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Chronic chromium exposure-induced changes in testicular histoarchitecture are associated with oxidative stress: study in a non-human primate (*Macaca radiata* Geoffroy)

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BACKGROUND: Reproductive toxicity of chromium is in dispute despite positive findings in rodents. Recently we reported epididymal toxicity of hexavalent chromium (CrVI) in bonnet monkeys and in this paper we report its testicular toxicity. METHODS: Adult monkeys (*Macaca radiata)* were given drinking water containing CrVI (100, 200, 400 p.p.m.) for 6 months and testes were removed for ultrastructural and biochemical analyses. RESULTS: CrVI treatment disrupted spermatogenesis, leading to accumulation of prematurely released spermatocytes, spermatids and uni- and multinucleate giant cells in the lumen of seminiferous tubules. Transmission electron microscopy revealed granulation of chromatin and vacuolation between acrosomal cap and manchette microtubules of elongated spermatids and in the Golgi area of round spermatids. Pachytene spermatocytes had fragmented chromatin and swollen mitochondria with collapsed cristae. Spermatocytes and spermatogonia in the basal compartment were unaffected. Macrophages containing phagocytosed sperm and dense inclusions in Sertoli cells were seen. Specific activities of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase and concentrations of the non-enzymatic antioxidants glutathione, vitamins A, C and E decreased, while concentrations of H_2O_2 and hydroxyl radicals increased in the testis of chromium-treated monkeys. Withdrawal of chromium treatment for 6 months normalized spermatogenesis and the status of pro- and antioxidants in the testis. CONCLUSIONS: CrVI disrupts spermatogenesis by inducing free radical toxicity, and supplementation of antioxidant vitamins may be beneficial to the affected subjects.

Key words: chromium/multinucleate spermatids/oxidative stress/testicular toxicity/testis ultrastructure

Introduction

Unabated pollution of the environment is considered to be a major reason for the decline of human semen quality over the years (Skakkebaek *et al*., 1991). Occupational, industrial, environmental, therapeutic and dietary exposures to a wide range of chemicals and heavy metals have harmful effects on male fertility (Cheek and McLachlan, 1998; Pant *et al*., 2003).

Hexavalent chromium (CrVI), used in more than 50 industries, is an important heavy metal pollutant (Barceloux, 1999). Several systemic toxicities of CrVI have been demonstrated in experimental animals *in vivo* and *in vitro* (Bagchi *et al*., 2002; Levina *et al*., 2003). However, reproductive toxicity of chromium has been underplayed since the report of Bonde (1993), which stated that low-level exposure to CrVI might not be a major hazard affecting spermatogenesis in stainless steel welders. Even in a recent review, Bonde (2002) emphasized the need for additional data to recognize the reproductive toxicity of chromium. Nevertheless, a number of investigations using laboratory animals have pointed out testicular toxicity of CrVI (Behari *et al*., 1978; Ernst, 1990; Saxena *et al*., 1990; Zahid *et al*., 1990; Murthy *et al*., 1991; Ernst and Bonde, 1992; Chowdhury and Mitra, 1995; Sutherland *et al*., 2000). Two recent reports also correlated chronic occupational exposure to CrVI to abnormal semen quality in men (Li *et al*., 2001; Danadevi *et al*., 2003), though the amount and type of CrVI used by Li *et al.* were questioned (Duffus, 2002).

Uptake of CrVI by the testis and its subsequent reduction to trivalent chromium (CrIII) are well known (Sipowicz *et al*., 1997; Sutherland *et al*., 2000). Recently we reported accumulation of uni- and multinucleate germ cells in the epididymal lumen of the monkeys treated with CrVI causing ductal obstruction (Aruldhas *et al*., 2004). We attributed this to the disruption of spermatogenesis and testicular histoarchitecture and this was tested in the present study.

In an attempt to understand the mechanism underlying the testicular toxicity of chromium, we also studied the status of antioxidants and pro-oxidants in the testes of these animals. The involvement of oxidative stress in heavy metal-induced cellular toxicity is known (Sugiyama, 1994). Maintenance of a critical balance between pro-oxidants and antioxidants is a physiological feature of a cell, to sustain its survival (Aitken, 1989; Yu, 1994). Production of free radicals/reactive oxygen species (ROS) by sperm (Iwasaki and Gagnon, 1992) and the adverse effect of excess ROS and peroxidation on sperm motility and viability were also reported (de Lamirande and Gagnon, 1992; Kim and Parthasarathy, 1998). The testis, epididymis, sperm and seminal plasma contain high activities of antioxidant enzymes, which protect sperm against the deleterious effects of ROS (Kobayashi *et al*., 1991; Zini *et al*., 1993; Potts *et al*., 2000). Increased concentrations of ROS and subnormal antioxidant status have been recorded in the seminal plasma of infertile men (Pasqualotto *et al*., 2000). In the light of the above background information, it is hypothesized that chronic exposure to chromium would disrupt spermatogenesis and testicular histoarchitecture by inducing oxidative stress. Bonde and Ernst (1992) considered only the nasal route of human exposure to CrVI in the welding industry, whereas workers in tanneries are subjected to direct exposure to chromium. A number of people who live around such industries suffer indirectly through contamination of drinking water due to percolation of untreated or incompletely treated effluents into the ground. There are several such large-scale leather industries in India and other developing countries, where pollution control laws may not be implemented effectively. Therefore, it would be pertinent to revisit the testicular toxicity of CrVI to man, adopting a protocol simulating oral exposure and using an animal model closer to the human, in order to understand and overcome the imminent problem of chromium-induced infertility/subfertility in such men.

In the earlier experimental studies, rabbits, rats and mice have been used as animal models to test the testicular toxicity of CrVI (Behari *et al*., 1978; Ernst, 1990; Saxena *et al*., 1990; Zahid *et al*., 1990; Murthy *et al*., 1991; Chowdhury and Mitra, 1995; Sutherland *et al*., 2000). Fine details of spermatogenesis, the relative duration of postnatal development and the onset of sexual maturation differ between rodents and primates (de Kretser and Kerr, 1994). Taking into consideration all the above facts, we tested the hypothesis in a non-human primate model, *Macaca radiata* subjected to chronic exposure to chromium through drinking water.

Materials and methods

The experimental protocol of the present study on a non-human primate model (*Macaca radiata*) was approved by the Institutional Animal Ethics Committee (IAEC) constituted under the auspices of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. At the end of the experiments, the monkeys were handed over to the CPCSEA for rehabilitation.

Animals

Adult male monkeys weighing 7–8 kg (6–8 years old as calculated by the dental formula), trapped by the Department of Forests and Wild Life, Government of Tamil Nadu State, for creating public nuisance, and kept in captivity, were procured with the permission of the Chief Wildlife Warden, Chennai, India. Animals were kept under quarantine, screened for infectious diseases and acclimatized to the animal house for 2 months before the experiments were initiated. The monkeys were maintained in cages (60 × 60 × 80 cm) individually, under natural temperature (28 ± 2°C), and light and dark schedules (12 ± 1 h) in a well-ventilated animal quarter. All animals were fed *ad libitum* with pellet diet (Brooke Bond India, Kolkatta, India), rice cooked with lentils, vegetables such as potatoes, carrots and beetroot, and seasonally available fresh fruits such as banana and guava.

Experimental design

The experimental design was the same as reported earlier (Aruldhas *et al*., 2004). Briefly, adult male bonnet monkeys (*Macaca radiata*) were divided into four groups, each consisting of six animals. Animals belonging to groups I, II and III were provided with drinking water containing CrVI (potassium dichromate) at concentrations of 100, 200 or 400 p.p.m. respectively, *ad libitum* for 180 days. Group IV consisted of control animals, which were provided with drinking water without chromium. Potassium dichromate was selected as it is highly soluble in water and is used in many industries. The concentrations of chromium were selected on the basis of a dose–response study with reference to reproductive toxicity, as judged by decline in sperm characteristics. Chromium at a concentration of 10 mg/l has been considered as safe for humans (Barceloux, 1999). Therefore, we tested increasing concentrations of potassium dichromate, starting a little above the safe level, i.e. 12.5, 25, 50, 100, 200, 400 and 800 mg/l (p.p.m.) for reproductive toxicity. The doses (100, 200, 400 mg/l) that impaired spermatogenesis (sperm count decreased by 50–70% of the control value of 350 to 375×10^6/ml) by 6 months with the least systemic toxicity were selected for the study. At the end of the experiments, testes from three monkeys in each of the experimental groups and the control group were removed surgically under deep sodium pentathol anaesthesia (28 mg/kg body weight, i.p. injection). The testes of remaining three monkeys in each group were removed after withdrawing CrVI treatment for a period of 180 days. Immediately after removing, the testes were weighed and used for histological and biochemical analyses. After removing the testes, the wound was closed using nylon stitches; antibiotics were applied every day and stitches were removed on the eighth day.

