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Animal Reproduction Science 86 (2005) 89–108

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## Evaluation of single intratesticular injection of calcium chloride for nonsurgical sterilization of male Black Bengal goats (*Capra hircus*): a dose-dependent study

Kuladip Jana<sup>a,b</sup>, P.K. Samanta<sup>a,\*</sup>, D. Ghosh<sup>b</sup>

<sup>a</sup> Department of Veterinary Surgery and Radiology, West Bengal University of Animal and Fishery Sciences, 64/1/14 Belgachia Road (Milk Colony), 37 & 68 K.B. Sarani, Calcutta 700037, West Bengal, India

<sup>b</sup> Reproductive Endocrinology and Family Welfare Research Unit, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore 721102, West Bengal, India

Received 5 September 2002; received in revised form 14 May 2004; accepted 25 May 2004

### Abstract

This study describes the induction of chemosterilization in three groups each of six adult male Black Bengal goats at 30 days after a single bilateral intratesticular injection of a calcium chloride ( $\text{CaCl}_2, 2\text{H}_2\text{O}$ ) solution at the doses of 10, 20 or 40 mg/kg body weight/testis, always in a 2 ml volume of normal saline. Another one group of animals received only 2 ml of normal saline per testis as a control. The induction of chemosterilization was measured using relative testicular weight as well as histomorphological parameters including seminiferous tubular architecture and germ cell association in seminiferous tubules along with morphology of the interstitial space. Biochemical markers included activities of testicular  $\Delta^5$ ,  $3\beta$ -hydroxysteroid dehydrogenase ( $\Delta^5$ ,  $3\beta$ -HSD),  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD), catalase, glutathione peroxidase (GPx), glutathione *S*-transferase (GST) and superoxide dismutase (SOD) as well as monitoring the level of testicular thiobarbituric acid reactive substances (TBARS), conjugated dienes and reduced glutathione (GSH) content along with plasma concentrations of testosterone, LH and FSH. Histomorphological measures of testes showed total necrosis of testicular tissue at 30 days after an injection of either 20 or 40 mg  $\text{CaCl}_2$  along with fibrosis in seminiferous tubules and interstitial spaces. Infiltration of leucocytes was observed with the 40 mg dose. Disintegration of germ cell arrangement in seminiferous tubules and washing out of germ

\* Corresponding author. Tel.: +91 33 25568329; fax: +91 98 30164986.

E-mail address: kuladip.jana@yahoo.com (P.K. Samanta).

cells from the tubules were noted with the 10 mg dose. Relative organ weights, plasma concentrations of testosterone, testicular activities of  $\Delta^5$ , 3 $\beta$ -HSD, 17 $\beta$ -HSD, catalase, GPx, GST, and SOD and testicular contents of GSH all were declined. Increases occurred in testicular TBARS, conjugated dienes and plasma concentrations of LH and FSH with each of the treatments by comparison with the control group. Plasma concentrations of cortisol and fasting blood sugar level as well as packed cell volume (PCV) and total plasma protein were recorded to monitor the changes of chronic stress in the experimental animals. Changes in these parameters were not significant. An intratesticular injection of calcium chloride at specified doses could be a suitable method of sterilization in preference to surgical castration of goats.

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*Keywords:* Goat; Calcium chloride; Chemosterilization; Androgenic key enzymes; Antioxidant enzymes; Stress; Testosterone

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## 1. Introduction

Castration of domestic male animals by open surgery can require post-operative care to minimize the risk of hemorrhage and infection although it is cost effective. Alternatives have been tested for non-surgical chemical sterilization including the injection of androgens (Matsumoto, 1988), progestagens (Swerdloff et al., 1992), antiandrogens (Dhar and Setty, 1990), anabolic steroids (Wu, 1988) and androgens plus progestagens (Wu and Aitken, 1989). However, these treatments have not been consistent. Gonadotrophin releasing hormone (GnRH) agonists have also been used (Trembley and Belanger, 1984; Dube et al., 1987) with better results but repeated treatment may be necessary. Immunization techniques have also been used to induce antibodies against gonadotrophins and GnRH (Dowsett et al., 1991; Gonzalez et al., 1989) with varied effectiveness and duration of azospermia. Adverse vaccination reactions have also been observed. 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 also been used. These agents cause 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 reversible 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) leading to secondary male behaviour, which caused management problems (Fordyce et al., 1989). A completely effective chemosterilizing agent has not been identified. Inducing sterilization by intratesticular injection of calcium chloride has been attempted in adult stray dogs (Samanta, 1998) and scrub bulls (Mitra and Samanta, 2000), but the results were not defined. We recently reported that an intratesticular injection of calcium chloride in the rat produced chemosterilization by the induction of free radicals in the testis (Jana et al., 2002). The present experiment was designed to explore the efficacy of calcium chloride for inducing chemosterilization in goats and to further delineate the possible mechanism of action of this agent for induction of male sterilization.

## 2. Materials and methods

### 2.1. Animals

Clinically healthy male Black Bengal goats (*Capra hircus*) ( $n = 24$ ) weighing 8 kg (range 6–10 kg), aged from 1.5 to 2 years with normal libido and serving ability in the breeding season (July–August) were acclimatized initially for 1 month in the animal house. They were dewormed with levamisole (7.5 mg/kg). The animal house had artificial lighting and controlled temperatures (22 °C, ranging from 19 to 25 °C). The goats were fed with a standard ration (2 parts wheat husk + 1 part ground-nut cake + 1 part crushed gram + 1 part crushed maize + and 2 parts green chop) and had continuous access to fresh water. *The Animal Ethics Committee of the Institute* duly approved the protocol of this experiment.

### 2.2. Experimental protocol

The maximum effective dose of calcium chloride for induction of chemosterilization was estimated by dividing the 24 animals randomly into four groups. Every animal in each of the three groups receiving calcium chloride was administered a single bilateral intratesticular injection of sterile analytical grade of calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , Merck, Mumbai, India) solution at 10, 20 or 40 mg/kg body weight in 2 ml of normal saline as well as 0.5 ml of 2% lignocaine hydrochloride (a local anesthetic agent, Zylocaine 2%, Astra IDL, Bangalore, India) for a average volume of 2.5 ml per testis. The animals in the control group each received a single bilateral intratesticular injection of 2 ml sterile normal saline as well as 0.5 ml of 2% lignocaine hydrochloride.

#### 2.2.1. Intratesticular injection of calcium chloride solutions

Each intratesticular injection was performed using a sterile 21 gauge needle directed from the codoventral aspect of each testis approximately 1 cm from the epididymal tail and towards the dorsocranial aspect of that testis so that the solution was deposited over the entire route by linear infiltration while withdrawing the needle from the proximal end to the distal end. Necessary care was taken to avoid the seepage of the solution from the injection site. Nothing more was done following an injection. The animals were kept under routine clinical observations.

#### 2.2.2. Scarification, collection of blood and reproductive organs

Castration was performed using a routine surgical procedure at 30 days after injection (O'Conner, 1938). The testes were collected and wet weights recorded. The right testis from each animal was used for histomorphological studies, while the left one was used for biochemical assays. Blood was collected from a jugular vein of each goat into heparinized tubes before castration in fasted animals within 8.00 a.m. to 8.30 a.m. These samples were analyzed immediately after collection for packed cell volume (PCV) and fasting blood sugar level. They were also centrifuged for 10 min at  $13,000 \times g$  for plasma separation then stored at  $-20^\circ\text{C}$  until assayed for testosterone, LH, FSH, cortisol and total protein concentrations.

### 2.3. Assays for plasma testosterone and cortisol concentrations

The plasma concentration of testosterone and cortisol were measured using an ELISA reader (Merck, Japan) according to the standard protocol given by *National Institute of Health and Family Welfare* (NIHFW, New Delhi, India) (Srivastava, 2002). The ELISA kit for testosterone was supplied by IBL (Hamburg, Germany) and the cortisol kit was supplied by NIHFW. Horseradish peroxidase was used as an enzyme-labeled antigen in both assays that made a competition with unlabelled antigen for binding with a limited number of antibody sites on the micro-plates (solid phase). Assays were performed following standardized instructions. Each testosterone concentration was calculated from a standard curve with five standards supplied by IBL whereas each cortisol concentration was calculated from six standards supplied by NIHFW with the absorbance of the standards and samples monitored against a blank at 450 nm. The stated cross-reaction of the testosterone antibody with dehydrotestosterone was 10% and the intra-assay CV was 6.2%. The stated cross-reaction of the cortisol antibody with corticosterone was 10% and intra-assay CV was 5.5%. All the samples were included in a single assay.

