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Reproductive toxicity of chromium in adult bonnet monkeys (*Macaca radiata* Geoffrey). Reversible oxidative stress in the semen

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Abstract

The present study was designed to test the hypothesis that oxidative stress mediates chromium-induced reproductive toxicity. Monthly semen samples were collected from adult monkeys (*Macaca radiata*), which were exposed to varying doses (50, 100, 200 and 400 ppm) of chromium (as potassium dichromate) for 6 months through drinking water. Chromium treatment decreased sperm count, sperm forward motility and the specific activities of antioxidant enzymes, superoxide dismutase and catalase, and the concentration of reduced glutathione in both seminal plasma and sperm in a dose- and duration-dependent manner. On the other hand, the quantum of hydrogen peroxide in the seminal plasma/sperm from monkeys exposed to chromium increased with increasing dose and duration of chromium exposure. All these changes were reversed after 6 months of chromium-free exposure period. Simultaneous supplementation of vitamin C (0.5 g/L; 1.0 g/L; 2.0 g/L) prevented the development of chromium-induced oxidative stress. Data support the hypothesis and show that chronic chromium exposure induces a reversible oxidative stress in the seminal plasma and sperm by creating an imbalance between reactive oxygen species and antioxidant system, leading to sperm death and reduced motility of live sperm.

Keywords: Chromium; Semen; Oxidative stress; Reative oxygen species; Catalase; Superoxide dismutase

Introduction

The decrease in human semen quality over the past several years is considered to be the result of deteriorating environmental conditions due to increased pollution (Cheek and McLachlan, 1998). Heavy metal pollutants like lead, cadmium and mercury are known to affect human reproductive health (Rodamilans et al., 1988; Skakkebæk et al., 1991). However, the reproductive toxicity of chromium, which is used in more than 50 industries including stainless steel welding, chrome plating, vulcanizing, brewing, leather tanning, paint, cement and ceramics (Morris et al., 1990; Barceloux, 1999; Stupar et al., 1999; Fowler, 2000), is not yet understood clearly. A report on metal workers indicated that stainless steel welders suffer an increased risk of reduced semen quality (Mortensen, 1989). However, a series of reports on stainless steel welders (Jelnes and Knudson, 1988; Bonde, 1990a, 1990b; 1993; Bonde and Ernst, 1992) failed to show any correlation between body chromium concentration and semen quality or fertility. However, a subsequent study on semen quality of metal welders concluded that these negative findings might not apply to populations with high level of exposure to welding fumes or other putative hazards (Hjollund et al., 1998). A recent study has reported a negative correlation between chromium and semen quality (Danadevi et al., 2003). Experimental studies on rodents exposed to different doses of chromium for varying durations strongly suggest an adverse effect of chromium on testicular and epididymal functions, as well as on semen quality (Ernst, 1990; Saxena et al., 1990; Murthy et al., 1991; Ernst and Bonde, 1992). Studies in the author's laboratory have also shown the adverse effect of chromium on male reproduction in rats (Subramanian, 2001). We have also reported an impaired function and histoarchitechture of the testis and epididymis of chromium-treated monkeys (Aruldhas et al., 2004, 2005). The

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observed inconsistency in the reproductive toxicity of chromium among existing clinical and experimental reports may therefore be due to the difference in the mode of intake, dose and duration of chromium exposure.

With this information in mind, the present study has been designed to understand the effect of continuous exposure of chromium on seminal parameters. In order to draw an appropriate conclusion on the probable adverse effects of chromium on human fertility, an animal model close to human being, i.e., a non-human primate, the bonnet monkey (*Macaca radiata* Geoffrey) has been employed in the present study. Since the major routes of exposure to chromium are air, soil and water (Barceloux, 1999), we have used a more appropriate route to expose the experimental animals to chromium, i.e., drinking water.