Tissue processing for light microscopy and transmission electron microscopy (TEM)

Tissues were not subjected to perfusion fixation for TEM, as samples from the same monkeys were also used for light microscopic and biochemical studies. The entire right testis was immersion-fixed in 2.5% glutaraldehyde in cacodylate buffer (Hess and Moore, 1993) immediately after removal. Thin slices of the testis were again fixed in the same fixative to ensure proper fixation. The tissues were postfixed in 1% osmium tetroxide and embedded in thin viscosity resin (Spurr's mix; Sigma, St Louis, MO, USA). Semithin sections (1μm) were obtained with an Ultratome (Reichert Jung, Vienna, Austria) and stained in toluidine blue O (TBO) for light microscopic observations. The diameter of the seminiferous tubules (20 randomly selected tubules from each animal) was measured using a calibrated ocular micrometer (Erma, Tokyo, Japan). Ultrathin sections were cut with an ultramicrotome (Leica Microsystems, Nussloch, GmbH Nussloch, Germany), stained with uranyl acetate and lead citrate, and observed in a Phillips 201-C (Amsterdam, Holland) TEM. Image analysis and processing were done using Axivision image analysis software (Carl Zeiss, Jena, Germany). Tissues from the recovery group of monkeys were subjected to light microscopic analysis only.

Biochemical analysis

Blood collection and chromium analysis

Blood samples were collected by vein puncture before starting, every month during and at the end of experimentation, and plasma was separated and used for estimation of chromium in an atomic absorption spectrophotometer (Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA), following the protocol of the manufacturer. All glassware used for chromium analysis was washed thoroughly in deionized and double-distilled water, after overnight soaking in nitric acid.

Antioxidant enzymes

Tissue preparation. Immediately after removal, the left testis from each monkey was washed in ice-cold saline and a 10% homogenate was prepared in 0.1 M Tris–HCl buffer (pH 7.4). To 1.0 ml of 1:10 diluted tissue extract, 0.25 ml absolute ethanol and 0.15 ml chloroform were added and the mixture was placed in a mechanical shaker for 15 min, centrifuged at 3000 g for 10 min at 4°C and the supernatant was used for the assay of antioxidant enzymes.

Superoxide dismutase (SOD) (EC 1.15.11). SOD activity was estimated according to the method of Marklund and Marklund (1974). The percentage inhibition of pyrogallol auto-oxidation at 470 nm was converted to units of enzyme activity. The amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation was considered as 1 unit of enzyme activity.

Catalase (EC No. 1.15.1.6). Catalase activity was quantified colorimetrically (Sinha, 1972) based on the intensity of the green colour developed due to the conversion of dichromate into chromic acetate. The amount of enzyme that uses 1 μM of H_2O_2 per minute is equivalent to 1 unit.

Glutathione peroxidase (GPx) (EC 1.11.1.9). GPx activity was determined colorimetrically by estimating the amount of reduced glutathione (GSH) oxidized per minute, using GSH as the standard (Rortruck *et al*., 1973).

Glutathione reductase (GR) (EC 1.6.4.2). GR activity was assayed colorimetrically by following the rate of reduction of oxidized glutathione (Stall and Vegel, 1969).

Glutathione-S-transferase (GST) (EC 2.5.1.18). GST activity was determined by estimating the amount of enzyme that catalysed the conjugation of a known amount of 1-chloro-2,4 dibenzene with GSH, as described by Habig *et al*. (1973).

Glucose-6-phosphate dehydrogenase (G-6-PDH) (EC 1.1.1.49). G-6-PDH activity was assayed spectrophotometrically by following the rate of oxidation of glucose-6-phosphate to 6-phosphogluconate, according to Beutler (1983).

γ-Glutamyl transpeptidase (γ-GT) (EC 2.3.2.2). γ-GT activity was estimated colorimetrically by quantifying the amount of *p*-nitroaniline released from γ-glutamyl-*p*-nitroanilide in a given time, using the method of Orlowski and Meister (1965) with *p*-nitroaniline as the standard.

Non-enzymic antioxidants

Reduced glutathione (GSH). GSH concentration was quantified colorimetrically by measuring the product of the reaction between GSH and DTNB (Moren *et al*., 1979).

Vitamin C. Vitamin C was estimated colorimetrically using ascorbate as the standard (Omaye *et al*., 1971).

Vitamin E. Vitamin E content was quantified by the colorimetric method of Desai (1984), using α-tocopherol as the standard.

Vitamin A. Vitamin A content was quantified by the colorimetric method of Bayfield and Cole (1974), using β-carotene as the standard.

Reactive oxygen species

Hydrogen peroxide (H_2O_2). H_2O_2 production was assessed spectrophotometrically (Holland and Storey, 1981) by estimating the oxidation product of ferrocytochrome.

Hydroxyl radical. The production of hydroxyl radical was quantified by the colorimetric method described by Puntarulo and Cederbaum (1988).

Statistics

Data were subjected to one-way analysis of variance, and whenever the *F* value was significant, the data were analysed by Duncan's multiple comparison test to find the within-group significance at the $P < 0.05$ level.

Results

Plasma chromium

The plasma concentration of chromium increased up to ten-fold in monkeys that were provided with drinking water containing chromium and it returned to the normal level in the recovery group (Table I).

Organ weight

The absolute weights of the testis did not show any appreciable change due to CrVI treatment. However, there was a statistically significant decrease in the relative weight of the testes of monkeys that were exposed to CrVI The weight of the testes in monkeys belonging to the withdrawal group showed a trend of recovery to control level (Table I). (Author please re-check this.

Histopathology

Light microscopic observations

Control testes. The seminiferous tubules in the testes of control monkeys had the typical organization (Figure 1a), with different generations of germ cells associated with Sertoli cells (Figure 1b). The Leydig cells also had their characteristic organization (Figure 1a).

Testes of chromium-treated monkeys. The seminiferous tubules of the chromium-treated monkeys were disorganized, with decreased diameter (0.61 ± 0.08, 0.52 ± 0.07 and 0.97 ± 0.11 mm for the 100, 200 and 400 p.p.m. groups, respectively, versus the control value of 1.32 ± 0.13 mm). Depletion of germ cells and hyperplasia of Leydig cells (Figure 1c–e) were the

Table I. Plasma chromium concentration (μg/l) and testicular weight (g/kg body weight) in monkeys treated with different doses of CrVI

Treatment	Plasma Cr	Testicular weight
Control	46.5 ± 2.10	3.95 ± 0.25
100 p.p.m.		
A	150.0 ± 6.3*	3.05 ± 0.20*
B	48.81 ± 2.5	3.68 ± 0.13
200 p.p.m.		
A	230.0 ± 10.5*	2.55 ± 0.10*
B	47.52 ± 2.8	3.50 ± 0.27
400 p.p.m.		
A	410.3 ± 20.2*	2.60 ± 0.17*
B	48.67 ± 3.1	3.75 ± 0.23

Each value is mean ± SEM of three values.
A, CrVI-treated (24 h after the 180th day of treatment).
B, withdrawal group (180 days after the last day of chromium treatment).

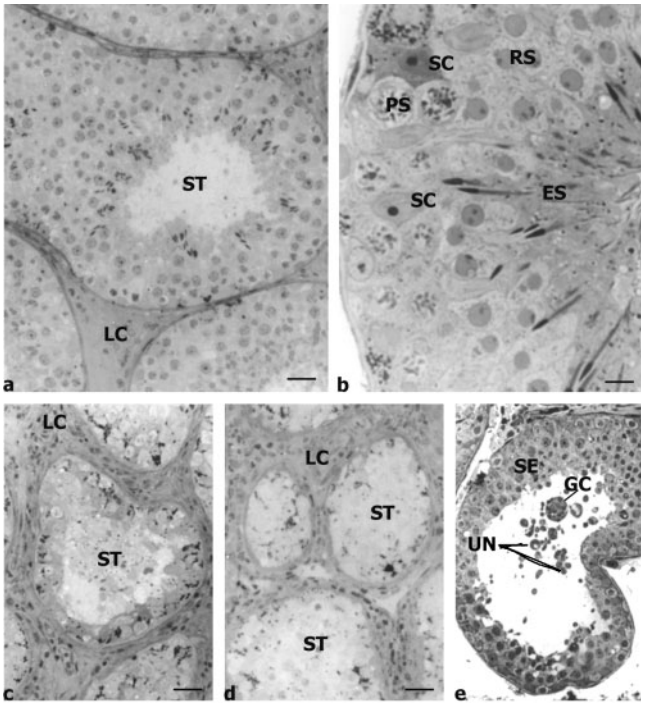

Figure 1. (a) Semithin section of testis of a control monkey showing seminiferous tubules (ST) and Leydig cells (LC). Scale bar, 20 μm. (b) Seminiferous epithelium of a control monkey showing Sertoli cells (SC) and the different generations of germ cells (PS, pachytene spermatocyte; RS, round spermatid; ES, elongating spermatid). Scale bar, 6 μm. (c, d) Seminiferous tubules (ST) and the Leydig cells (LC) of CrVI-treated monkeys (c, 100 p.p.m.; d, 200 p.p.m.) showing regression of seminiferous tubules and degeneration of seminiferous epithelium. Scale bar, 20 μm. (e) A seminiferous tubule of a CrVI-treated (400-p.p.m.) monkey showing mild effect in the seminiferous epithelium (SE) with uninucleated germ cells (UN) and multinucleate giant cells (GC) in the lumen. Scale bar, 20 μm.