### 2.4. Assays of plasma LH and FSH concentrations

Plasma concentrations of LH and FSH were assayed with a double antibody radioimmuno-assay using rabbit anti-ovine LH (Mori and Kano, 1984) and FSH (Walkden-Brown et al., 1994) antiserum. Highly purified ovine LH and FSH were iodinated with 1 mCi  $^{125}\text{I}$  (BARC, Mumbai, India; Greenwood et al., 1963) using freshly prepared chloramine T (Sigma, St. Louis, MO, USA). NIH-0LH-S20 and NIH-0FSH-S12 (*National Hormone and pituitary programme*, NIH, Baltimore, MA, USA) were used as standard for LH and FSH assay, respectively. Caprine anti-rabbit  $\gamma$ -globulin was used as second antibody. The minimal detectable concentration was 0.38 ng/ml for LH and 0.13 ng/ml for FSH assay. All the samples were assayed on one day. The intra assay co-efficient of variation was 6.8%.

### 2.5. Estimation of total plasma protein concentrations and fasting blood sugar levels

Total plasma protein concentration was measured according to the standard method of Lowry et al. (1951) and the level was expressed as gm/dl. Fasting blood sugar level was measured using a single touch glucometer (Blood Life Scan, Johnson & Johnson, Milpitas, CA, USA) and the concentration was expressed as mg/100 ml.

### 2.6. Assays of testicular key androgenic enzyme activities

Testicular tissue of each animal was used for studying the activities of  $\Delta^5$ ,  $3\beta$ -hydroxysteroid dehydrogenase ( $\Delta^5$ ,  $3\beta$ -HSD) and  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD). Testicular  $\Delta^5$ ,  $3\beta$ -HSD activity was assayed spectrophotometrically according to the procedure of Talalay (1962). The activity of testicular  $17\beta$ -HSD was measured in UV spectrophotometer according to the procedure of Jarabak et al. (1962). One unit of enzyme activity for  $\Delta^5$ ,  $3\beta$ -HSD and  $17\beta$ -HSD was considered to be the amount causing a change in absorbance of 0.001/min at 340 nm.

### 2.7. Assays of testicular catalase and glutathione peroxidase (GPx) activities

Testicular tissue was homogenized in 10% (w/v) chilled 50 mM Tris–HCl (Merck) buffer (pH 7.0) containing 0.16 M KCl and the homogenate was centrifuged at  $10,000 \times g$  for 20 min at 4 °C. The supernatant was used for catalase assay and the assay was performed according to the standard protocol given by [Beers and Sizer \(1952\)](#). The catalase activity of the supernatant was monitored following a decrease in absorbance of H<sub>2</sub>O<sub>2</sub> at 240 nm (extinction coefficient  $\epsilon = 46.6 \text{ M}^{-1} \text{ cm}^{-1}$ ). The activity of testicular glutathione peroxidase (GPx) was determined according to the modified procedure described by [Paglia and Valentine \(1967\)](#). GPx present in the sample catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide; in the presence of glutathione reductase (GSSG-R) and NAD(P)H. The oxidized glutathione (GSSG) is immediately converted to the reduced form (GSH) with a concomitant oxidation of NAD(P)H to NADP<sup>+</sup>. The decrease in absorbance of NAD(P)H was measured at 340 nm. GPx activity was expressed as nM NAD(P)H oxidized/min/mg of protein. Estimation of the protein in the samples was done using a standard method ([Lowry et al., 1951](#)).

### 2.8. Assays of testicular superoxide dismutase (SOD) and glutathione S-transferase (GST) activities

Testicular tissue was homogenized in chilled 100 mM sodium phosphate buffer saline (pH 7.4) to give a tissue concentration 10% (w/v) for the measurement of the activities of superoxide dismutase (SOD) and glutathione S-transferase (GST). The SOD activity was measured spectrophotometrically according to the standard protocol ([Paoletti and Macoli, 1990](#)). One unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of 50% NAD(P)H oxidation and expressed as units/mg of protein. Glutathione S-transferase activity was measured spectrophotometrically ([Habig et al., 1974](#)) using 1-choloro-2,4-dinitrobenzene (CDNB, Sigma) as substrate. The formation of product CDNB, S-2,4-dinitro-phenyl glutathione, was monitored by measuring the net increase in absorbance at 340 nm against blank. The enzyme activity was calculated using the extinction co-efficient  $\epsilon = 9.6 \text{ M}^{-1} \text{ cm}^{-1}$  and expressed as nM of product formed/min/mg of protein. The amount of protein present in the tissue was measured by the method of [Lowry et al. \(1951\)](#).

### 2.9. Estimations of lipid peroxidation from thiobarbituric acid reactive substances (TBARS) and conjugated dienes

The testicular tissue was homogenized (10% (w/v)) in ice-cold phosphate buffer (0.1 M, pH 7.4) and the homogenate was centrifuged at  $15,000 \times g$  in 4 °C for 3 min. The supernatant was used for the estimation of TBARS and conjugated dienes. TBARS was determined by the reaction of thiobarbituric acid (TBA; Merck, Germany) with malondialdehyde (MDA), a product formed due to the peroxidation of lipids, according to the method of [Ohkawa et al. \(1979\)](#). The amount of TBARS formed was measured by taking the absorbance at 530 nm (extinction coefficient,  $\epsilon = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ) 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  $1000 \times g$  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.

### 2.10. *Quantifications of testicular glutathione (GSH) content*

A 10% (w/v) homogenate of the testicular tissue in 5% (w/v) metaphosphoric acid was centrifuged at  $1000 \times g$  for 30 min at room temperature, and the deproteinized supernatant was used to assay of GSH. This was measured by monitoring the absorbance at 412 nm in a UV spectrophotometer (Hitachi), which reflects the rate of reduction of 5,5-dithiobis-2-nitrobenzoate (DTNB) to 2-nitro-5-thiobenzoate following the standard method given by Zakowski and Tappel (1978).

### 2.11. *Histopathological studies on the testes*

The right testis from each animal was fixed in Bouin's fixative and embedded in paraffin wax. A section  $5 \mu\text{m}$  thick was cut from the middle portion of each testis, stained with hematoxylin–eosin and examined under light microscopy at  $100\times$  and  $400\times$  magnifications. The structures of the seminiferous tubules and interstitial spaces in the testis were examined.

### 2.12. *Statistical analysis*

One-way analyses of variance by combined with a multiple two-tail 't'-test with the Bonferroni modification were used for statistical analysis of the data (Das, 1998). Differences were considered significant when  $P < 0.05$ ;  $P < 0.01$ ;  $P < 0.001$ . Accordingly, a statistical software package (SPSS) was used.

## 3. Results

Every animal tolerated the intratesticular injections of calcium chloride. They did not suffer from any agitation fever or marked inflammatory swelling of the testis except for a slight increase of firmness of a testis on palpation. Mild testicular swelling was evident in every goat by 24 h following injection. Swelling was maximum in treated animals up to 48–72 h following injection, then gradually decreased at 3 weeks. At 4 weeks after the 40 mg calcium chloride injection, only a small testicular remnant could be palpated. Food consumption was not affected among the four groups of animals throughout the experimental schedule. Every one of the goats injected with calcium chloride survived in a good healthy condition through out the experimental period.