Chromium can exist in several oxidation states ranging from −2 to +6, of which the trivalent (+3) and hexavalent (+6) forms are of biological importance (Morris et al., 1990). Hexavalent chromium, which exists as an oxyanion (e.g., CrO_4^-), can readily enter the cell compared to the trivalent form. Once inside the cell, the hexavalent form is ultimately reduced to the trivalent form, through the formation of reactive intermediates like pentavalent and tetravalent forms (DeFlora et al., 1990). Hexavalent chromium induces cell injuries including DNA lesions, chromosomal damage, lipid peroxidation and cytotoxicity (Sugiyama, 1992; Shi et al., 1999). Chromium intermediates directly interact with DNA producing DNA–DNA cross-links and DNA–protein cross-links (Misra et al., 1994). Hexavalent chromium can react with H_2O_2 to form pentavalent form and $^{\cdot}OH$ through Fenton-type or Haber–Weiss reactions leading to DNA strand breaks and 8-hydroxy substitutions in DNA (Aiyar et al., 1990). In the present study, we have also made an attempt to understand the putative mechanism by which chromium may induce reproductive toxicity by analyzing the status of various antioxidants and pro-oxidants since oxidative stress due to an imbalance between antioxidants and free radicals has been implicated in various pathological conditions including male infertility (Nakazawa et al., 1996; Sanocka et al., 1996; Sharma and Agarwal, 1996). The involvement of ROS and antioxidants in heavy-metal-induced cell damage has also been clearly shown (Sugiyama, 1994). Finally, the present study also attempts to test the reversibility of chromium toxicity and the prophylactic effect of antioxidant vitamin supplementation along with chromium, on the reproductive toxicity of the latter. The specific hypothesis tested in the present study is "Exposure of mature male monkeys to chromium containing water adversely affects their semen quality through impairment in the oxidant–antioxidant status of the seminal plasma and sperm". It is also hypothesized that the effects of chromium on semen are reversible, and simultaneous supplementation with antioxidant vitamins may have a prophylactic effect on the semen quality.

Materials and methods

The experimental protocol of the present study on a non-human primate animal model (*M. radiata* Geoffrey) was approved by the Institutional Animal Ethical committee for Studies on Experimental Animals and by the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSCA), Ministry of Social Justice and Empowerment, Government of India.

Experimental design. Adult male bonnet monkeys (*M. radiata*) weighing 7–9 kg, trapped by the department of Forest and Wild Life, Government of Tamil Nadu, India for creating public nuisance and kept in captivity, were procured with the permission from Chief Wildlife Warden, Chennai. Animals were kept under quarantine and acclimatized to our animal house condition for 2 months before experimentation. Monkeys were maintained in spacious cages (60 × 60 × 80 cm) individually, under normal 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 standard pellet diet (Brooke Bond India Ltd), cooked rice with lentils and vegetables like carrot, potatoes, beetroots and fresh fruits available in the season (banana, guava). Experimental monkeys were given different concentrations of hexavalent chromium (50-, 100-, 200- and 400-ppm chromium, as potassium dichromate) through drinking water for 6 months. The maximum concentration of chromium employed is 400 ppm since increasing the concentration of chromium beyond 400 ppm caused reduced consumption of food and water leading to death within 3 months of treatment. One batch of monkeys exposed to 400-ppm chromium was given simultaneous vitamin C supplementation (0.5 g/L or 1.0 g/L or 2.0 g/L) for 6 months. Another batch of monkeys exposed to 400-ppm chromium for 6 months was left free of chromium treatment for a further period of 6 months to study the reversibility of chromium toxicity. Monthly semen samples were collected by gently rubbing the penis with fingers, which arouse the animal sexually, leading to erection and ejaculation. The technician used separate sterile gloves, while handling each animal. This procedure was practiced to avoid any discomfort to the animal. Sperm and seminal plasma were separated, after assessing sperm count and sperm forward motility, by centrifugation at 3000×*g* for 15 min. Spermatozoa were resuspended in physiological saline at a concentration of 100 × 10^6 cells/ml. Spermatozoa were then lysed by hypotonic treatment (0.4% saline for 30 min) and centrifuged at 6000×*g* for 20 min. The supernatant was used for various assays (Keyhani and Storey, 1973).

Plasma chromium. Blood samples were collected at 1-month intervals, plasma separated and the concentration of plasma chromium was estimated by atomic absorption spectrophotometry. The values were expressed as μg/mL. All the laboratory wares used for blood collection and processing were soaked overnight in analytical grade nitric acid and washed three times with deionized water.

Sperm count and straight line velocity (VSL). The concentration of sperm in the semen was quantified by using Neubauer's chamber (WHO, 1992). The time taken by a spermatozoon to travel 20 oculometer divisions was noted using a stopwatch. An average of 25 sperm/sample were studied, and the mean was calculated and expressed as micrometer traveled per second.

Antioxidant enzymes. Superoxide dismutase (SOD) (E.C. No. 1.15.11) was assessed by the colorimetric method of Sinha (1972). Catalase (E.C. No. 1.15.1.6) activity was estimated by the standard colorimetric method of Marklund and Marklund (1974). SOD activity is expressed as units/ml of seminal plasma and units/10^6 sperm. Catalase activity is expressed as amount of H_2O_2 consumed/min/ml of seminal plasma and amount of H_2O_2 consumed/min/10^6 sperm.

