDA) and conjugated dienes were significantly elevated ($p < 0.01$) at doses of 5 mg, 10 mg or, even more markedly, 20 mg of calcium chloride in respect to the controls. Calcium chloride treatment at the dose of 2.5 mg did not cause any remarkable change in the concentrations of these free radical products (Table II).

Effect on epididymal sperm count

The epididymal sperm count decreased significantly ($p < 0.001$) at doses of 5 mg, 10 mg or 20 mg of calcium chloride in comparison to the controls. Calcium chloride treatment at the dose of 2.5 mg did not cause any significant change ($p > 0.05$) in the epididymal sperm count compared to the controls (Table I).

Changes in histopathology of testis

Testicular sections from the control group showed normal arrangements of the germ cells in the seminiferous tubules, with distinct peritubular space and interstitial cells of Leydig (Figure 6). Intratesticular injection of 2.5 mg calcium chloride solution caused

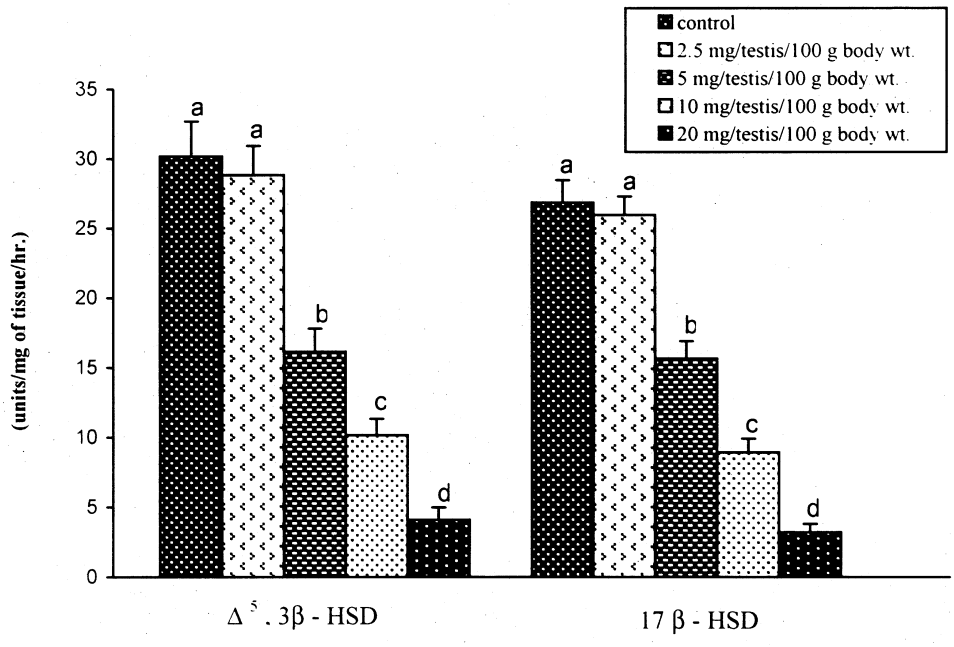

Figure 4. Graphical representation of the effect of an intratesticular injection of calcium chloride on testicular androgenic enzyme activities in albino rats. Data are mean ± SE, n = 12; ANOVA followed by multiple two-tailed t-tests. Figures with different superscripts (a, b, c, d) on each bar indicate the probability that they differ significantly ($^{b,c}p < 0.01$, $^{d}p < 0.001$, from the respective control)

no remarkable alteration in the histological architecture of the seminiferous tubules and interstitial cells of Leydig in comparison to the controls (Figure 7). The dose of 5 mg of calcium chloride was associated with necrosis of seminiferous tubules, which were loaded with degenerative germ cells, along with calcium deposition. Some mature and elongated spermatozoa were present in the interstitital space (Figure 8). Intratesticular injection of 10 mg of calcium chloride in solution resulted in a significant degree of necrosis in the germinal epithelium of the seminiferous tubules and interstitial cells of Leydig, along with the appearance of fibrous tissue in the interstitial spaces. Some of the seminiferous tubules showed scattered foci of calcium deposition and all the tubules were atrophied (Figure 9). At the highest dose (20 mg) for the intratesticular injection of calcium chloride solution, there was complete disarrangement of the tubular architecture, without any distinct boundary between the tubular and extratubular compartments, along with infiltration of a large number of leukocytes throughout the testicular tissue. Degeneration of the interstitial cells of Leydig, along with the appearance of fibrous tissue, was visible throughout the testicular tissue (Figure 10).