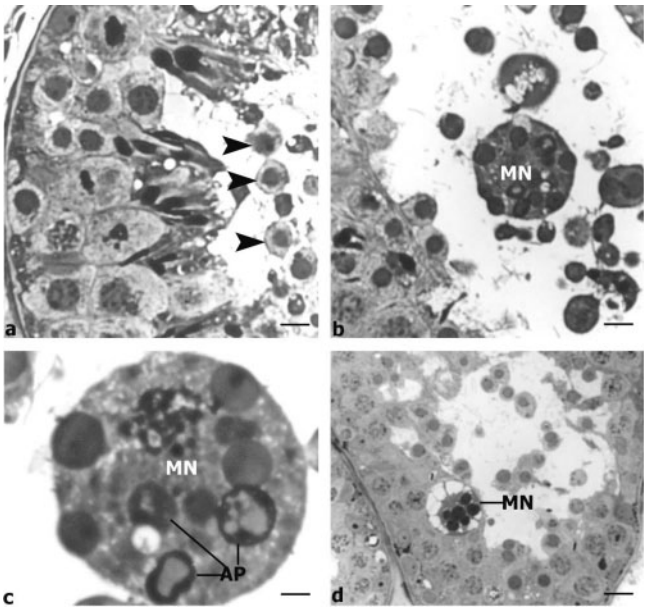

Figure 2. (a) Seminiferous epithelium of a CrVI-treated monkey (200 p.p.m.) showing premature release of round spermatids from the Sertoli cells (arrowheads). Scale bar, 6 μm. (b) Seminiferous tubular lumen of a CrVI-treated monkey (200 p.p.m.) showing occurrence of a multinucleate giant cell (MN). Scale bar, 6 μm. (c) A multinucleate giant cell (MN) produced in the seminiferous tubule of a CrVI- treated monkey (100 p.p.m.) with some of the nuclei indicating apoptotic morphology (AP). Scale bar, 3 μm. (d) A multinucleate giant cell (MN) with vacuolated cytoplasm and pycnotic nuclei present in the seminiferous epithelium of a CrVI-treated monkey (100 p.p.m.). Scale bar, 10 μm.

common features in all the experimental groups. Spermatids were the most affected germ cells in most of the tubules and were totally absent in a few tubules. Most of the germ cells in the adluminal compartment were prematurely released into the lumen, resulting in Sertoli cell fibrosis. Even those spermatids that adhered to the epithelium had vacuoles around them, indicating that they were detaching from the Sertoli cell (Figure 2a). Of the round and elongating spermatids, the former appeared to be the earlier target for chromium as the round spermatids were missing or being lost in many of those tubules which should possess two generations of spermatids, whereas the elongating spermatids were present (Figure 2a).

In several tubules, which were in the process of losing round spermatids, the adluminal compartment contained multinucleate giant cells (Figure 1e). The organization of the nuclei of the multinucleate giant cells suggested they were spermatids (Figure 2b), and in a few such giant cells the nuclei were abnormally shaped, with marginalized chromatin, creating a hollow in the centre (Figure 2c). In rare cases, the cytoplasm of the multinucleate spermatids appeared vacuolated and the nuclei were pycnotic (Figure 2d).The lumen was invariably filled with prematurely released germ cells and cell debris (Figures 1c, e and 2b, d).

In several postzygotene spermatocytes, the chromatin appeared fragmented and discontinuous (Figure 3a), whereas in those with chromosomes in the metaphase plate the spindle fibres were not discernible (Figure 3b). Cells resembling macrophages were noticed in the adluminal compartment (Figure 3a). In most of the prematurely released germ cells, the chromatin was either intact or marginalized; in a few instances hydropic swelling and necrosis were also noticed (Figure 3c). Depending upon the extent of the loss of germ cells, the seminiferous epithelium reflected prominence of Sertoli cells and a few such tubules reflected the Sertoli cell-only syndrome (Figure 3d), but closer examination revealed the presence of spermatogonia in the basal compartment Accumulation of electron-dense granules in the basal and perinuclear cytoplasm of Sertoli cells was a common manifestation of chromium toxicity (Figure 3c, d).

In the recovery group of monkeys (i.e. 6 months after the withdrawal of chromium exposure) the diameter (100 p.p.m., 1.18 ± 0.12 mm; 200 p.p.m., 1.22 ± 0.08 mm; 400 p.p.m., 1.25 ± 0.14 mm) and the histological organization of the seminiferous tubules were comparable to those of control monkeys. Spermatogenesis recovered fully, though a few prematurely released germ cells were still noticed in the lumen (Figure 4a, b).

TEM observations

Control testes. Low-power electron micrographs of the testes of control monkeys revealed closely packed Sertoli cells

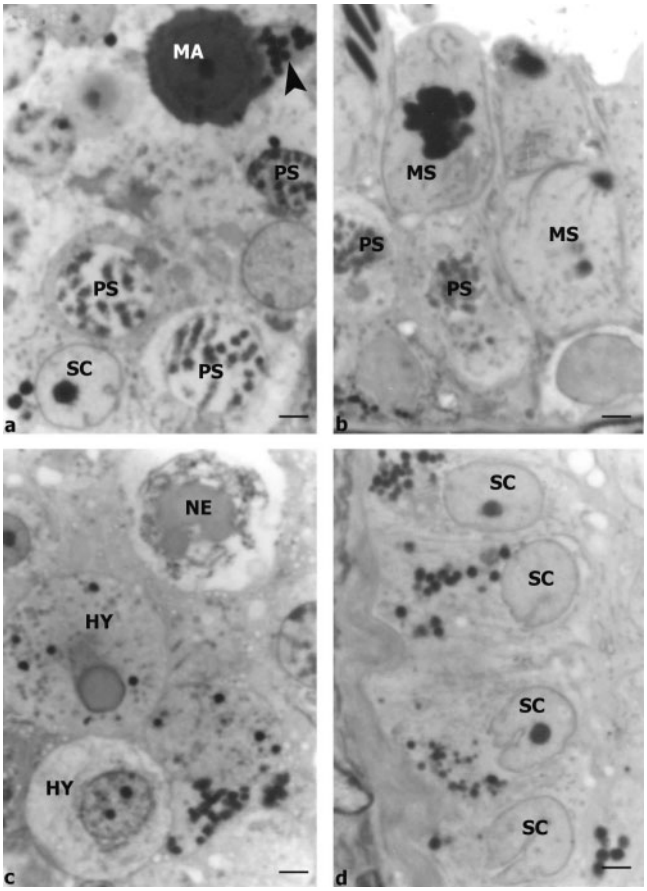

Figure 3. (a) Seminiferous epithelium of a CrVI-treated monkey (100 p.p.m.) showing fragmented chromatin in pachytene spermatocytes (PS). Picture also shows occurrence of a macrophage (MA) associating with a cell in metaphase of meiotic division (arrowhead). Scale bar, 3 μm. (b) Seminiferous epithelium of a CrVI-treated monkey (200 p.p.m.) showing disruption of spindle fibres in meiotic metaphase cells (MS). Pachytene spermatocytes (PS) are also seen. Scale bar, 3 μm. (c) Seminiferous epithelium of a CrVI-treated monkey (100 p.p.m.) showing death of round spermatids through necrosis (NE) following hydropic swelling (HY). Scale bar, 3 μm. (d) Seminiferous epithelium of a CrVI-treated monkey (200 p.p.m.) showing a continuous row of Sertoli cells (SC), with dense granules in the cytoplasm, without any trace of germ cells. Scale bar, 3 μm.