Non-enzymatic antioxidant. Reduced glutathione (GSH) concentration in sperm and seminal plasma was also quantified colorimetrically (Moren et al., 1979). The concentration of GSH is expressed as μg/ml of seminal plasma and μg/10^6 sperm.

Reactive oxygen species. The hydrogen peroxide (H_2O_2) production was assessed by the spectrophotometric method of Holland and Storey (1981). H_2O_2 concentration is expressed as μmol/ml of seminal plasma and μmol/10^6 sperm.

Statistical analysis. Data from monkeys treated with different doses of chromium were subjected to two-way analysis of variance followed by Student–Newman–Keul's (SNK) test to assess the time- and duration-dependent effect of

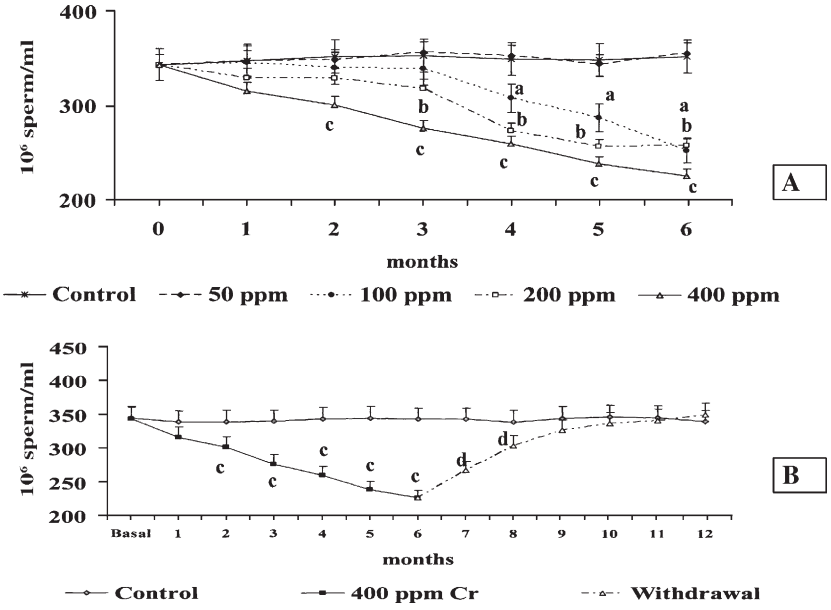

Fig. 1. Concentration of spermatozoa in monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Animals were exposed to different concentrations of chromium, as potassium dichromate, through drinking water for 6 months. Each value is mean ± SEM. $n = 3$. a, b, c and d denote statistical significance at $P < 0.05$. a—basal vs. 100 ppm; b—basal vs. 200 ppm; c—basal vs. 400 ppm; d—400 ppm vs. withdrawal.

chromium. Data from animals subjected to withdrawal regime and vitamin C co-supplementation were analyzed using one-way analysis of variance and SNK test. The statistical evaluations were performed using the computer package SPSS.

Results

Sperm count

Sperm count was unaltered in monkeys exposed to 50-ppm chromium, whereas higher doses of chromium dwindled the same in a duration- and dose-dependent manner. Daily treatment with 100-ppm chromium decreased the sperm concentration significantly (~11%) by the end of the fourth month. While 200-ppm chromium decreased the sperm count by the third month of exposure (~13%), 400-ppm chromium decreased the same by the end of the second month (~13%) (Fig. 1A). At the end of the sixth month, there was a 25% reduction in 100- and 200-ppm-chromium-treated rats and 30% reduction in 400-ppm-chromium-treated rats. By 1 month of withdrawal of chromium treatment, an increase in sperm count was noticed and normality was achieved by the third month of withdrawal (Fig. 1B). Simultaneous vitamin C supplementation to chromium (400 ppm)-treated monkeys from the beginning of the experiments prevented the reduction in sperm count. All the three doses of vitamin C were equally effective in preventing chromium-induced changes, and therefore only data from the highest dose employed are shown (Table 1).