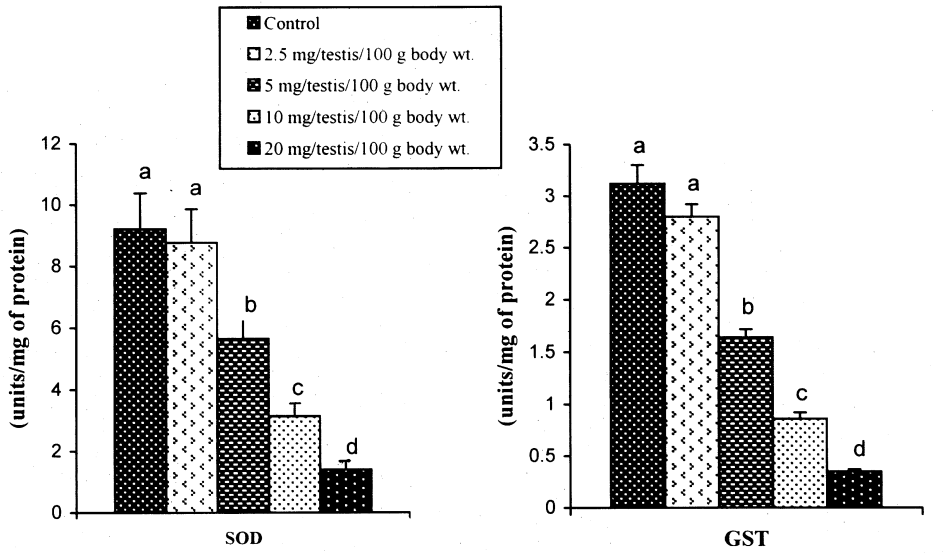

Figure 5. Graphical representation of the effect of an intratesticular injection of calcium chloride on the testicular activities of SOD and GST in albino rats. Data are mean ± SE, n = 12; ANOVA followed by multiple two-tailed t-test. Figures with different superscripts (a, b, c, d) on each bar indicate the probability that they differ significantly ($^{b,c}p < 0.01$, $^{d}p < 0.001$, from the respective control)

DISCUSSION

When the dose-dependent effects of calcium chloride for induction of chemosterilization in adult albino rats were studied, the minimum effective dose was considered to be 5 mg, as both the biochemical and histological paramters related to chemosterilization showed significant alterations in respect to the controls at that dose. The optimum effective doses were considered to be 10 mg and 20 mg, as the most desired level of results to chemosterilization were noted in all the relevant biochemical and histological parameters in male animals at these doses. Drastic changes were noted in the relevant parameters for induction of chemosterilization from 20 mg calcium chloride and there would seem to be little point in using a greater amount for this purpose. No significant response was noted in the relevant indicators for evaluating chemosterilization at the dose of 2.5 mg, so this was considered to be ineffective.

The graded necrosis in the seminiferous tubules that was noted is in agreement with the previous studies on this chemical agent (Samanta, 1998; Mitra and Samanta, 2000). This necrotizing property of calcium chloride was also reported by Koger (1976), Albers and Theilen (1985) and McGinnis and colleagues (1999).