### 3.1. *Changes in relative testicular weight*

A single intratesticular injection of calcium chloride at doses of 10, 20 or 40 mg led to a graded diminution ( $P < 0.01$ ) in relative testicular weight compared to the animals in the

Table 1

Effect of single bilateral intratesticular injection of calcium chloride on relative testicular weight, packed cell volume (PCV), total plasma protein concentration, fasting blood sugar level, reduced glutathione (GSH) content of testis in male Black Bengal goats (*Capra hircus*)

Condition	Relative testicular weight (mg/100 g body wt.)	Packed cell volume (PCV) (%)	Total plasma protein (gm/dl)	Fasting blood sugar level (mg/dl)	Reduced glutathione (GSH) (nmol/mg of protein)
Control	1250 ± 40 <sup>a</sup>	31.8 ± 0.4 <sup>a</sup>	7.13 ± 0.07 <sup>a</sup>	73.5 ± 1.1 <sup>a</sup>	80.2 ± 4.3 <sup>a</sup>
10 mg/kg body weight/testis	890 ± 30 <sup>b</sup>	31.5 ± 0.5 <sup>a</sup>	7.21 ± 0.06 <sup>a</sup>	73.8 ± 0.8 <sup>a</sup>	56.0 ± 3.2 <sup>b</sup>
20 mg/kg body weight/testis	280 ± 18 <sup>c</sup>	31.7 ± 0.3 <sup>a</sup>	7.42 ± 0.08 <sup>a</sup>	74.0 ± 1.5 <sup>a</sup>	24.1 ± 2.2 <sup>c</sup>
40 mg/kg body weight/testis	110 ± 8 <sup>d</sup>	31.3 ± 0.3 <sup>a</sup>	7.38 ± 0.02 <sup>a</sup>	74.5 ± 1.6 <sup>a</sup>	15.1 ± 1.8 <sup>d</sup>

Data presented as mean ± S.E.,  $n = 6$ . Different superscripts (a, b, c, d) on each vertical column differ from each other significantly (<sup>b,c</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$ , as compared with their respective control).

control group. This parameter exhibited a greater level of diminution at the maximum dose in respect to either the medium or low dose treatments (Table 1).

### 3.2. Effect on plasma concentrations of testosterone and cortisol

Plasma concentrations of testosterone were decreased in a graded manner ( $P < 0.01$ ) at doses from 10 to 40 mg calcium chloride treatments in comparison to treated controls. A low concentration of testosterone was noted with the highest dose of calcium chloride. The treatment did not cause any remarkable change in plasma concentrations of cortisol at any of the doses in comparison to controls ( $P > 0.05$ ; Fig. 1).

### 3.3. Effect on plasma concentrations of LH and FSH

The calcium chloride treatment was associated with graded elevations ( $P < 0.01$ ) in the concentrations of LH and FSH in comparison to control animals (Fig. 2).

### 3.4. Effect on fasting blood sugar levels, PCV and total plasma protein concentrations

There were no alterations in fasting blood sugar level, PCV or total plasma protein concentration in any of the treated groups with respect to the control group as well as among the three treated groups ( $P > 0.05$ ; Table 1).

### 3.5. Effect on testicular $\Delta^5$ , $3\beta$ -HSD and $17\beta$ -HSD activities

The activities of  $\Delta^5$ ,  $3\beta$ -HSD and  $17\beta$ -HSD in testicular tissue in response to treatment with calcium chloride were decreased ( $P < 0.01$ ) with every dose. These effects were dose related ( $P < 0.001$ ; Fig. 3).

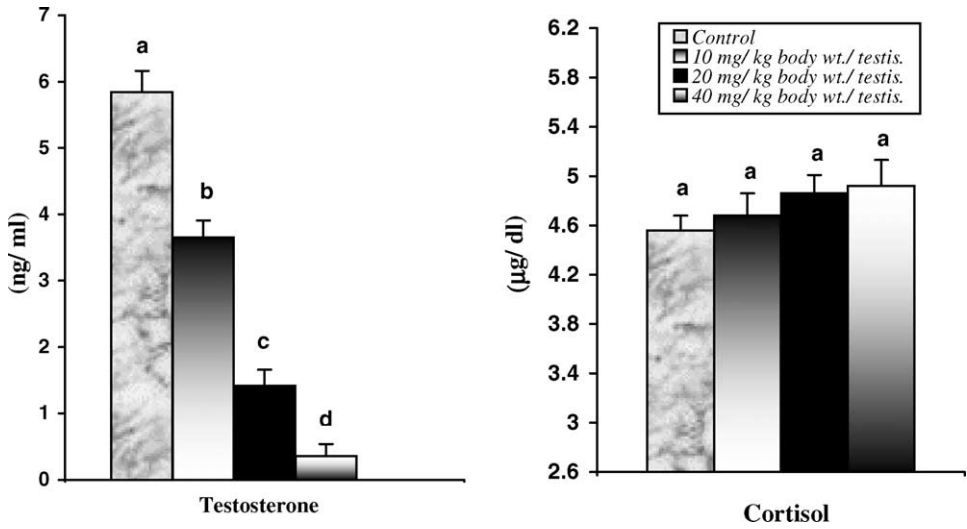


Fig. 1. A graphical representation of the effects of an intratesticular injection of calcium chloride on plasma concentrations of testosterone and cortisol in male Black Bengal goats. Data are presented as mean  $\pm$  S.E.,  $n = 6$ . Different superscripts (a, b, c, d) on each bar differ from each other significantly ( $^aP > 0.05$ ,  $^b, ^cP < 0.01$ ,  $^dP < 0.001$ , as compared with their respective control).

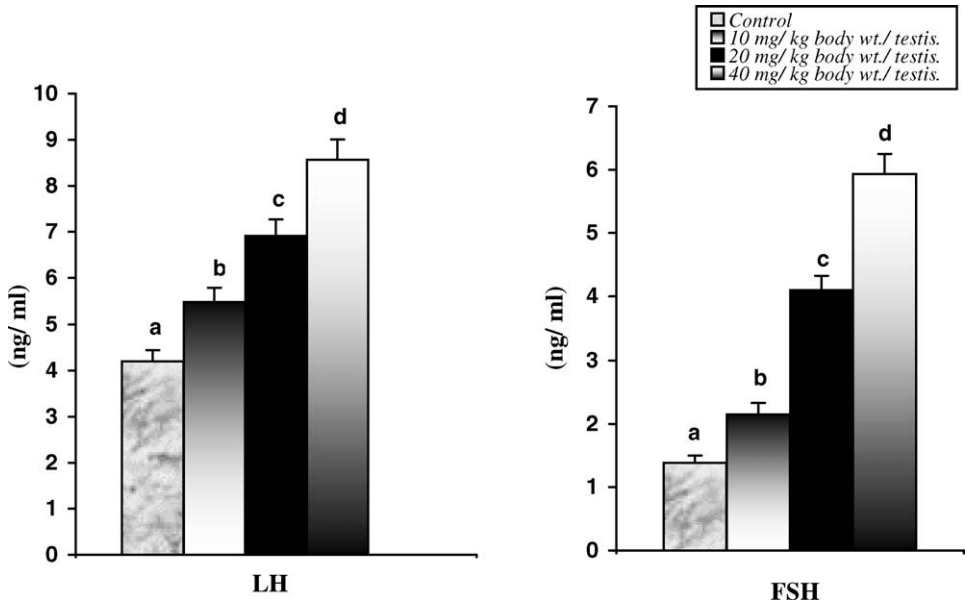


Fig. 2. A graphical representation of the effects of an intratesticular injection of calcium chloride on plasma concentrations of LH and FSH in male Black Bengal goats. Data are presented as mean  $\pm$  S.E.,  $n = 6$ . Different superscripts (a, b, c, d) on each bar differ from each other significantly ( $^b, ^cP < 0.01$ ,  $^dP < 0.001$ , as compared with their respective control).