Straight line velocity (VSL)

Like sperm count, sperm motility was also unaltered in monkeys given 50-ppm chromium for 6 months. On the other hand, sperm motility decreased in those monkeys provided with higher concentrations of chromium in a duration- and dose-dependent manner. Decreased sperm motility became significant by the end of 3 months of 100-ppm chromium exposure (~10%), by the third month in 200-ppm-chromium-treated group (~15%) and by the second month in those monkeys given water containing 400-ppm chromium (~12%) (Fig. 2A). By the

Table 1
Effect of vitamin C supplementation along with 400-ppm chromium through drinking water for 6 months on seminal sperm count and sperm forward motility

	Basal	1 month	2 months	3 months	4 months	5 months	6 months
Sperm count (10^6 sperm/ml)							
Control	343.57 ± 19.65	348.15 ± 21.24	352.19 ± 10.10	353.11 ± 9.56	349.92 ± 12.10	348.76 ± 6.54	352.34 ± 10.64
400-ppm chromium	343.57 ± 19.65	315.48 ± 9.58	301.15 ± 8.97[a]	276.037 ± 7.77[a]	259.703 ± 8.07[a]	238.3 ± 7.21[a]	226.02 ± 6.01[a]
400-ppm chromium + vitamin C 2.0 g/L	343.57 ± 19.65	342.25 ± 6.58	348.68 ± 7.86	351.09 ± 8.41	351.54 ± 7.24	349.67 ± 6.54	350.21 ± 8.24
Sperm forward motility (μm traveled/s)							
Control	264 ± 4.97	263.10 ± 5.12	264.50 ± 3.26	262.19 ± 6.59	267.70 ± 4.56	265.18 ± 3.26	268.19 ± 2.56
400-ppm chromium	264 ± 4.97	234.5 ± 10.55	232 ± 8.42[a]	213.5 ± 11.01[a]	168.3 ± 8.11[a]	160.5 ± 4.93[a]	154.6 ± 7.21[a]
400-ppm chromium + vitamin C 2.0 g/L	264 ± 4.97	259.97 ± 4.51	264.35 ± 2.45	263.18 ± 3.16	264.65 ± 4.26	258.65 ± 5.21	270.06 ± 4.69

Each value is mean ± SEM, $n = 3$.
[a] Statistical difference compared to control at $P < 0.05$.

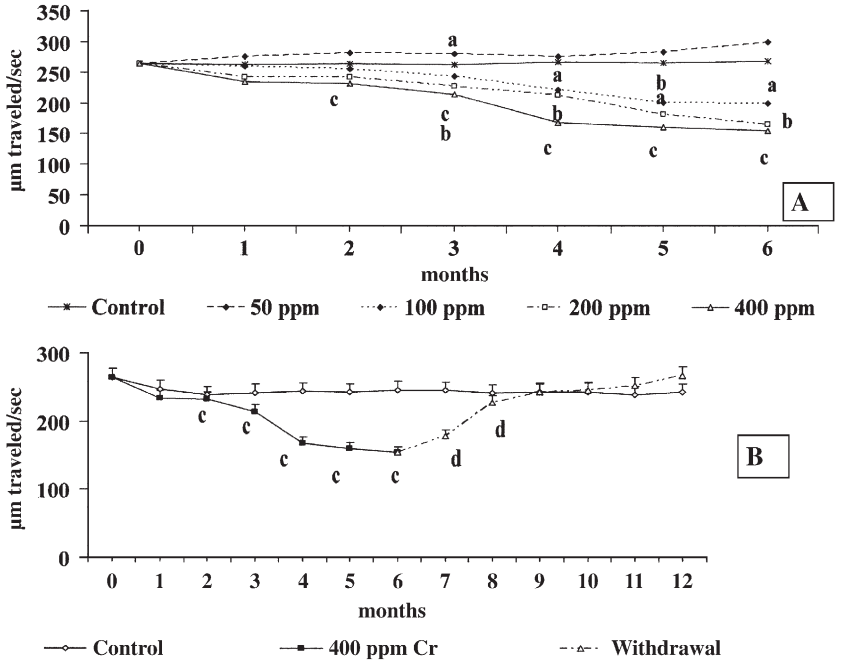

Fig. 2. Straight-line velocity of spermatozoa in monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Explanations are the same as given under Fig. 1.

end of the sixth month, the reduction observed in 100-, 200- and 400-ppm chromium was ~25, 20 and 35%, respectively. Withdrawal of chromium treatment restored normal sperm motility by the third month of chromium-free period (Fig. 2B). Chromium-induced suppression of sperm motility was prevented by simultaneous supplementation of vitamin C. No significant dose-dependent effect was evident among the three doses of vitamin C employed, and therefore only data from the highest dose employed are shown (Table 1).