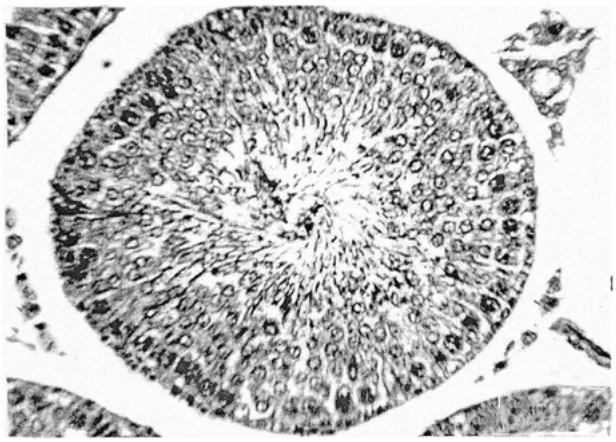

Figure 6. Photomicrograph of a testicular cross-section showing the normal arrangement of germ cells in a seminiferous tubule with distinct interstitial cells of Leydig

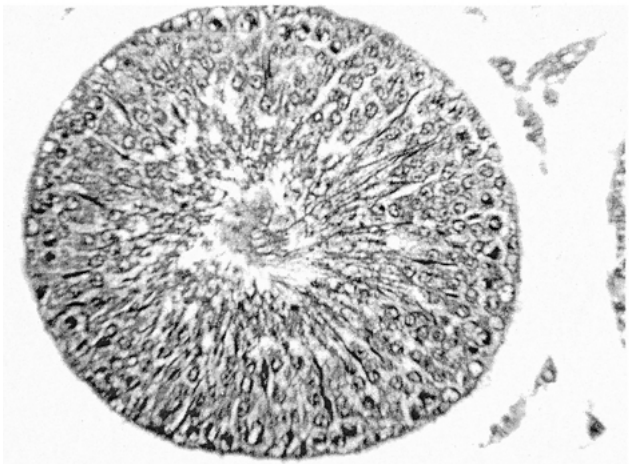

Figure 7. Photomicrograph of a testicular cross-section showing the normal arrangement of germ cells in a seminiferous tubule with distinct interstitial cells of Leydig despite the administration of 2.5 mg of calcium chloride/100 g body weight per testis

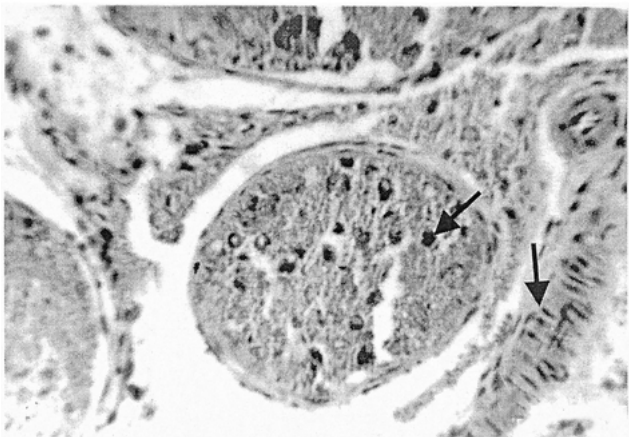

Figure 8. Photomicrograph of a testicular cross-section showing the degenerative germ cells with calcium deposition in a seminiferous tubule and presence of elongated matured spermatozoa outside the seminiferous tubule following the administration of 5 mg of calcium chloride/100 g body weight per testis. (Arrows indicate calcium deposition in seminiferous tubule, and, at the interstitial space, the presence of matured elongated spermatozoa)

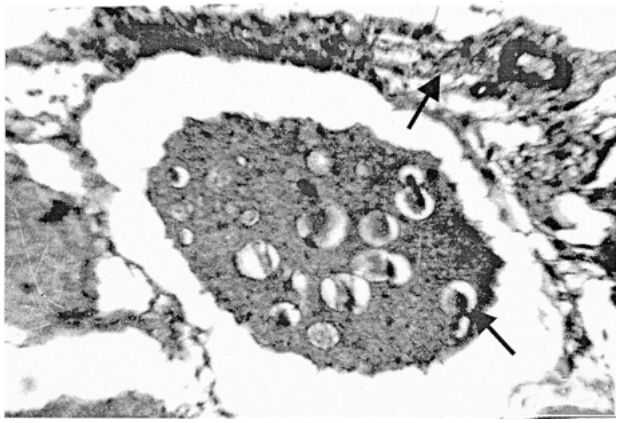

Figure 9. Photomicrograph of a testicular cross-section showing the necrosis in a seminiferous tubule and interstitital cells of Leydig and calcium deposition into the tubules along with fibrous tissue in the interstitial space following treatment with 10 mg of calcium chloride/100 g body weight per testis. (Arrows indicate calcium deposition in seminiferous tubule and the appearance of fibrous tissue in the interstitial space)