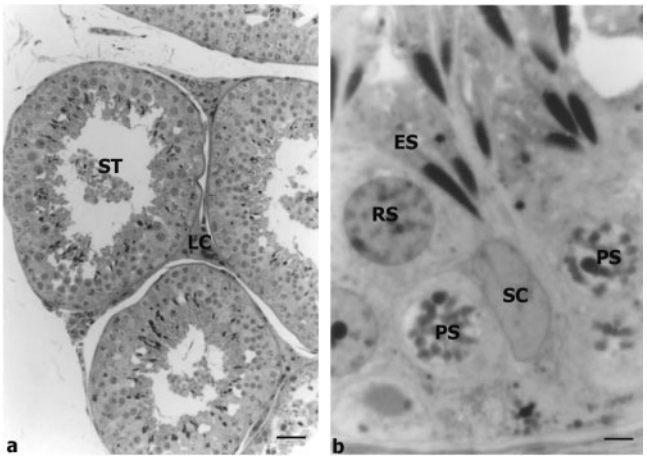

Figure 4. (a) Seminiferous tubules (ST) of a monkey in the recovery group showing almost complete recovery of spermatogenesis; the Leydig cells (LC) also appear normal. Scale bar, 20 μm. (b) Epithelium in Figure 4a magnified, showing normal Sertoli cells (SC), pachytene spermatocytes (PS), round spermatids (RS) and elongating spermatids (ES). Scale bar, 3 μm.

contacting the basement membrane. The spermatogonia and spermatocytes up to preleptotene stage were present in the basal compartment, whereas spermatocytes beyond preleptotene stage and spermatids were present in the adluminal compartment of Sertoli cells. The cell types reflected their characteristic ultrastructural organization (Figure 5a).

Testes of chromium-treated monkeys: elongating spermatids. Sertoli cells in which sperm heads were embedded developed vacuoles around the latter and had electron-dense granules around the posterior aspect of the sperm nucleus. The cytoplasm of the differentiating midpiece was also vacuolated (Figure 5b). Other important manifestations in spermatids of the monkeys exposed to chromium included: (i) chromatin becoming heterogeneous and granular; (ii) the appearance of a dense, plaque-like acrosomal cap; (iii) association of the nuclear cap with microtubule-like structures; (iv) the presence of a large vacuole at the junction between the acrosomal cap and the manchette microtubules; (v) vacuoles in the developing midpiece (Figure 5c); (vi) formation of the manchette microtubules into a continuous patch (Figure 5d); and (vii) the midpiece lagging in pace behind that of the head (Figure 5e).

Round spermatids. In the 400 p.p.m. chromium-treated group the round spermatids were prematurely released from the Sertoli cells, with little pathological change (Figure 6a), whereas in the 100 and 200 p.p.m. groups the round spermatids developed vacuoles in the cytoplasm, particularly in the Golgi area (Figure 6b). The mitochondria were swollen, with collapsed cristae (Figure 6c).

Spermatocytes and spermatogonia. In spermatocytes at the advanced pachytene stage, the chromatin appeared fragmented and the mitochondria underwent hydropic swelling accompanied by collapse of cristae (Figure 6d). A few spermatocytes showed necrotic changes with thoroughly damaged chromatin (Figure 7a). However, the pale and dark spermatogonia and preleptotene spermatocytes manifested little pathological change (Figure 7b,c), though mitochondria were slightly damaged in the pale spermatogonia (Figure 7d).

Sertoli cells. Sertoli cells of CrVI-treated monkeys accumulated large electron-dense bodies in the basal and perinuclear cytoplasm, and at a higher magnification these bodies appeared to be pale, transitional and dark lysosomes (Figure 8a). In certain rare cases, deformed sperm heads were seen in a vacuole in the Sertoli cell cytoplasm (Figure 8a). The structure of inter-Sertoli cell junctions (Figure 8b) and the Sertoli cell–germ cell junctions appeared to be affected (Figures 6a and 8b).

Multinucleate giant cells and their origin. Ultrastructural analysis revealed the multinucleate giant cells to be spermatids possessing two or more nuclei in a common cytoplasm. The presence of an acrosome in the nuclear cap in some of these cells suggested the initiation of spermiogenesis, though in respect of the different constituent cells this was not uniform (Figure 9a).

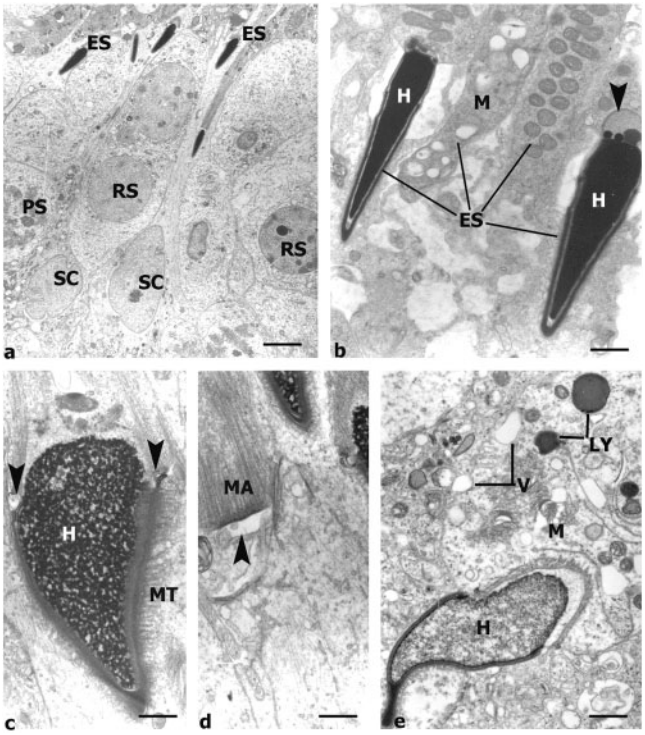

Figure 5. (a) A low-power transmission electron micrograph (TEM) of the seminiferous epithelium of a control monkey showing Sertoli cells (SC) and different generations of germ cells, viz., pachytene spermatocytes (PS), round spermatids (RS) and elongating spermatids (ES). Scale bar, 3 μm. (b) TEM showing presence of vacuoles in and around elongating spermatids (ES) in a CrVI-treated monkey (100 p.p.m.). H, head; M, midpiece with cytoplasmic vacuolation. Arrowhead points to a large vacuole containing electron-dense granules. Scale bar, 1μm. (c) TEM of an elongating spermatid of a CrVI-treated monkey (200 p.p.m.) showing head (H) with dispersed chromatin, and microtubule-like structures (MT) connected to the head. Arrowheads point to vacuolated areas at the junction of manchette microtubules with the head cap. Scale bar, 1 μm. (d) TEM of an elongating spermatid showing manchette microtubules (MA) occurring as a continuous patch, with a large vacuole at the point of origin (arrowhead). Scale bar, 1 μm. (e) TEM showing an unusual organization of the elongating spermatid, with much advanced development of the head (H), leaving behind the development of midpiece (M), containing vacuoles (V) and lysosomes (LY). Scale bar, 1 μm.

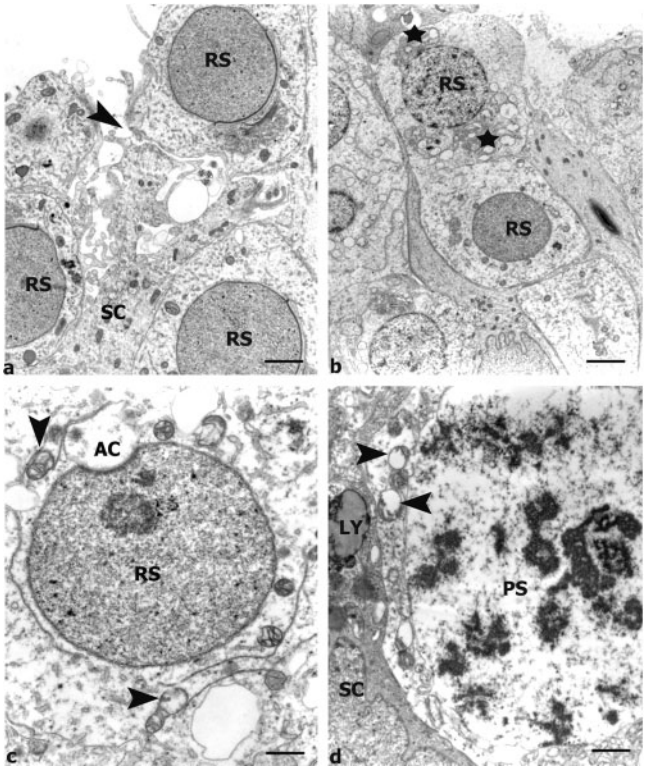

Figure 6. (a) TEM showing detachment (arrowhead) of round spermatids (RS) from the Sertoli cell (SC). Scale bar, 1.5 μm. (b) TEM showing appearance of vacuoles in the Golgi area and mitochondria of spermatocytes (asterisks). Scale bar, 2.5 μm. (c) TEM showing swelling of mitochondria and collapse of their cristae (arrowhead) of a round spermatid (RS). The developing acrosome (AC) is also affected. Scale bar, 0.5 μm. (6) TEM showing chromatin fragmentation and mitochondrial swelling (arrowhead) in a pachytene spermatocyte (PS) remaining attached to a Sertoli cell (SC), which has abundant lysosomes (LY). Scale bar, 0.2 μm.