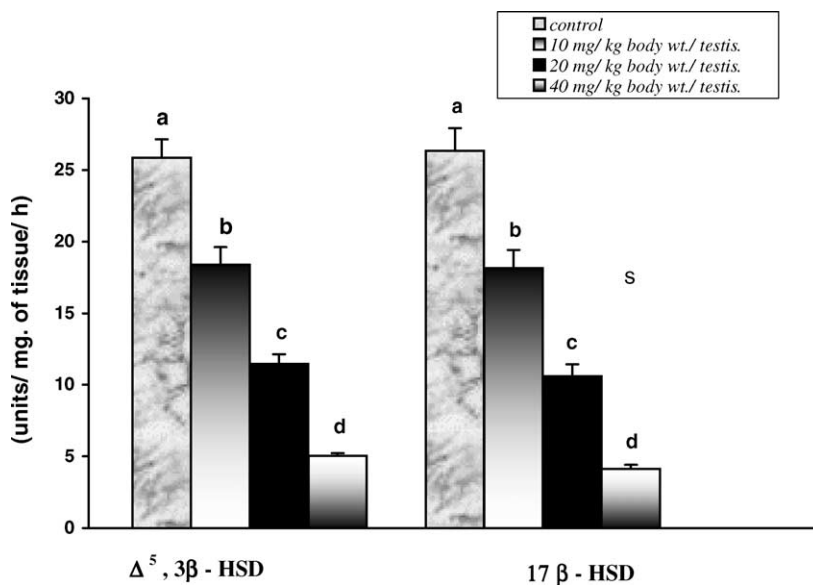


Fig. 3. A graphical representation of the effects of an intratesticular injection of calcium chloride on testicular androgenic key enzyme activities in male Black Bengal goats. Data are presented as mean  $\pm$  S.E.,  $n = 6$ . Different superscripts (a, b, c, d) on each bar differ from each other significantly (<sup>b,c</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$ , as compared with their respective control).

### 3.6. Effect on testicular catalase, glutathione peroxidase (GPx), glutathione S-transferase (GST) and superoxide dismutase (SOD) activities

A dose related decrease was associated with testicular catalase, GPx, GST and SOD activities. The activity of these antioxidant enzymes was diminished ( $P < 0.01$ ) with both the 20 mg and 40 mg treatments. The dose of 40 mg caused a greater degree of inhibition ( $P < 0.001$ ) in the activity of these enzymes. Significant inhibition ( $P < 0.01$ ) in these testicular enzyme activities was noted for every dose from 10 to 40 mg calcium chloride (Figs. 4 and 5).

### 3.7. Changes in testicular content of thiobarbituric acid reactive substances (TBARS) and conjugated dienes

Testicular content of TBARS and conjugated dienes were elevated ( $P < 0.01$ ) after treatment with 10 mg or 20 mg calcium chloride in respect to the controls, but the 40 mg dose exerted a greatest degree of elevation ( $P < 0.001$ ) in the testicular content of TBARS and conjugated dienes when compared to the controls. Levels of TBARS and conjugated dienes in testicular tissue were elevated ( $P < 0.01$ ) between any of the two groups of 10, 20 or 40 mg of calcium chloride (Fig. 6).

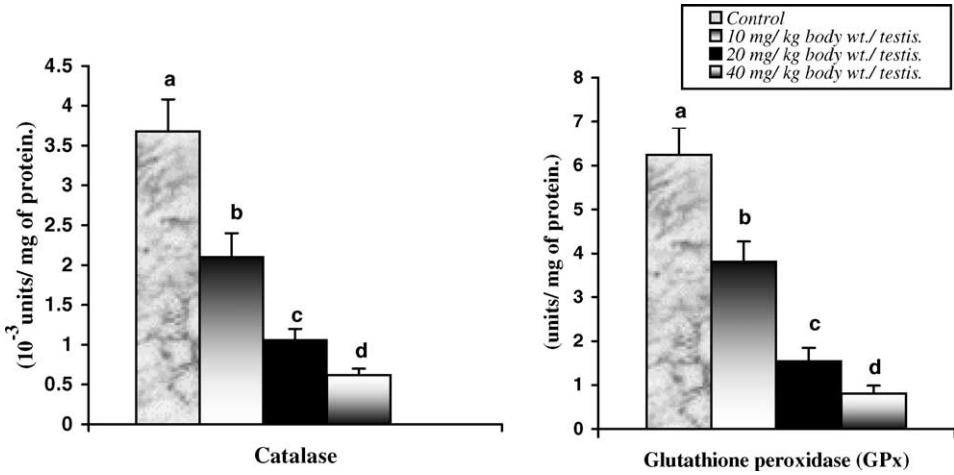


Fig. 4. A graphical representation of the effects of an intratesticular injection of calcium chloride on testicular activities of catalase and glutathione peroxidase (GPx) in male Black Bengal goats. Data are presented as mean  $\pm$  S.E.,  $n = 6$ . Different superscripts (a, b, c, d) on each bar differ from each other significantly (<sup>b,c</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$ , as compared with their respective control).

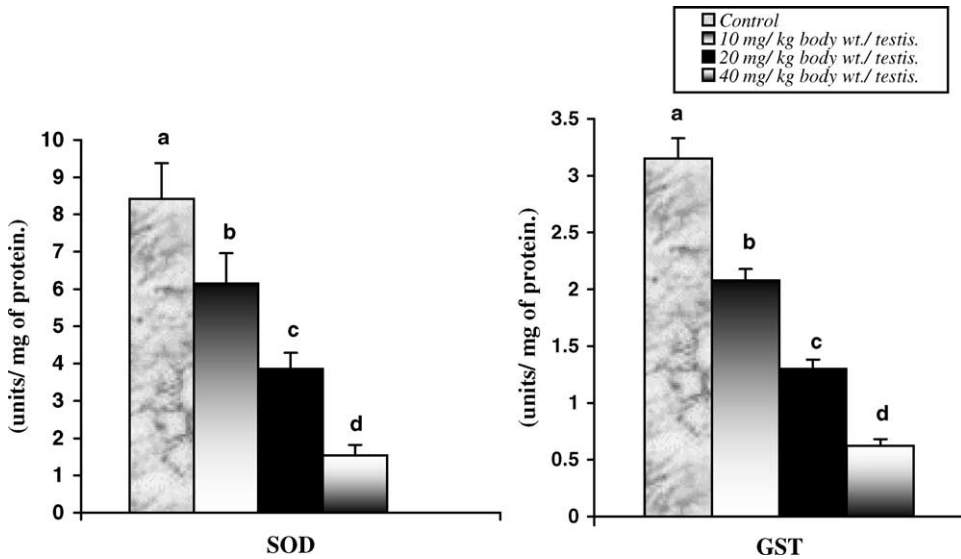


Fig. 5. A graphical representation of the effects of an intratesticular injection of calcium chloride on testicular activities of superoxide dismutase (SOD) and glutathione S-transferase (GST) in male Black Bengal goats. Data are presented as mean  $\pm$  S.E.,  $n = 6$ . Different superscripts (a, b, c, d) on each bar differ from each other significantly (<sup>b,c</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$ , as compared with their respective control).

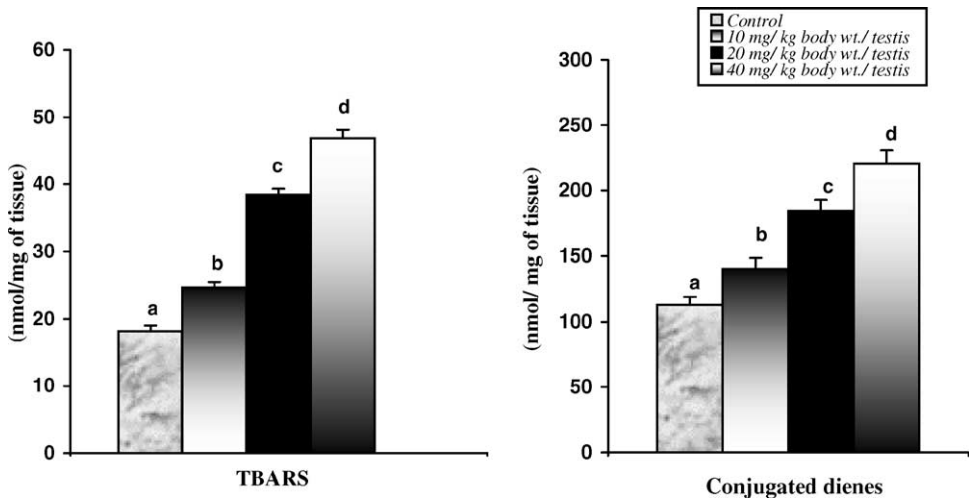


Fig. 6. A graphical representation of the effects of an intratesticular injection of calcium chloride on plasma concentrations of TBARS and conjugated dienes in male Black Bengal goats. Data are presented as mean  $\pm$  S.E.,  $n = 6$ . Different superscripts (a, b, c, d) on each bar differ from each other significantly (<sup>b,c</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$ , as compared with their respective control).