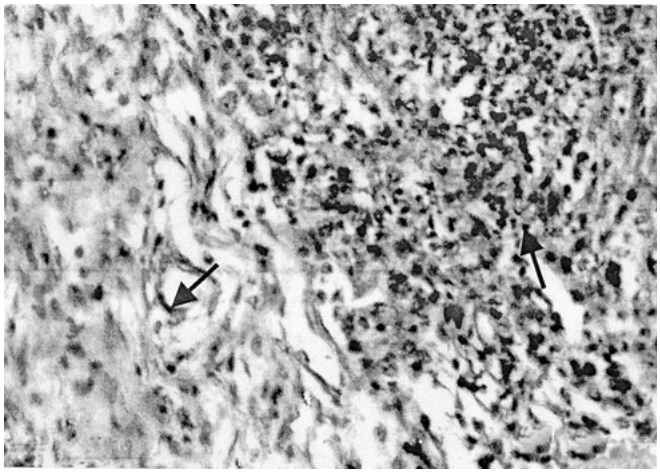

Figure 10. Photomicrograph of a testicular cross-section showing complete derangement of the seminiferous tubular architecture and the presence of leukocytes throughout the tubular and extra-tubular zone along with the presence of fibrous tissue following administration of 20 mg of calcium chloride/100 g body weight per testis. (Arrows indicate the presence of leukocytic infiltration and fibrous tissue both in the tubular and in the peritubular zones)

The degeneration of the germ cells, along with the derangement of the seminiferous tubules and the migration of mature spermatozoa into the interstitial space following effective doses of calcium chloride, may be associated with the reduced plasma concentration of testosterone, as the structural morphology and normal physiology of the seminiferous tubules and the arrangement and confinement of spermatozoa in the tubules are regulated by testosterone (Bertlett *et al.*, 1986; Sharpe *et al.*, 1988; Kerr *et al.*, 1993).

The graded and significant diminution in the plasma concentration of testosterone in response to graded doses of calcium chloride was supported by the graded diminution in the activities of testicular Δ^5,3β-HSD and 17β-HSD, as these are the key enzymes for testicular androgenesis (Ohba *et al.*, 1982; Ishii-Ohba *et al.*, 1986; Ghosh *et al.*, 1990).

In addition to this direct effect of calcium chloride on androgenesis, at all the effective doses, the plasma concentrations of LH and FSH were significantly increased in comparison to the controls. This may have been due to the withdrawal of the negative feedback effect of testosterone on the hypothalamo-pituitary unit (Plant, 1982; Tillbrook and Clark, 2001). This high concentration of LH may be another cause of the degeneration of germ cells (Rivier *et al.*, 1979; Kerr and Sharpe, 1986). The apparent induced chemosterilization by calcium chloride was also supported by this elevation in the concentrations of LH and FSH in the plasma, as an increased secretion of gonadotrophins (LH and FSH) is noted following castration in many species of animals (Damassa *et al.*, 1976; Tillbrook and Clark, 1995).

The diminution in the plasma concentration of testosterone after treatment with an effective dose of calcium chloride was supported by the diminution in the prostato-somatic, seminal vesiculo-somatic and epididymal-somatic indices, as the growth of these accessory sex organs is controlled by testosterone (Barkley and Goldmass, 1977; Ghosh *et al.*, 1983; Coffey, 1988). The significant diminution in epididymal sperm counts also reflects the low plasma concentration of testosterone, as the production of sperm in the testis, as well as the maturation of sperm in the epididymis, are controlled by testosterone (Sharpe *et al.*, 1988, 1992; Steinberger, 1971, 1975).