Occasionally, the nuclear envelope in the binucleate spermatid was indented at varying degrees, and the mitochondria that were destined to develop into the mitochondrial sheath accumulated at this pole of the nucleus (Figure 9b). The formation of bi- and multinucleate spermatids appeared to be due to incomplete cytokinesis (Figure 9c), as a result of which two nuclei shared a common nuclear cap with a common Golgi apparatus lying on top of it (Figure 9d).

Macrophages in the seminiferous epithelium. The occurrence of macrophages in the seminiferous epithelium was confirmed in the TEM study, and such macrophages were seen to align close to sperm heads that detached from the Sertoli cell (Figure 10a). In some cases, damaged macrophages containing germ cell fragments were found within the Sertoli cell cytoplasm (Figure 10b).

Biochemical analysis

Antioxidant enzymes

Chromium treatment for 6 months led to a dose-dependent decrease in the specific activities of testicular SOD, catalase, GPx, GR and G-6-PDH, with the maximum decrease in monkeys exposed to 400 p.p.m. chromium (Figures 11 and 12). The specific activity of γ-GT (Figure 11) decreased in the testes of monkeys exposed to 400 p.p.m. chromium alone. Unlike the other enzymes, GST activity (Figure 12) increased in the testes of chromium-treated monkeys in all groups.

Non-enzymatic antioxidants

The concentration of reduced glutathione (Figure 12) increased in the testes of monkeys exposed to chromium, whereas the concentration of antioxidant vitamins A, C and E (Figure 13) decreased.

Free radicals

The concentrations of H_2O_2 and OH^- increased significantly in the testes of monkeys exposed to chromium, in a dose-dependent manner; the maximum concentration was recorded in monkeys exposed to 400 p.p.m. chromium (Figure 14).

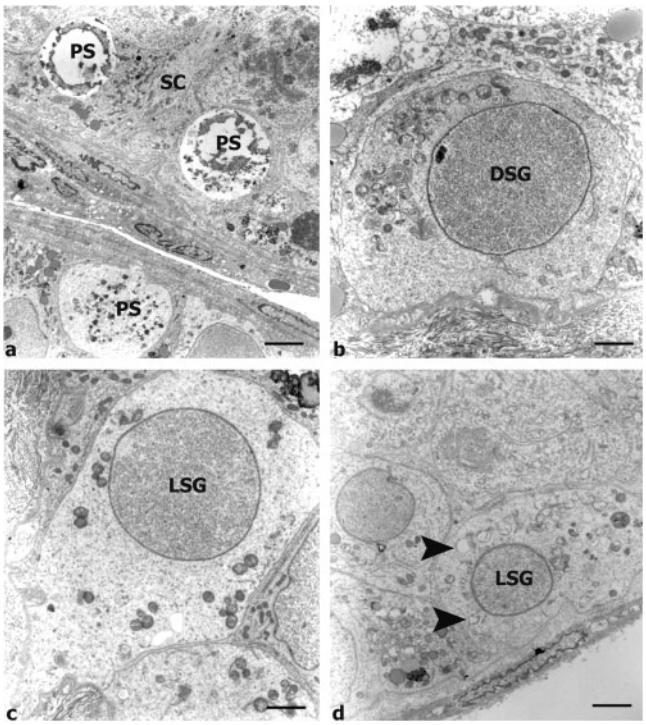

Figure 7. (a) TEM showing necrosis of pachytene spermatocytes (PS) remaining attached to Sertoli cell (SC) in CrVI- treated monkey (200 p.p.m.). Scale bar, 3 μm. (b) TEM of an intact dark spermatogonium (DSG) in the seminiferous epithelium of a CrVI- treated monkey (200 p.p.m.). Scale bar, 1.5 μm. (7) TEM of an intact light spermatogonium (LSG) in the seminiferous epithelium of a CrVI-treated monkey (200 p.p.m.). Scale bar, 1.5 μm. (7) TEM of a light spermatogonium (LSG) in a CrVI-treated monkey (200 p.p.m.), showing slight damage to mitochondria (arrowheads). Scale bar, 2 μm.

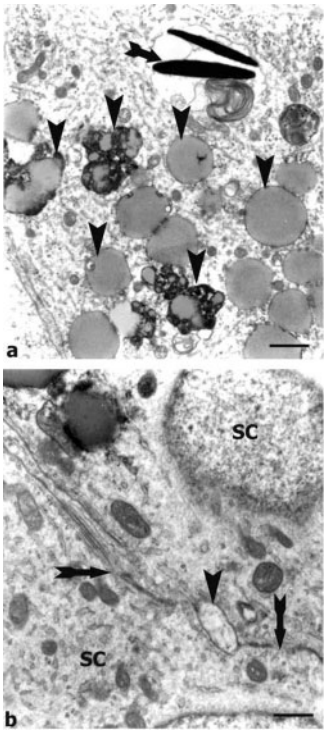

Figure 8. (a) TEM of basal cytoplasm of a Sertoli cell, showing the electron-dense inclusions to be polymorphic forms of lysosomes (arrowheads). Picture also shows sperm heads inside an endocytic vesicle (arrow). Scale bar, 0.4 μm. (b) TEM showing disruption of inter-Sertoli cell (SC) junctions (arrows). Arrowhead points to a large gap between the Sertoli cells. Scale bar, 0.4 μm.

Discussion

Data on plasma chromium ascertain the supraphysiological levels of the metal in circulation in experimental monkeys. The histopathological changes in the testis presented here and those of the epididymis reported earlier (Aruldhas *et al*., 2004) in monkeys exposed to CrVI ascertain the male reproductive toxicity of this heavy metal. The important observation of the present study is that chromium intoxication affects germ cells in the adluminal compartment to a great extent, leaving those in the basal compartment and the basal portion of the Sertoli cells unaffected, providing scope for recovery of spermatogenesis. The reversibility of chromium-induced histopathological changes in the seminiferous tubules is clearly distinct from the normal pattern of testicular histology observed in monkeys of the withdrawal group. The normozoospermia observed in these monkeys by the sixth month of withdrawal period (data not shown here) ascertains the recovery of normal spermatogenesis in these animals.

CrVI as a genotoxic agent

The histopathological changes observed in the testis of chromium-treated monkeys suggest the cytotoxic effect of the metal. The impact of chromium on meiotic germ cells (spermatocytes) is interesting because the chromosomes appeared to undergo breakage in two ways, subsequent to pairing in the zygotene stage. In most instances, chromosomes of the pachytene spermatocytes underwent fragmentation and clumping, leading to karyolysis and necrosis. In a few cells, which had developed the metaphase plate, the chromosomes failed to proceed towards the poles and underwent fragmentation to form dot-like chromatoid bodies.

Chromium is known to induce chromosomal aberrations such as single-strand breaks and 8-oxo-guanosine substitutions (Sugiyama *et al*., 1993; Wise *et al*., 1993; Qi *et al*., 2000). Direct interaction of reactive chromium intermediates with DNA, leading to DNA–chromium binding, DNA–DNA crosslinks and DNA–protein crosslinks are known (Misra *et al*., 1994). Interaction of chromium with GSH results predominantly in chromium–DNA binding and strand breaks (Aiyar *et al*., 1991). Therefore, the increased concentration of H_2O_2, OH^- and GSH observed in the present study may favour the operation of such a mechanism in the testes of monkeys exposed to CrVI, resulting in damage to the chromosomes/chromatin of meiotic/postmeiotic germ cells.

The data on antioxidants and free radicals in the present study indicate free radical toxicity in the testis of chromium-treated monkeys, as there was a significant increase in the concentration of H_2O_2 and OH^- and subnormal activity of most of the antioxidant enzymes tested, accompanied by decreased concentrations of the antioxidant vitamins A, C and E. Thus, chronic exposure to chromium appears to result in oxidative stress in the testis due to poor scavenging of free radicals The histopathological changes in the testis suggest that spermatocytes

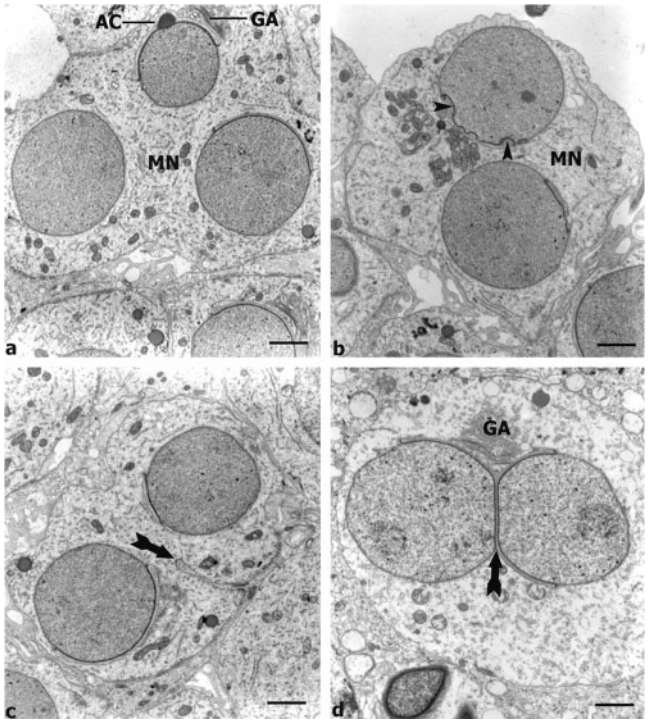

Figure 9. (a) TEM of a multinucleate giant spermatid (MN). The constituent cells are at different steps in spermiogenesis. AC, acrosomal vesicle; GA, Golgi apparatus. Scale bar, 1.5 μm. (b) TEM of a multinucleate giant spermatid (MN) showing indented nuclear envelope (arrowheads) and the mitochondria crowding around that area. Scale bar, 1.5 μm. (c) TEM showing incomplete cytokinesis between spermatids (arrow). Scale bar, 1.5 μm. (d) TEM of a binucleate spermatid, without any trace of cytokinesis (arrow). There is a single Golgi apparatus (GA) on the top of the two. Scale bar, 1 μm.