### 3.8. Changes in testicular content of reduced glutathione (GSH)

The testicular content of GSH was decreased ( $P < 0.01$ ) in a graded manner in response to calcium chloride treatment in each of the doses with respect to the controls (Table 1).

### 3.9. Changes in histomorphology of testis

The microscopic structure of seminiferous epithelium among animals in the control group showed a normal arrangement of germ cells in the seminiferous tubules (spermatogenesis) with distinct interstitial spaces (Fig. 7). The intratesticular injection of 10 mg calcium chloride produced disintegration of the germ cell association in seminiferous tubules and washing out of the germ cells from the tubules. Some of the tubules showed the elimination of all germ cells and the presence of only spermatogonia and Sertoli cells. Nevertheless, the induced damage was variable and tubules were not affected uniformly in the lowest dose (Fig. 8). Histomorphological analysis of testis of animals treated with 20 mg calcium chloride showed significant morphological changes, including atrophy of the seminiferous tubules, significant necrosis in the seminiferous epithelium and the interstitial cells as well as sloughing of necrosed cells into the tubular lumen combined with fibrosis in interstitial spaces (Fig. 9). The intratesticular injection of 40 mg calcium chloride resulted in total necrosis in seminiferous tubules and interstitial Leydig cells, with replacement by a fibrocollagenous band. Complete de-arrangement of tubular architecture without any distinct boundary between the tubular and extra tubular compartments along with infiltration of leucocytes through out the testicular tissue were noted. There was no identification of mature or immature germ cells in testicular sections (Fig. 10a and b).

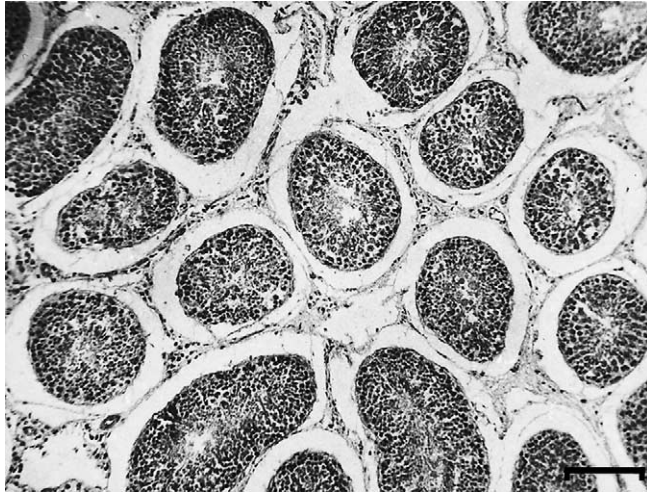


Fig. 7. A photomicrograph of a testicular section of a male Black Bengal goat (*Capra hircus*) showing the normal arrangement of germ cells in the seminiferous tubules (spermatogenesis) with distinct interstitial space. Bar = 100  $\mu$ m.

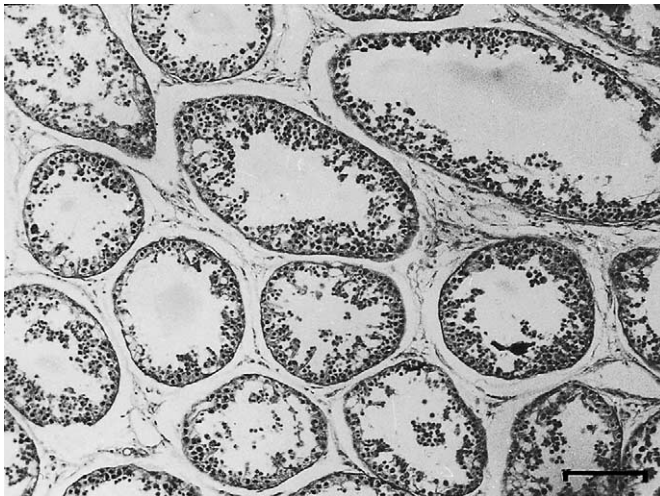


Fig. 8. Photomicrograph of a testicular section following an intratesticular injection of 10 mg calcium chloride in a male Black Bengal goat (*Capra hircus*) showing disintegration of germ cell associations along with washing out of germ cells from the seminiferous tubules. Bar = 100  $\mu$ m.

#### 4. Discussion

The present study identified a dose-dependent relationship when calcium chloride was used to induce sterilization in the male Black Bengal goat (*C. hircus*). The maximum

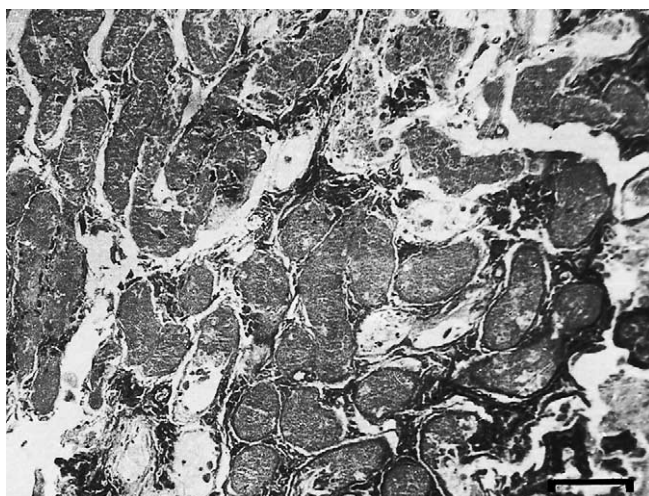


Fig. 9. Photomicrograph of a testicular section following an intratesticular injection of 20 mg calcium chloride in a male Black Bengal goat (*Capra hircus*) showing a high degree of coagulative necrosis in seminiferous tubules and interstitial Leydig cells as well as atrophy of the tubules and fibrosis in to the interstitial spaces. Bar = 100  $\mu$ m.

responses both in the biochemical and histological parameters related to chemosterilization were noted at high (40 mg) and medium (20 mg) doses. Calcium chloride induces necrosis of entire testicular tissue. This is in agreement with previous studies with this chemical agent on the testis in the rat (Jana et al., 2002), and in other domestic animals (Samanta, 1998; Mitra and Samanta, 2000). Testicular palpation after calcium chloride injection indicated severe degeneration of testicular tissue, which would likely have rendered the animals infertile. It is believed that the initial testicular swelling is due to edema followed by necrosis of the testicular tissue leads to atrophy of testicular gland parenchyma. Testicular histology also showed the degenerative changes associated with the graded doses of calcium chloride. The disintegration of germ cell association in seminiferous tubules and washing out of germ cells from the seminiferous tubules were noted even with the lowest dose, though the tubular compartment remained distinct with respect to the extra-tubular compartment. The drastic necrosis in seminiferous tubules along with atrophy of the tubules was noted with the intermediates medium dose. The tubular compartment at this dose was also distinct in respect to paritubular space. But after the high dose of calcium chloride treatment, complete degeneration of germ cells together with the absence of a distinct boundary of seminiferous tubules with respect to the interstitial spaces was observed. At this dose, appearance of leucocytes was noted in both tubular and extra tubular compartment. These changes may be due to the necrotizing properties of calcium chloride as reported by others (Koger, 1976; Albers and Theilen, 1985; McGinnis et al., 1999). In addition, germ cell degeneration by calcium chloride has been associated with low plasma concentrations of testosterone, a prime regulator for the maintenance of structural morphology as well as the normal physiology of seminiferous tubules (Bertlett et al., 1986; Kerr and Sharpe, 1986; Sharpe et al., 1988, 1992; Kerr et al., 1993). The induction of testicular degeneration by this chemical