The degeneration of the interstitial cells of Leydig caused by the graded doses of calcium chloride may have led to the reduction in the plasma testosterone concentration (Johnson and Thomson, 1987). The low concentration of plasma testosterone in the rats treated with calcium chloride is further evidenced by the qualitative study on the testicular sections, in which the significant fibrosis seen in the interstitial spaces of seminiferous tubules may be due to a low concentration of testosterone (Jegou and Sharpe, 1993). The efficacy of calcium chloride in inducing chemosterilization is supported by the necrosis of the seminiferous tubules and interstitital cells of Leydig and the significant fibrosis at the interstitial space, which are inconsistent with previous studies using other chemical agents for chemosterilization in male animals (Parizek, 1960; Johnson, 1977; Immegart and Threlfall, 2000).

The infiltration of leukocytes into the seminiferous tubules and interstitial spaces after treatment with the highest dose of calcium chloride may be due to the testicular tissue damage or degeneration, as this may release large amounts of chemotactic factors responsible for the ingress of leukocytes (Heath and Arowolo, 1987). This testicular damage was supported by the graded reduction in the testicular somatic index in the groups treated with calcium chloride, as this parameter is an accepted measure of damage to testicular tissue (Foote *et al.*, 1986).

Another way in which chemosterilization may be induced by calcium chloride is through the generation of large amounts of free radicals or their products in the testicular tissue, as free radical production in the testis results in a low level of testosterone (Peltola *et al.*, 1996; Chainy *et al.*, 1997). Calcium chloride is an effective chemical agent for inducing the generation of free radicals in tissues (Watanabe *et al.*, 1990; Kakkar *et al.*, 1992). Free radicals can cause the destruction of all cellular structures and of lipids by lipid peroxidation (Halliwell and Gutteridge, 1985). The extent of lipid peroxidation and consequently of tissue damage can be assessed by the measurement of MDA and conjugated dienes (Gutteridge and Halliwell, 1990; Gutteridge, 1995). The fact that intratesticular injection of calcium chloride is associated with free radical production and lipid peroxidation in the testis is reflected in the high testicular content of MDA and conjugated dienes. An alternative way in which free radicals may be generated in the testis is through the infiltration of leukocytes, as testicular free radical production is closely associated with infiltration of leukocytes (Aitken and West, 1990; Sikka, 2001).

Elevation of the formation of free radicals in the testis treated with calcium chloride is also supported by the diminution in the testicular peroxidase, SOD and GST activities, as these are the important scavenging enzymes against free radicals in male gonads (Peltola *et al.*, 1992; Samanta *et al.*, 1999; Rao and Shaha, 2000). Moreover, the

low activities of testicular SOD and GST in the treated rats may be due to the low level of testosterone (Hatayama *et al.*, 1986; Chainy *et al.*, 1997). Therefore, it may be deduced that the testicular degeneration in this experiment was in part due to the generation of free radicals in the testis, as free radicals are inhibitors for spermatogenesis (Alvarez and Story, 1984; Aitken, 1994) and testicular androgenesis (Georgiou *et al.*, 1987). The low level of GST activity in the testis is another cause of the low sperm count in the epididymis and of the degenerative necrosis of seminiferous tubules, as GST is an important regulator of germ cell proliferation (Aravinda *et al.*, 1996).

The stress indicators, including the concentrations of plasma corticosterone (Munck *et al.*, 1984), prolactin (Seltzer *et al.*, 1986) and fasting blood glucose, and the rectal temperature (Cohen *et al.*, 1990) were measured to ascertain whether the calcium chloride treatment was associated with any chronic stress in the experimental animals. Almost any type of stress will cause an increase in the secretion of corticosterone in rats (Guyton and Hall, 1996; Das, 2000) and an increase in the rectal temperature (Cohen *et al.*, 1990). It is well established that corticosterone is a good indicator of stress (Willett and Erb, 1972; Munck *et al.*, 1984; Buckingham *et al.*, 1996). Stress also induces secretion of prolactin, mediated by hypothalamic histaminergic neurons (Knigge *et al.*, 1988). As there were no significant alterations in the plasma concentrations of corticosterone, prolactin or fasting blood glucose in the treated animals with respect to the controls, this method of chemosterilization does not appear to be associated with a chronic stress response. However, as the rectal temperature was increased significantly on the third day after the injection with effective doses of calcium chloride, it may be assumed that these doses did induce some acute stress in the treated animals, though this diminished soon thereafter.

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