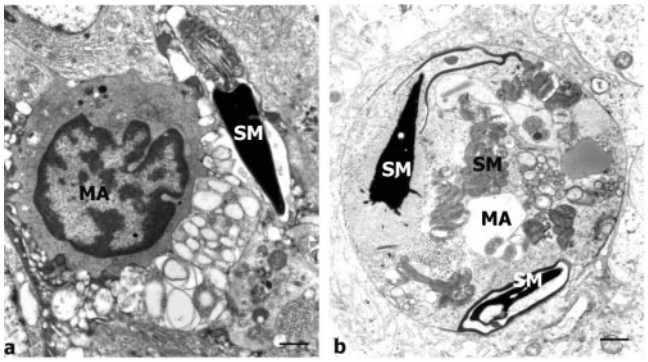

Figure 10. TEM showing the occurrence of macrophage in the seminiferous epithelium of CrVI-treated monkeys. (a) The macrophage (MA) is in close proximity to a damaged sperm (SM), as seen in the vacuoles surrounding it. Scale bar, 1 μm. (b) The macrophage (MA) has engulfed spermatozoa (SM). Scale bar, 0.7 μm.

and spermatids are susceptible to chromium toxicity, whereas spermatogonia are resistant to the hostile environment of increased free radicals in the tissue. GSH is a major cellular reductant, and plays a pivotal role against the nefarious actions of hydroxyl radicals (Pal *et al*., 1993). The increased concentration of GSH observed in the testis of monkeys exposed to chromium probably acted as a protector of spermatogonia. The mechanism underlying such a cell-specific effect of GSH may be another area of future research for a better understanding of the mechanism of the testicular toxicity of chromium.

γGT couples γ-glutamyl moiety to a suitable amino acid acceptor for transport into the cell and makes it suitable for the intracellular synthesis of GSH (Markey *et al*., 1998). γGT also mediates the cleaving of the dipeptidyl cysteinyl glycine, which provides cells with cysteine, a rate-limiting factor for the synthesis of GSH (Pal *et al*., 1993). GR converts oxidized glutathione into GSH (Sen, 1997). Therefore, the reduced activity of γGT (specifically in 400 p.p.m. chromium-treated monkeys) and GR (in all groups) observed in the present study suggests decreased degradation as a possible mechanism underlying increased concentration of GSH in chromium-treated monkeys. Presumably, this is a defence mechanism against chromium-induced free radical toxicity. Increased activity of GST in all groups and γGT in monkeys exposed to 50 p.p.m. CrVI (data not shown) also indicates an attempt by the testis to overcome the toxic effect of chromium. GST catalyses the conjugation of the electrophilic xenobiotics to the -SH group of glutathione, and thus increases their water solubility to facilitate excretion (Sen, 1997). Therefore, the enhanced activity of testicular GST observed in chromium-treated monkeys may be a pointer to augmented detoxification of xenobiotic compounds.

Thus, it is clear that testicular tissue tends to overcome the adverse effects of the free radical toxicity induced by chronic exposure to chromium. Spermatogonia and preleptotene spermatocytes appear to be the preferred cell types for such protective measures in the testis, which help the revival of spermatogenesis after the withdrawal of chromium treatment.

Reversibility of the toxic effect of chromium on the testis was vividly reflected in the normal organ weight and histoarchitecture of the testis and normal sperm counts (data not shown here) in monkeys that were kept free of chromium exposure for 6 months. It is well established that it takes about 10 weeks for a spermatozoon to complete testicular and post-testicular maturation and to be ready for ejaculation in monkeys (Amann *et al*., 1976; Shrape, 1994). In fact, normozoospermia was achieved in experimental monkeys in the present study by the end of 3 months after the withdrawal of chromium treatment (Aruldhas *et al*., 2000).

In Wistar rats exposed to chromium water for 30 days, concurrent supplementation with vitamin C or vitamins C and E protected the testis from the adverse effects of chromium, as evident from the normal histoarchitecture and antioxidant enzymes (Subramanian, 2001). Vitamin A and E are also essential for the maintenance of normal spermatogenesis (Huang and Hembree, 1979). Vitamin E deficiency was reported to induce spermatogenic arrest (Bensoussan *et al*., 1998). Therefore, decreased concentrations of vitamins A, E and C in the testes of chromium-treated monkeys might also have contributed to the premature release of germ cells into the lumen. This proposal was ascertained by the finding of the maintenance of normozoospermia in monkeys supplemented with any one of these vitamins while concurrently treated with chromium (data not shown).

Multinucleate giant cells

The multinucleate spermatids appearing in the seminiferous epithelium of chromium-treated monkeys, irrespective of the

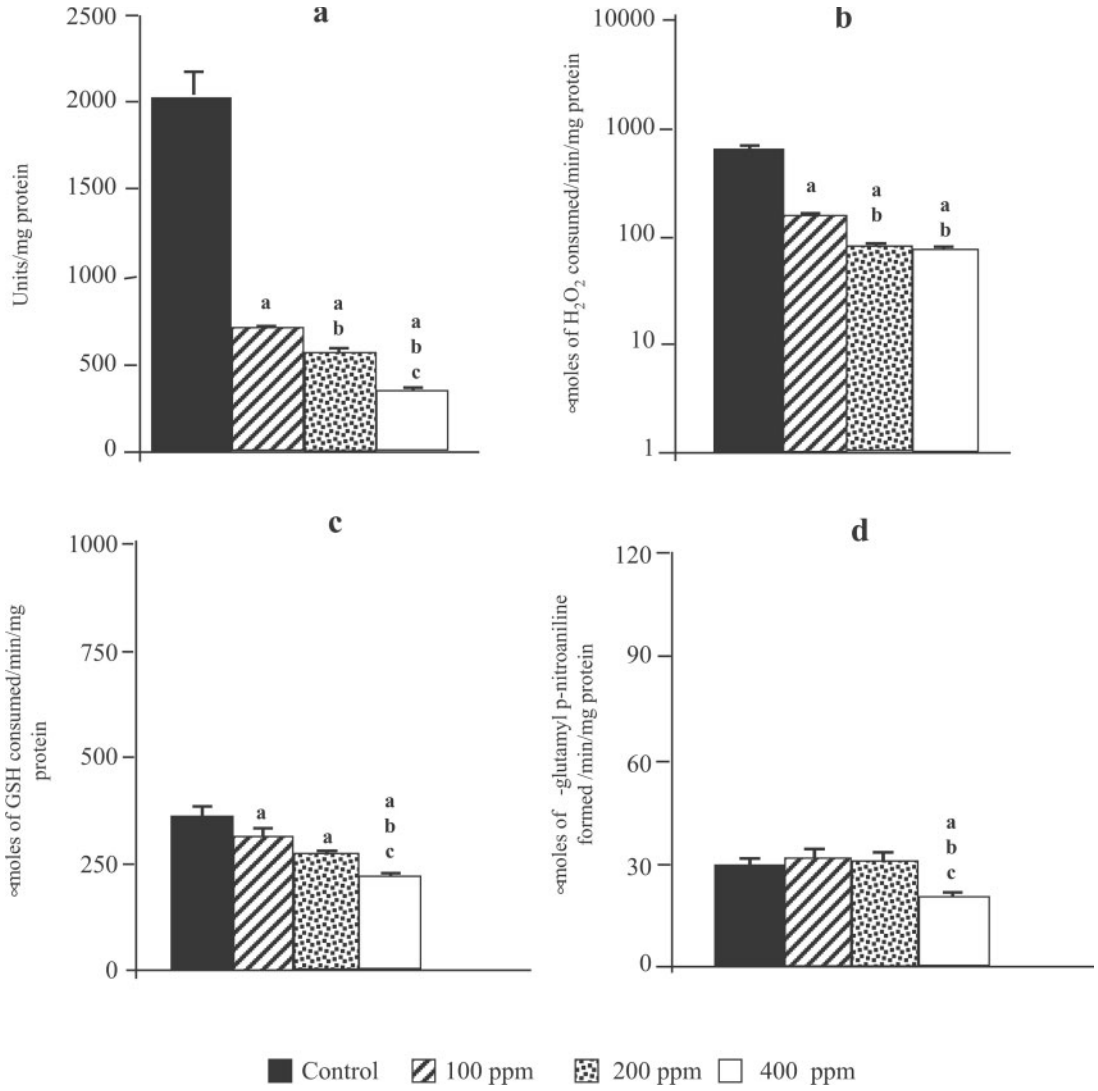

Figure 11. Effect of chronic chromium exposure on the specific activities of testicular superoxide dismutase (a), catalase (b), glutathione peroxidase (c) and γ-glutamyl-transpeptidase (d). Each bar represents the mean and the vertical line above denotes the SEM. $n = 3$. Statistical significance of differences among groups at $P < 0.05$: [a]Control versus experimental; [b]100 p.p.m. vs 200/400 p.p.m.; [c]200 p.p.m. versus 400 p.p.m.