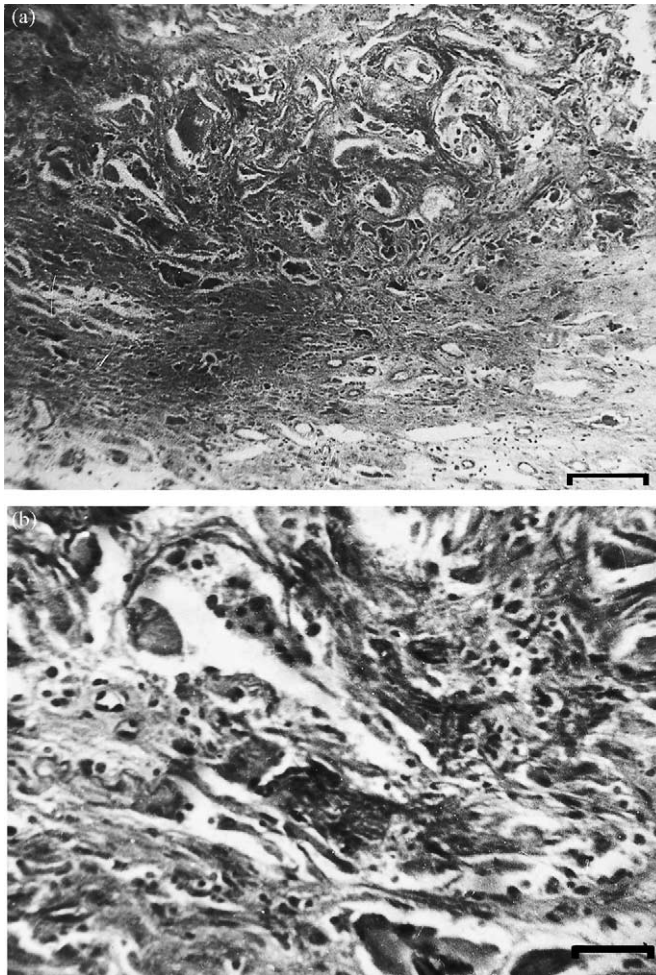


Fig. 10. (a) Photomicrograph of a testicular section following an intratesticular injection of 40 mg calcium chloride in a male Black Bengal goat (*Capra hircus*) showing complete derangement of seminiferous tubular architecture along with the presence of fibrous tissue and leucocytes infiltration through out testicular tissue. Bar = 100  $\mu$ m. (b) Representative photomicrograph of a testicular section following an intratesticular injection of 40 mg calcium chloride in a male Black Bengal goat (*Capra hircus*) showing complete derangement of seminiferous tubular architecture along with fibrosis into the testicular parenchyma. Bar = 25  $\mu$ m.

agent is supported by the diminution in relative testicular weight in treated goats, when this is an accepted measure of testicular damage (Foote et al., 1986).

The graded and significant diminutions in the plasma concentrations of testosterone in response to graded doses of calcium chloride were correlated to graded diminutions in the activities of testicular  $\Delta^5$ , 3 $\beta$ -HSD and 17 $\beta$ -HSD, especially as these are key enzymes for testicular androgenesis (Ishii-Ohba et al., 1986; Ohba et al., 1982; Ghosh et al., 1990).



In addition to this direct effect of calcium chloride on androgenesis, the plasma concentrations of LH and FSH were significantly increased with each one of the effective doses. This may have been due to withdrawal of the negative feedback effect of testosterone on the hypothalamo–pituitary axis (Tillbrook and Clarke, 2001; Plant, 1982). The apparent induced chemosterilization by calcium chloride was also supported by elevations in the concentrations of LH and FSH. An increased secretion of gonadotrophins (LH and FSH) was noted following castration in other species of animals (Tillbrook and Clarke, 1995; Damassa et al., 1976).

The degeneration in the interstitial cells of Leydig by the graded doses of calcium chloride may have led to a reduction in plasma testosterone concentrations (Johnson and Thomson, 1987). The low concentrations of plasma testosterone in these groups treated with calcium chloride was further evidenced by the qualitative study of testicular sections in which significant fibrosis was seen with the medium and highest doses. These have been due to a low concentration of testosterone (Jegou and Sharpe, 1993). The efficacy of calcium chloride in inducing chemosterilization was supported by the necrosis of the seminiferous tubules and interstitial cells, along with the significant fibrosis. These effects are consistent with previous studies using other chemical agents for chemosterilization (Parizek, 1960; Johnson, 1977; Immegart and Threlfall, 2000). Infiltration of leucocytes into the seminiferous tubules and interstitial spaces after treatment with the highest dose may have been due to damage of the testicular tissue or to degeneration that may have released large amounts of chemotactic factors responsible for the ingress of leucocytes (Heath and Arowolo, 1987).

Another way by which chemosterilization may be induced with calcium chloride could be through the generation of large amounts of free radicals, or their products in the testicular tissue. Free radical production in the testis results in a low level of testosterone (Peltola et al., 1996; Chainy et al., 1997). Calcium chloride is also an important 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 the associated tissue damage can be assessed by the measurement of TBARS and conjugated dienes (Gutteridge and Halliwell, 1990; Gutteridge, 1995). The fact that an intratesticular injection of calcium chloride was associated with free radical production and lipid peroxidation in the testis was reflected by the high testicular content of TBARS and conjugated dienes. The alternative way by which free radicals may be generated in the testis would be due to the infiltration of leucocytes. Testicular free radical production was closely associated with the infiltration of leucocytes (Aitken and West, 1990; Sikka, 2001).

This elevation in the formation of free radicals in testes treated with calcium chloride is also supported by the diminution in testicular catalase, GPx, SOD and GST activities, as these are considered important scavenging enzymes against free radicals in male gonads (Peltola et al., 1992; Samanta et al., 1999; Rao and Shaha, 2000). Besides these enzymatic antioxidants, calcium chloride treatment was also associated with a lowered content of testicular reduced glutathione (GSH), as important non-enzymatic antioxidant in testis (Grosshans and Calvin, 1985). The low activities of testicular catalase, GPx, SOD and GST along with a low content of GSH among the treated goats may have been due to the lowered concentrations of testosterone (Hatayama et al., 1986; Peltola et al., 1996; Chainy et al., 1997). It may be assumed that testicular degeneration in this experiment was due to

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; Jana et al., 2002). In addition, the reduced levels of GST activity and GSH content in the testes would be another cause for the degenerative necrosis of seminiferous tubules. GST and GSH are important regulators for proliferation and differentiation of germ cells and provide protection of germ cells against harmful effects of free radicals (Teaf et al., 1985; Aravinda et al., 1996; Gopalakrishnan and Shaha, 1998). However, Bauche et al. (1994) showed that Sertoli and peritubular cells have a high content of GSH and a higher activity of GST. Degeneration of these cells by calcium chloride treatment could be a possible cause of the lower content of GSH and GST activity in the treated testis.

The stress indicators including the concentrations of plasma cortisol, total plasma protein, fasting blood sugar level and PCV were measured to ascertain whether the calcium chloride treatment was associated with any chronic stress response in the experimental animals. Almost any type of stress will cause an increase in the secretion of cortisol in the goat (Guyton and Hall, 1996; Das, 2000). Cortisol is established as an indicator of stress (Cohen et al., 1990; Willett and Erb, 1972; Munck et al., 1984; Buckingham et al., 1996). As there were no significant alterations in the plasma concentrations of cortisol, fasting blood sugar level, PCV or total plasma protein concentrations in the animals treated with calcium chloride with respect to the controls, this method of chemosterilization does not appear to be associated with any chronic stress response.

It is suggested that the chemosterilization developed by an intratesticular injection of calcium chloride might be caused by a high rate of free radicals generated in the testicular tissues and a low level of testosterone.

## 5. Conclusion

An intratesticular injection of calcium chloride was effective and economical for the sterilization of male Black Bengal goats (*C. hircus*). It was free from chronic stress and will contribute a simple alternative method to surgical castration.

## Acknowledgements

The authors are grateful for the financial support provided by the Indian Council of Agriculture Research (ICAR), Govt. of India, Krishi Bhavan, Pusa, New Delhi 110001. The authors are also thankful to Prof. M.K. Bhowmik, Director of Research, Extension and Farms and Prof. D.K. Dey, Head, Department of Surgery & Radiology, West Bengal University of Animal & Fishery Sciences, Calcutta for their interest in this study.

## References

- Aitken, R.J., 1994. A free radical theory of male infertility. *Reprod. Fertil. Dev.* 6, 19–24.
- Aitken, R.J., West, K.M., 1990. Analysis of the relationship between ROS production and leukocyte infiltration in fraction of human semen separated on percoll gradients. *Int. J. Androl.* 13, 433–451.