Superoxide dismutase (SOD)

SOD activity in seminal plasma and sperm was not altered in monkeys exposed to 50-ppm chromium whereas, higher

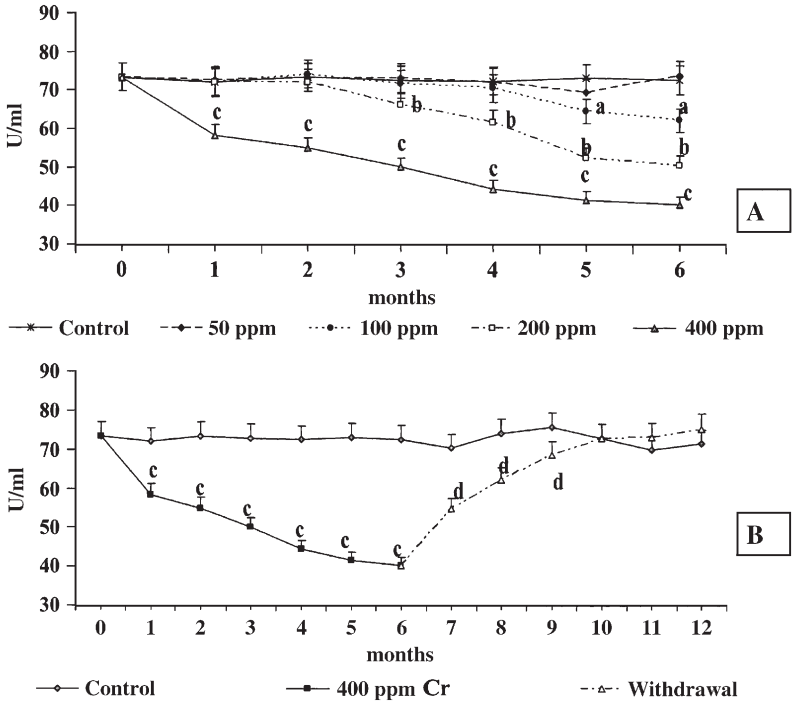

Fig. 3. Superoxide dismutase activity in the seminal plasma of monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Explanations are the same as given under Fig. 1.

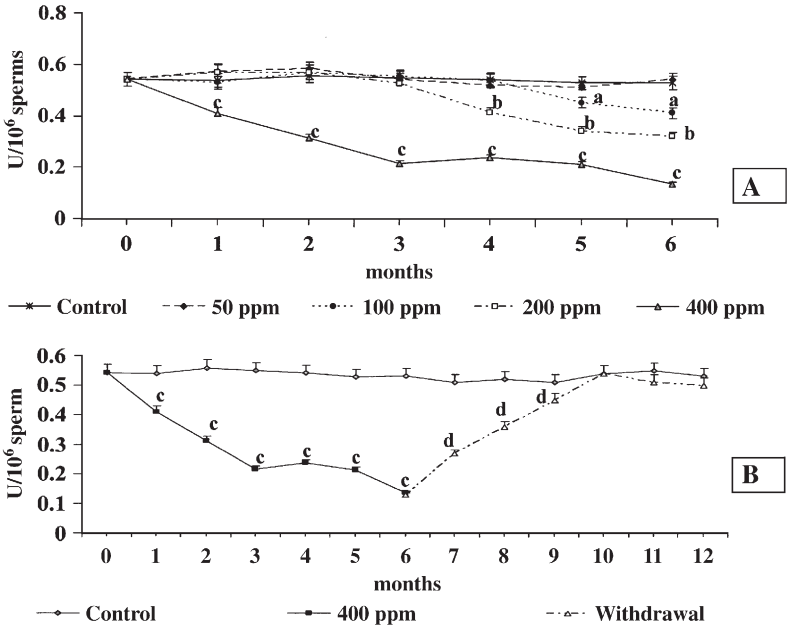

Fig. 4. Superoxide dismutase activity in spermatozoa of monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Explanations are the same as given under Fig. 1.

doses suppressed the same. Treatment with 100-ppm chromium caused a significant suppression of SOD activity in the seminal plasma (~12%) and sperm (~16%) by the end of 5 months. While 200-ppm chromium diminished seminal plasma SOD activity by the third month of exposure (~10%), sperm from these monkeys recorded a diminution (~20%) only at the end of the fourth month. However, 400-ppm chromium decreased the enzyme activity in both the seminal plasma (~20%) and sperm (~25%) by the first month (Figs. 3A, 4A). At the end of 6 months of exposure, there was a reduction of about 15%, 30% and 45% in seminal plasma of 100-, 200-, and 400-ppm-chromium-treated monkeys, respectively. In sperm from these monkeys, the reduction was ~25%, 40% and 75%, respectively. Normal SOD activity was restored in the seminal plasma and sperm after 4 months of post-chromium-exposure-free period (Figs. 3B, 4B). Vitamin