dose, may explain another mechanism of chromium toxicity, independent of its genotoxic effect. It is also important to note that the formation of multinucleated giant cells and the degenerative features described in the present study are common in animals exposed to a wide range of toxic compounds (Russel *et al*., 1990). This may be due to disruption of the cytoplasmic bridges connecting germ cell clones or failure of cytokinesis (Stanley and Akbarsha, 1992). The binucleate spermatids with the cap of the two nuclei remaining fused and the incomplete cytokinesis in a binucleate spermatid, observed in the present study, are clear indications of failure of cytokinesis as the probable mechanism underlying the generation of multinucleate spermatids in the context of chromium toxicity.

Premature loss of germ cells and Sertoli cell toxicity

The results of the present study clearly point to the germ cells present in the adluminal compartment of the seminiferous epithelium as the principal target of chromium toxicity in the testis. The manifestations are clearly pathological and the affected cells have become non-viable or dead and hence do not go through the process of spermatogenesis and/or spermiogenesis. Such cells are lost from any epithelium through either necrosis or apoptosis (Levin *et al*., 1999). The seminiferous epithelium responds to such a situation with premature release of the affected cells, which reach the epididymal duct for further processing and removal (Agnes and Akbarsha, 2001; Aruldhas *et al*., 2004). The TEM observations in the present study suggest occasional necrosis in the testis of chromium-treated monkeys. Though DNA labelling and *in situ* staining by the TUNEL method are the most reliable techniques for the confirmation of apoptosis, marginalization of nuclear chromatin is a morphological indication of apoptosis (Flickinger *et al*., 1999), the mode of cell death caused by chromium (Blankenship *et al*., 1994).

ROS produced during the reduction of CrVI was shown to be responsible for initiation of p53-induced apoptosis (Ye *et al*., 1999). Data on enzymatic and non-enzymatic antioxidants

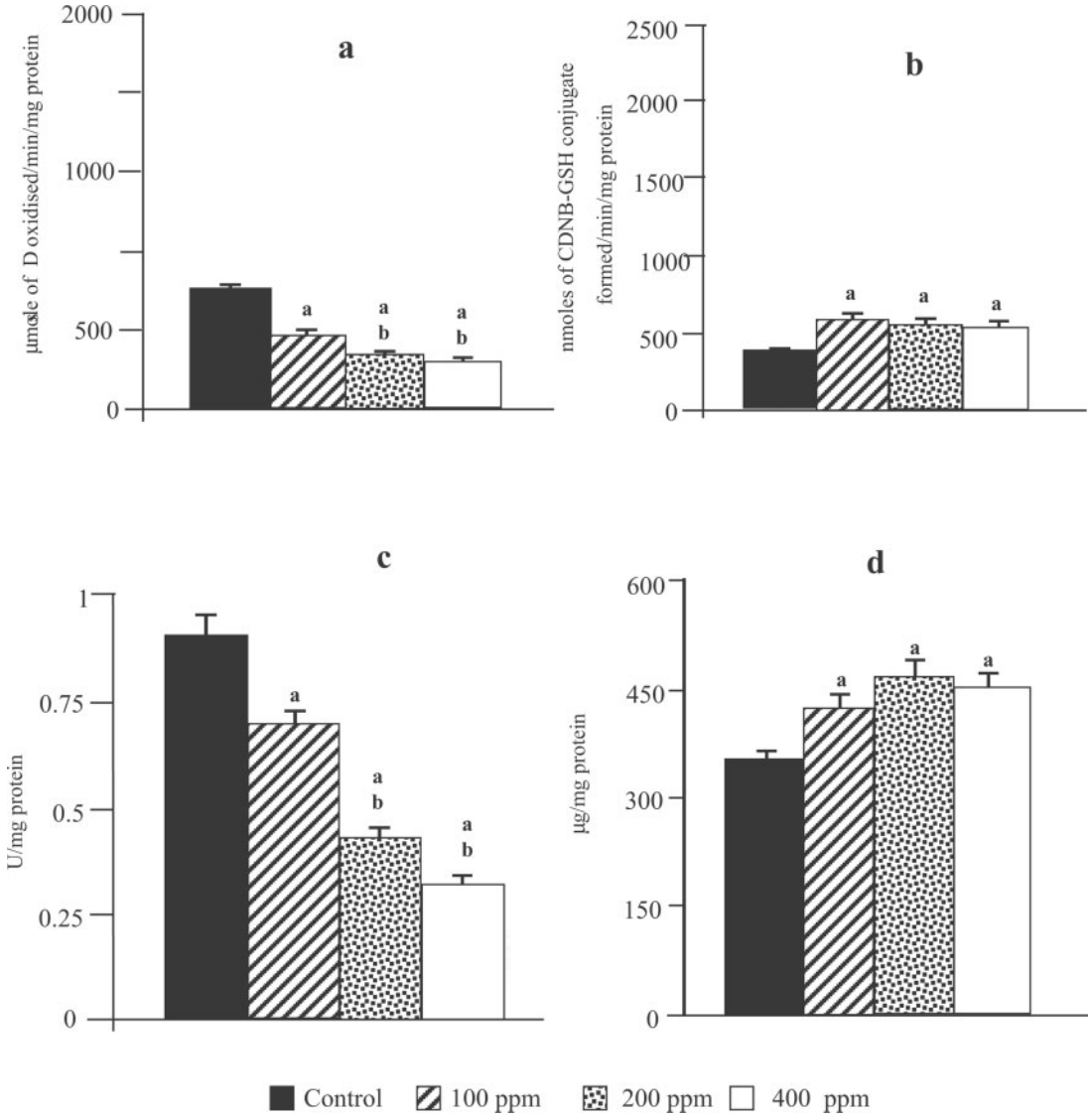

Figure 12. Effect of chronic chromium exposure on the specific activities of testicular glutathione reductase (a), glutathione-S-transferase (b), glucose-6-phosphate dehydrogenase (c) and concentrations of reduced glutathione (d). Each bar represents the mean and the vertical line above denotes the SEM. $n = 3$. Letters a, b and c denote statistical significance of differences among groups at $P < 0.05$: [a]control versus experimental; [b]100 p.p.m. versus 200/400 p.p.m.; [c]200 p.p.m. versus 400 p.p.m.

and free radicals clearly establish the development of oxidative stress in the testis of CrVI-treated monkeys. Therefore, it may be logical to propose that ROS-mediated apoptosis may occur in the testis of CrVI-treated monkeys. This could be another mechanism in the system towards maintenance of a viable epithelium to provide scope for revival of spermatogenesis, apart from the preservation of spermatogonia.

The participation of Sertoli cells in the process of premature loss of germ cells is indispensable. The germ cells in the adluminal compartment are held in position by the characteristic inter-Sertoli cell junctions and the Sertoli cell–germ cell junctions (Bardin *et al.* 1994). These junctional complexes are maintained by cytoskeletal elements and the ectoplasmic specialization of the Sertoli cell cytoplasm (Bardin *et al*., 1994). Once a germ cell has arrived at the adluminal compartment, it is released only at the time of spermiation in the normal course (de Kretser and Kerr, 1994). During toxic manifestations, as in the present case, the Sertoli cells may release the germ cells prematurely, and this would necessarily involve alterations in the junctional complexes and the cytoskeletal framework of the Sertoli cells (de Kretser and Kerr, 1994). When such a response to chromium toxicity is imminent, the Sertoli cell itself becoming a target of the toxicant cannot be ruled out. The changes occurring in the mitochondria of the Sertoli cell and the accumulation of pale, transitional and dark lysosomes in the basal aspect of the Sertoli cell cytoplasm, as observed in the present study, may be reflections of such consequences of chromium toxicity.