- Albers, G.W., Theilen, G.H., 1985. Calcium chloride for treatment of subcutaneous lipomas in dogs. *J. Am. Vet. Med. Assoc.* 186, 492–494.
- Alvarez, J.G., Story, B.T., 1984. Assessment of cell damage caused by spontaneous lipid peroxidation in rabbit spermatozoa. *Biol. Reprod.* 30, 323–331.
- Aravinda, S., Gopalakrishnan, B., Shaha, C., 1996. Testicular GST in germ cell proliferation. In: Sengupta, J., Ghosh, D. (Eds.), *Cellular and Molecular Signaling in Reproduction* New Age International Publishers, New Delhi, pp. 1–10.
- Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* 195, 130–140.
- Bauche, F., Fonchard, M., Jegou, B., 1994. Antioxidant system in rat testicular cells. *FEBS Lett.* 349, 392–396.
- Bertlett, J.M.S., Kerr, J.B., Sharpe, R.M., 1986. The effect of selective destruction and regeneration of rat Leydig cells on the intratesticular distribution of testosterone and morphology of the seminiferous epithelium. *J. Androl.* 7, 240–253.
- Buckingham, J.C., Christain, H.C., Gillies, G.E., Philip, J.G., Taylor, A.D., 1996. The hypothalamo–pituitary–adrenal immune axis. In: Marsh, J.A., Kandel, M.D. (Eds.), *The Physiology of Immunity*. CRC Press, New York, pp. 331–354.
- Chainy, G.B.N., Samantaray, S., Samanta, L., 1997. Testosterone induced changes in testicular antioxidant system. *Andrologia* 29, 343–350.
- Cohen, R.D.H., King, B.D., Thomas, L.R., Janzen, E.D., 1990. Efficacy and stress of chemical versus surgical castration of cattle. *Can. J. Anim. Sci.* 70, 1063–1072.
- Damassa, D.A., Kobashigawa, D., Smith, E.R., Davidson, J.M., 1976. Negative feedback control of LH by testosterone: a quantitative study in male rats. *Endocrinology* 99, 736–742.
- Das, D., 1998. Analysis of variance. In: Das, D., Das, A. (Eds.), *Statistics in Biology and Psychology*. Academic Publisher, Calcutta, pp. 250–282.
- Das, D., 2000. Hormones and neurotransmitters. In: Das, D. (Ed.), *Biochemistry*. Academic Publishers, Calcutta, pp. 595–600.
- Dhar, J.D., Setty, B.S., 1990. Effect of a nonsteroidal antiandrogen, anadron, on the reproductive system and fertility in male rats. *Contraception* 42, 121–128.
- Dixit, V.P., Lohiya, N.K., Arya, M., Argrawal, M., 1973. Chemical sterilization of male dogs after a single testicular injection of ‘Danazol’. *Folia Biol.* 23, 305–310.
- Dowsett, K.F., Pattie, W.A., Knott, L.M., 1991. A preliminary study of immunological castration in colts. *J. Reprod. Fertil. Suppl.* 44, 183–190.
- Dube, D., Assaf, A., Pelletier, G., 1987. Morphological study of the effects of an GnRH agonist on the canine testis after 4 months of treatment and recovery. *Acta. Endocrinol. (Copenh.)* 116, 413–417.
- Foote, R.H., Schermerhorn, E.C., Simikili, M.E., 1986. Measurement of semen quality, fertility and reproductive hormones to assess dibromochloropropane (DBCP) effects in live rabbits. *Fundam. Appl. Toxicol.* 6, 628–637.
- Fordyce, G., Hodge, P.B., Beaman, N.J., Leing, H.R., Compro, C., Sheherd, R.K., 1989. An evaluation of calf castration by intratesticular injection of a lactic acid solution. *Aust. Vet. J.* 66, 272–276.
- Georgiou, M., Parkins, L.M., Payne, A.H., 1987. Steroid synthesis – dependent, oxygen – mediated damage of mitochondrial and microsomal cytochrome P-450 enzymes in rat Leydig cell cultures. *Endocrinology* 121, 1390–1399.
- Ghosh, D., Chowdhury, A., Biswas, N.M., Ghosh, P.K., 1990. Effect of lithium chloride on testicular steroidogenic and gamatogenic functions in male albino rats. *Life Sci.* 46, 127–137.
- Gopalakrishnan, B., Shaha, C., 1998. Inhibition of sperm glutathione S-transferase leads to functional impairment due to membrane damage. *FEBS Lett.* 422, 296–300.
- Gonzalez, A., Allen, A.F., Post, A.K., 1989. Immunological approaches to contraception in dogs. *J. Reprod. Fertil. Suppl.* 39, 189–198.
- Greenwood, F.O., Hunter, W.M., Glover, J.S., 1963. The preparation of <sup>131</sup>I labeled human growth hormone of high specific activity. *Biochem. J.* 89, 114–123.
- Grosshans, K., Calvin, H.I., 1985. Estimation of glutathione in purified populations of mouse testis germ cells. *Biol. Reprod.* 33, 1197–1205.
- Gunn, S.A., Gould, T.C., 1970. Cadmium and other mineral elements. In: Johnson, A.D., Gomes, W.R., Van Demark, N.L. (Eds.), *The Testis*, vol. III. Academic Press, New York, pp. 377–481.

- Gutteridge, J.M.C., 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin. Chem.* 41, 1819–1828.
- Gutteridge, J.M.C., Halliwell, B., 1990. The measurement and mechanism of lipid peroxidation in biological system. *Trends Biochem. Sci.* 15, 129–135.
- Guyton, A.C., Hall, J.E., 1996. The adrenocortical hormones. In: *Textbook of Medical Physiology*. W.B. Saunders Company, Philadelphia.
- Habig, W.H., Pabst, M.J., Jacoby, W.B., 1974. Glutathione *S*-transferase: the first step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–7139.
- Halliwell, B., Gutteridge, J.M.C., 1985. *Free Radicals in Biology and Medicine*. Clarendon Press Inc., Oxford.
- Hatayama, I., Satoh, K., Sato, K., 1986. Development and hormonal regulation of the major form of hepatitis glutathione *S*-transferase in male mice. *Biochem. Biophys. Res. Commun.* 140, 581–588.
- Heath, E., Arowolo, R., 1987. The early histopathologic effects of intratesticular injection with hyperosmolar glycerol, glucose or NaCl solutions. *Andrologia* 19, 654–661.
- Immegart, H.M., Threlfall, W.R., 2000. Evaluation of intratesticular injection of glycerol for non-surgical sterilization of dogs. *Am. J. Vet. Res.* 61, 544–549.
- Ishii-Ohba, H., Saiki, N., Inano, H., Takemori, B., 1986. Purification and properties of testicular 3 $\beta$ -hydroxysteroid dehydrogenase and 5-ene-4-ene-isomerase. *J. Steroid Biochem.* 25, 255–260.
- Jana, K., Samanta, P.K., Ghosh, D., 2002. Dose dependent response to an intratesticular injection of calcium chloride for induction of chemosterilization in adult albino rats. *Vet. Res. Commun.* 26, 651–673.
- Jarabak, J., Adams, J.A., Williams–Ashman, H.G., Talalay, P., 1962. Purification of a 17 $\beta$ -hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. *J. Biol. Chem.* 237, 345–357.
- Jegou, B., Sharpe, R.M., 1993. Paracrine mechanism in testicular control. In: Dekrester, D. (Ed.), *Molecular Biology of Male Reproduction*. Academic Press, London, pp. 271–295.
- Johnson, A.D., 1977. The influence of cadmium on the testis. In: Johnson, A.D., Gomes, W.R. (Eds.), *The Testis*, vol. IV. Academic Press, New York, pp. 1191–2001.
- Johnson, L., Thomson Jr., D.L., 1987. Effect of seasonal variation in Leydig cell number on the volume of smooth endoplasmic reticulum in Leydig cells and intratesticular testosterone content in stallions. *J. Reprod. Fertil.* 81, 227–231.
- Kakkar, P., Mehrotra, S., Viswanthan, P.N., 1992. Inter-relation of active oxygen species, membrane damage and altered calcium functions. *Mol. Cell. Biochem.* 111, 11–15.
- Kar, A.B., 1961. Chemical sterilization of male rhesus monkeys. *Endocrinology* 69, 1116–1119.
- Kar, A.B., Kamboj, V.P., Goswami, A., 1965. Sterilization of male rhesus monkeys by iron salts. *J. Reprod. Fertil.* 9, 115–117.
- Kerr, J.B., Sharpe, R.M., 1986. Effects and interaction of LH and LHRH agonist on testicular morphology and function in hypophysectomized rats. *J. Reprod. Fertil.* 76, 175–192.
- Kerr, J.B., Millar, M., Maddocks, S., Sharpe, R.M., 1993. Stage dependent changes in spermatogenesis and Sertoli cells in relation to the onset of spermatogenic failure following withdrawal of testosterone. *Anat. Rec.* 235, 547–559.
- Koger, L.M., 1976. Calcium chloride for the prevention of horn bud growth in calves. *J. Am. Vet. Med. Assoc.* 169, 219–221.
- Lowry, O.H., Resebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin–phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Matsumoto, A.M., 1988. Is high dosage of testosterone an effective male contraceptive agent? *Fertil. Steril.* 50, 324–328.
- McGinnis, K.M., Wangi, K.K., Genegy, M.E., 1999. Alterations of extra-cellular calcium elicits selective modes of cell death and protease activation in SH–SY5Y human neuroblastoma cells. *J. Neurochem.* 72, 1853–1863.
- Mitra, B., Samanta, P.K., 2000. Testicular degeneration of scrub bulls by calcium chloride. *Ind. J. Vet. Surg.* 21, 37–38.
- Mori, Y., Kano, Y., 1984. Changes in plasma concentrations of LH, Progesterone and Oestradiol in relation to the occurrence of luteolysis, oestrous and time of ovulation in the Shiba goat (*Capra hircus*). *J. Reprod. Fertil.* 72, 223–230.
- Munck, A., Guyre, P., holbrook, N., 1984. Physiological function of glucocorticoids during stress and their relation to pharmacological actions. *Endocr. Rev.* 5, 25–49.