Table 2
Effect of vitamin C supplementation along with 400-ppm chromium through drinking water for 6 months on the activities of superoxide dismutase (SOD) and catalase

	Basal	1 month	2 months	3 months	4 months	5 months	6 months
Seminal plasma SOD (U/mg protein)							
Control	73.38 ± 1.25	71.98 ± 2.13	73.46 ± 2.5	72.77 ± 1.64	72.46 ± 3.56	73.19 ± 2.46	72.67 ± 2.35
400-ppm chromium	73.38 ± 1.25	58.363 ± 3.24[a]	54.967 ± 4.87[a]	50.08 ± 3.99[a]	44.463 ± 4.87[a]	41.657 ± 3.9[a]	40.37 ± 3.77[a]
400-ppm chromium + vitamin C 2.0 g/L	73.38 ± 1.25	75.29 ± 3.1	74.68 ± 2.1	71.26 ± 1.95	72.29 ± 3.12	71.18 ± 3.24	72.48 ± 2.45
Sperm SOD (U/10^6 cells)							
Control	0.54 ± 0.012	0.54 ± 0.023	0.56 ± 0.018	0.55 ± 0.164	0.542 ± 0.031	0.529 ± 0.017	0.53 ± 0.015
400-ppm chromium	0.543 ± 0.012	0.41 ± 0.028[a]	0.313 ± 0.02[a]	0.217 ± 0.022[a]	0.237 ± 0.017[a]	0.213 ± 0.015[a]	0.137 ± 0.011[a]
400-ppm chromium + vitamin C 2.0 g/L	0.54 ± 0.012	0.52 ± 0.024	0.54 ± 0.026	0.53 ± 0.027	0.51 ± 0.019	0.54 ± 0.031	0.51 ± 0.032
Seminal plasma catalase (μmol of H_2O_2 consumed/min/mg protein)							
Control	0.684 ± 0.031	0.682 ± 0.025	0.673 ± 0.036	0.679 ± 0.024	0.673 ± 0.025	0.678 ± 0.036	0.672 ± 0.027
400-ppm chromium	0.684 ± 0.031	0.648 ± 0.042	0.63 ± 0.037[a]	0.604 ± 0.051[a]	0.58 ± 0.044[a]	0.534 ± 0.043[a]	0.513 ± 0.031[a]
400-ppm chromium + vitamin C 2.0 g/L	0.684 ± 0.031	0.682 ± 0.025	0.695 ± 0.028	0.685 ± 0.029	0.684 ± 0.041	0.695 ± 0.032	0.976 ± 0.029
Sperm catalase (μmol of H_2O_2 consumed/min/10^6 cells)							
Control	0.051 ± 0.002	0.052 ± 0.002	0.051 ± 0.002	0.053 ± 0.002	0.052 ± 0.002	0.055 ± 0.0016	0.052 ± 0.0024
400-ppm chromium	0.051 ± 0.002	0.044 ± 0.003[a]	0.037 ± 0.002[a]	0.0293 ± 0.001[a]	0.0213 ± 0.003[a]	0.0163 ± 0.002[a]	0.0133 ± 0.001[a]
400-ppm chromium + vitamin C 2.0 g/L	0.051 ± 0.002	0.05 ± 0.002	0.053 ± 0.002	0.051 ± 0.002	0.049 ± 0.002	0.05 ± 0.0015	0.052 ± 0.0019

Each value is mean ± SEM, $n = 3$.
[a] Statistical difference compared to control at $P < 0.05$.