One of the important histopathological observations in the present study is that the germ cells of the adluminal compartment alone are irrevocably affected, whereas those in the basal compartment remained unaffected. It is an established fact that

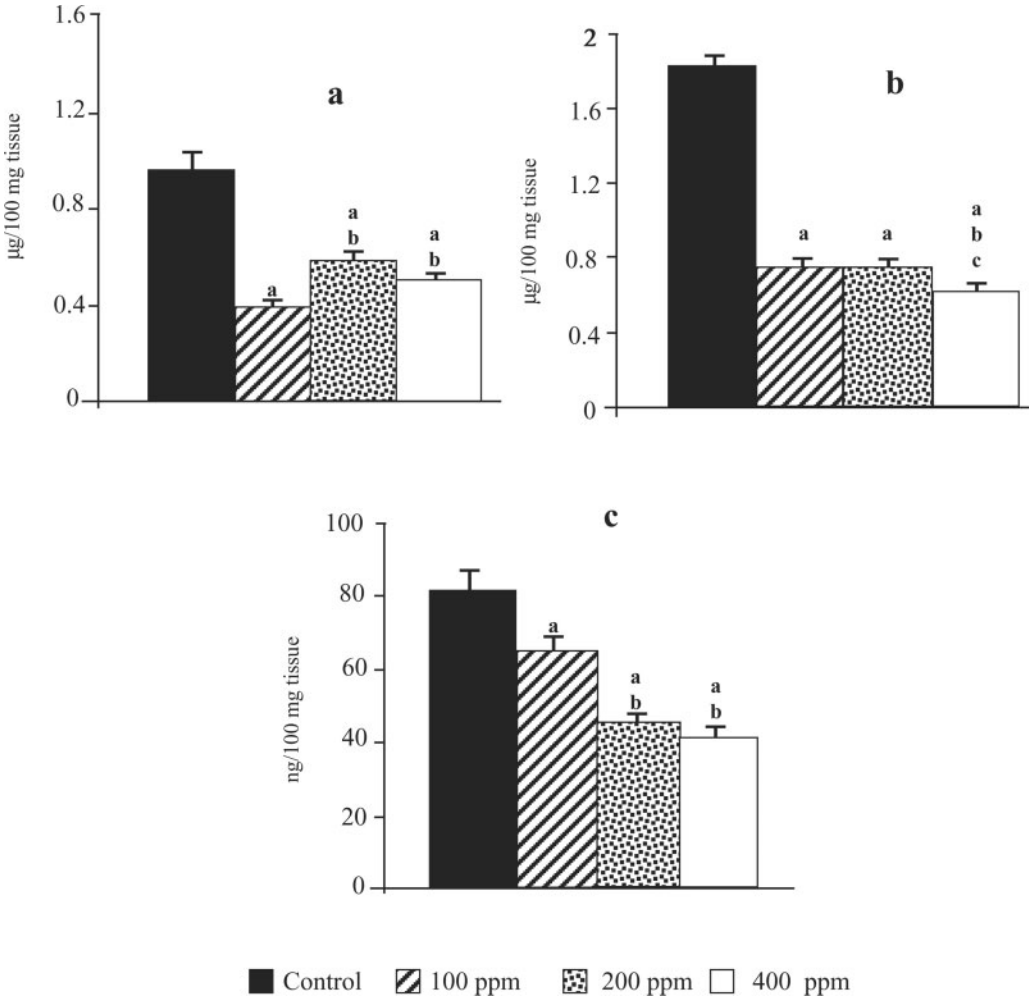

Figure 13. Effect of chronic chromium exposure on testicular concentrations of vitamins C (a), A (b) and E (c). Each bar represents the mean and the vertical line above denotes the SEM. $n = 3$. Letters a, b and c denote statistical significance of differences among groups at $P < 0.05$: [a]control versus experimental; [b]100 p.p.m. versus 200/400 p.p.m.; [c]200 p.p.m. versus 400 p.p.m.

nutritive and/or regulatory substances reach germ cells of the adluminal compartment through the Sertoli cell (Bardin *et al*., 1994), and chromium may also take the same route. Germ cells at different stages of development require specific nutrients, such as lactate, pyruvate, fatty acids and regulatory peptides (de Kretser and Kerr, 1994). Chromium toxicity probably affects the supply of such specific nutrients and/or regulatory substances required by meiotic/postmeiotic germ cells, resulting in their premature death.

Macrophages in the seminiferous epithelium

Because of the thick tunic around the seminiferous tubules and the consequent absence of a direct blood supply (Setchell *et al*., 1994), macrophages cannot enter the seminiferous tubules under normal circumstances (Russell *et al*., 1990). Thus, the present study is unique in reporting macrophages in the seminiferous epithelium of CrVI-treated monkeys. Chromium treatment has probably damaged the blood–testis barrier (Pereira *et al*., 2002), paving the way for the entry of macrophages into the seminiferous tubules. It is an established fact that Sertoli cells phagocytose the residual bodies and the other cell debris under physiological conditions as well as in certain pathological states (Russell *et al*., 1990). The presence of degenerating spermatozoa in vacuoles in the Sertoli cell cytoplasm of chromium-treated monkeys indicates the operation of such a mechanism of cleaning cell debris in the testes of these animals. The presence of macrophages inside the seminiferous tubules may enhance cleansing the damaged cellular materials from the tubules along with the apical portions of the Sertoli cells, as the latter could not assimilate such large volumes of degenerating cells, a situation which can attract macrophages (Russell *et al*., 1991).

Thus, from the present study on the testis and our earlier findings on the epididymis of monkeys subjected to chronic chromium exposure (Aruldhas *et al*., 2004), and in the light of recent reports of poor reproductive health in men experiencing occupational exposure to chromium (Li *et al*., 2001; Danadevi *et al*., 2003), it may be concluded that occupational and/or environmental exposure to CrVI can affect the male reproductive health of primates. Our results support the hypothesis that chromium-induced changes in the histoarchitecture of the testis

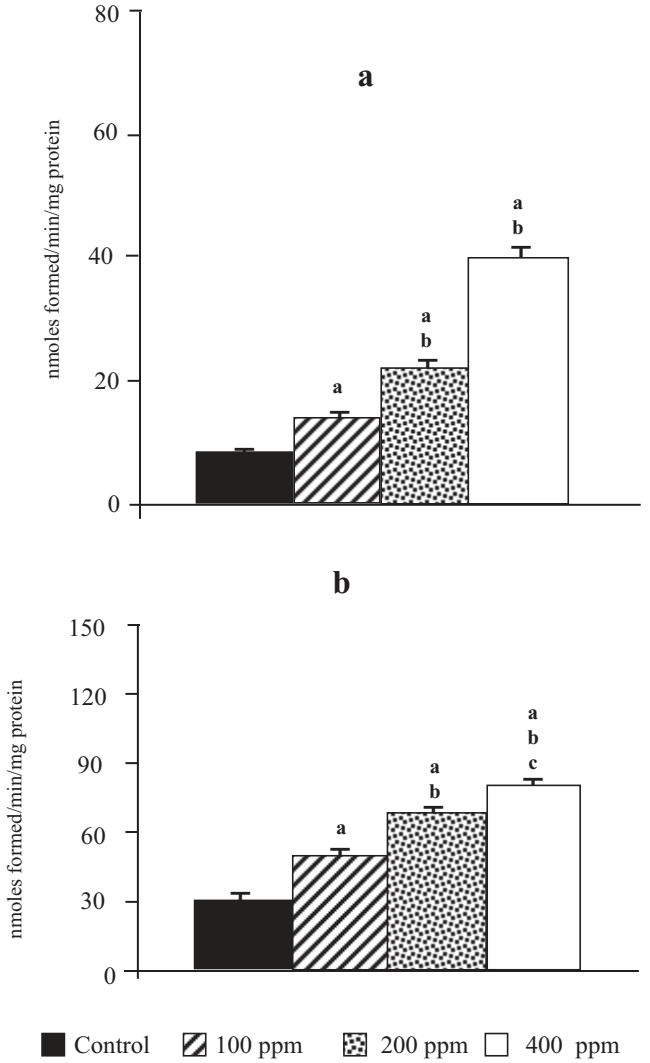

Figure 14. Effect of chronic chromium exposure on testicular hydroxyl radical (a) and hydrogen peroxide (b) concentrations. Each bar represents the mean and the vertical line above denotes the SEM. $n = 3$. Letters a, b and c denote statistical significances of differences among groups at $P < 0.05$: [a]control versus experimental; [b]100 p.p.m. versus 200/400 p.p.m.; [c]200 p.p.m. versus 400 p.p.m.

are due to increased ROS, leading to oxidative stress in the organ, and that these changes can be prevented by supplementation with antioxidants and are reversible. Since antioxidant supplementation has been advocated for infertile men (Irvine, 1996), it becomes likely that the management of reproductive health of men who have occupational exposure to chromium may benefit from supplementation of antioxidants.

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