- Naz, R.K., Talwar, G.P., 1981. Immunological sterilization of male dogs by BCG. *Int. J. Androl.* 4, 111–128.
- Ohba, H., Inano, H., Tamaoki, B., 1982. Kinetic mechanism of porcine testicular 17 $\beta$ -hydroxysteroid dehydrogenase. *J. Steroid Biochem.* 17, 381–389.
- Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95, 351–358.
- O’Conner, J.J., 1938. *Dollar’s Veterinary Surgery*. Bailliere Tindall, London.
- Paoletti, F., Macoli, A., 1990. Determination of superoxide dismutase activity by purely chemical system based on NAD(P)H oxidation. In: Packer, L., Glazer, A.N. (Eds.), *Methods in Enzymology*, vol. 186. Academic Press, New York, pp. 209–220.
- Paglia, D.E., Valentine, W.N., 1967. Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* 70, 158–169.
- Parizek, J., 1960. Sterilization of the male by cadmium salts. *J. Reprod. Fertil.* 1, 294–309.
- Peltola, V., Huhtaniemi, I., Ahotupa, M., 1992. Antioxidant enzyme activity in the maturing rat testis. *J. Androl.* 13, 450–455.
- Peltola, V., Huhtaniemi, I., Metsa-ketla, T., Ahotupa, M., 1996. Induction of lipid peroxidation during steroidogenesis in rat testis. *Endocrinology* 137, 105–112.
- Plant, T.M., 1982. Effects of orchidectomy and testosterone replacement treatment on pulsatile luteinizing hormone secretion in the adult Rhesus monkey (*Macaca mulatta*). *Endocrinology* 110, 1905–1913.
- Rao, A.V.S.K., Shaha, C., 2000. Role of glutathione S-transferase in oxidative stress-induced male germ cell apoptosis. *Free Radic. Biol. Med.* 29, 1015–1027.
- Samanta, P.K., 1998. Chemosterilization of stray dogs. *Ind. J. Anim. Health* 37, 61–62.
- Samanta, L., Roy, A., Chainy, G.B.N., 1999. Changes in rat testicular antioxidant defense profile as a function of age and its impairment by hexachlorocyclohexane during critical stages of maturation. *Andrologia* 31, 83–90.
- Sharpe, R.M., Donachie, K., Cooper, I., 1988. Re-evaluation of the intratesticular level of testosterone required for quantitative maintenance of spermatogenesis in the rat. *J. Endocrinol.* 117, 19–26.
- Sharpe, R.M., Maddocks, S., Millar, M., Saunders, P.T.K., Kerr, J.B., Mckinnell, C., 1992. Testosterone and spermatogenesis: identification of stage dependent, androgen-regulated proteins secreted by adult rat seminiferous tubules. *J. Androl.* 13, 172–184.
- Sikka, S.C., 2001. Relative impact of oxidative stress on male reproductive function. *Curr. Med. Chem.* 8, 851–862.
- Slater, T.L., 1984. Overview of methods used for detecting lipid peroxidation. In: Packer, L., Glazer, A.N. (Eds.), *Methods in Enzymology*, vol. 105. Academic Press, New York, pp. 283–293.
- Srivastava, T.G., 2002. Enzyme linked immunosorbent assay for steroid hormones. In: Orientation training course on research methodology in reproductive biomedicine. National Institute of Health and Family Welfare, New Delhi, India.
- Swerdlloff, R.S., Wang, C., Bhasin, S., 1992. Developments in the control of testicular function. *Baillier’s Clin. Endocrinol. Metab.* 6, 451–483.
- Talalay, P., 1962. Hydroxysteroid dehydrogenase. In: Colowick, S.P., Kaplan, N.O. (Eds.), *Methods in Enzymology*, vol. 5. Academic Press, New York, pp. 512–516.
- Teaf, C.M., Harbison, R.D., Bishop, B., 1985. Germ cell mutagenesis and GSH depression in reproductive tissue of the F-344 rat induced by ethen methanesulfonate. *Mutat. Res.* 144, 93–98.
- Tillbrook, A.J., Clarke, I.J., 1995. Negative feedback regulation of the secretion and actions of GnRH in male ruminants. *J. Reprod. Fertil. Suppl.* 49, 297–306.
- Tillbrook, A.J., Clarke, I.J., 2001. Negative feedback regulation of the secretion and actions of gonadotrophins releasing hormone in males. *Biol. Reprod.* 64, 735–742.
- Trembley, Y., Belanger, A., 1984. Reversible inhibition of gonadal functions by a potent gonadotrophin releasing hormone agonist in adult dog. *Contraception* 30, 483–497.
- Walkden-Brown, S.W., Restall, B.J., Norton, B.W., Scaramazzi, R.J., Martin, G.B., 1994. Effect of nutrition on seasonal patterns of LH, FSH and testosterone concentration, testicular mass, sebaceous gland volume and odour in Australian Casimira goats. *J. Reprod. Fertil.* 102, 351–360.
- Watanabe, H., Kobayashi, A., Yamamoto, T., Suzuki, S., Hayashi, H., Yamazaki, N., 1990. Alteration of human erythrocyte membrane fluidity by oxygen-derived free radicals and calcium. *Free Radic. Biol. Med.* 8, 507–514.
- Wiebe, J.P., Barr, K.J., Buckingham, K.D., 1989. Sustained azospermia in the squirrel monkey, *Saimiri sciureus*, resulting from a single intratesticular glycerol injection. *Contraception* 39, 447–457.

- Willett, L.B., Erb, R.E., 1972. Short term changes in plasma corticoids in dairy cattle. *J. Anim. Sci.* 34, 103–111.
- Wu, F.C.W., 1988. Male contraception: current status and future prospects. *Clin. Endocrinol.* 29, 443–465.
- Wu, F.C.W., Aitken, R.J., 1989. Suppression of sperm function by depot-medroxyprogesterone acetate and testosterone enanthate in steroid male contraception. *Fertil. Steril.* 29, 443–465.
- Zakowski, J.J., Tappel, A.L., 1978. A semi-automated system for measurement of glutathione in the assay of glutathione peroxidase. *Anal. Biochem.* 89, 430–436.