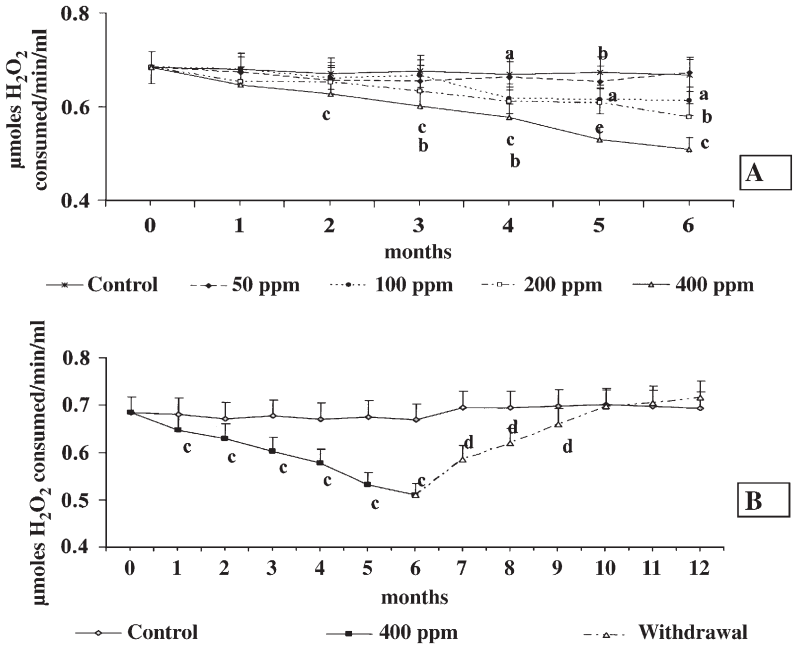

Fig. 5. Catalase activity in the seminal plasma of monkeys exposed to chromium for 6 months (A) and withdrawn of chromium treatment (B). Explanations are the same as given under Fig. 1.

C supplementation countered the adverse effect of chromium on seminal plasma and sperm SOD activity. No significant dose-dependent effect was observed among the three doses of vitamin C, and therefore only data from the highest dose employed are shown (Table 2).

Catalase

Treatment with 50-ppm chromium did not evoke any change in seminal plasma or sperm catalase activity. In monkeys exposed to 100-ppm chromium, the catalase activity in the

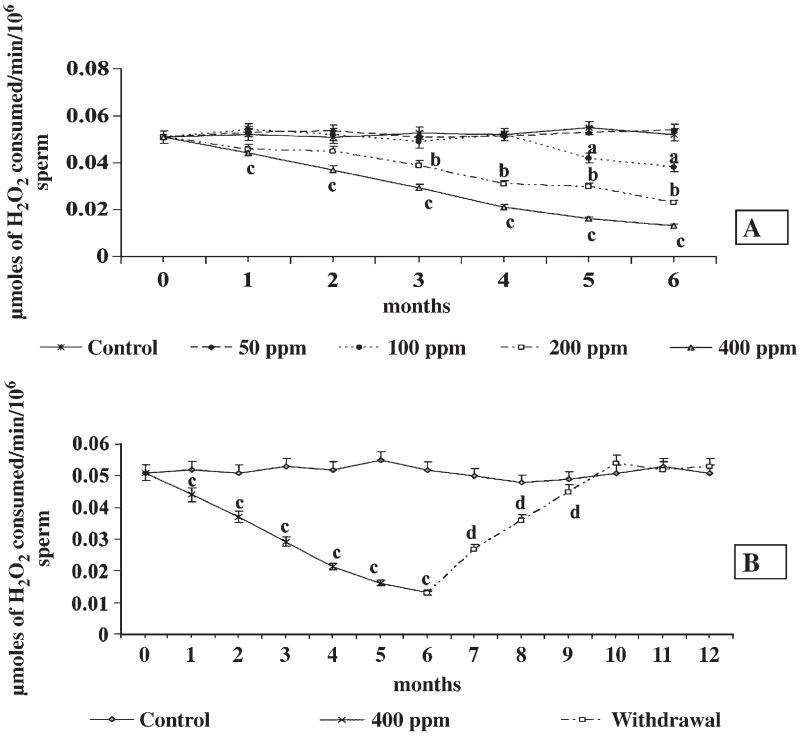

Fig. 6. Catalase activity in spermatozoa of monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Explanations are the same as given under Fig. 1.

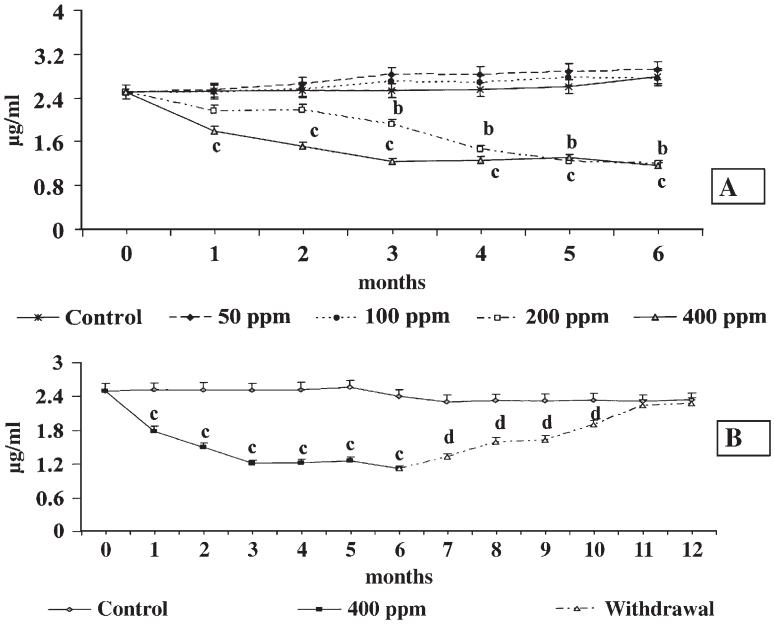

Fig. 7. Concentration of reduced glutathione in the seminal plasma of monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Explanations are the same as given under Fig. 1.

seminal plasma and in sperm was suppressed by the fourth month (10%) and fifth month (17%), respectively. However, decreased catalase activity in seminal plasma (~9%) and sperm (~25%) was observed by the end of 3 months in those monkeys treated with 200-ppm chromium. A similar trend was evident in seminal plasma (~9%) catalase activity by the second month and by the end of the first month in the sperm (~27%) from 400-ppm-chromium-treated monkeys (Figs. 5A, 6A). After 6 months of chromium exposure, the catalase activity in the sperm and seminal plasma from 100-, 200- and 400-ppm-chromium-treated rats was reduced to ~10%, 15% and 25%, and 25%, 55% and 74%, respectively. Withdrawal of chromium treatment increased the catalase activity in both seminal plasma and sperm at the end of 1 month, and normality was attained after 4 months of chromium-free period (Figs. 5B, 6B). Chromium-induced reduction in seminal plasma and sperm catalase activity was prevented by simultaneous vitamin C supplementation. No dose-dependent effect was observed

Table 3
Effect of vitamin C supplementation along with 400-ppm chromium through drinking water for 6 months on the reduced glutathione (GSH) concentration and hydrogen peroxide

	Basal	1 month	2 months	3 months	4 months	5 months	6 months
Seminal plasma GSH (µmol/ml)							
Control	2.499 ± 0.16	2.510 ± 0.16	2.500 ± 0.06	2.480 ± 0.08	2.488 ± 0.14	2.520 ± 0.14	2.350 ± 0.14
400-ppm chromium	2.499 ± 0.16	1.774 ± 0.07 [a]	1.486 ± 0.06 [a]	1.183 ± 0.07 [a]	1.195 ± 0.06 [a]	1.222 ± 0.1 [a]	1.067 ± 0.09 [a]
400-ppm chromium + vitamin C 2.0 g/L	2.499 ± 0.16	2.169 ± 0.09	2.194 ± 0.12	2.194 ± 0.04	2.324 ± 0.21	2.214 ± 0.16	2.167 ± 0.08
Sperm GSH (µmol/10^6 cells)							
Control	2.956 ± 0.012	2.987 ± 0.06	2.976 ± 0.05	2.899 ± 0.1	2.921 ± 0.15	2.989 ± 0.05	3.011 ± 0.08
400-ppm chromium	2.956 ± 0.012	2.093 ± 0.15	1.85 ± 0.07 [a]	1.617 ± 0.07 [a]	1.322 ± 0.08 [a]	1.0053 ± 0.04 [a]	0.854 ± 0.05 [a]
400-ppm chromium + vitamin C 2.0 g/L	2.956 ± 0.012	2.919 ± 0.08	2.894 ± 0.012	2.896 ± 0.0.14	3.015 ± 0.09	2.915 ± 0.14	2.998 ± 0.015
Seminal plasma H_2O_2 (µmol/min/ml)							
Control	7.19 ± 0.32	6.97 ± 0.24	7.1 ± 0.19	7.09 ± 0.09	6.97 ± 0.11	7.11 ± 0.07	7.01 ± 0.23
400-ppm chromium	7.19 ± 0.32	7.813 ± 0.33 [a]	7.887 ± 0.21 [a]	8.21 ± 0.18 [a]	8.54 ± 0.38 [a]	8.733 ± 0.21 [a]	8.92 ± 0.19 [a]
400-ppm chromium + vitamin C 2.0 g/L	7.19 ± 0.32	7.17 ± 0.26	7.17 ± 0.26	7.19 ± 0.18	7.18 ± 0.08	7.16 ± 0.11	7.24 ± 0.08
Sperm H_2O_2 (µmol/min/10^6 cells)							
Control	3.91 ± 0.05	3.93 ± 0.08	3.99 ± 0.04	4.01 ± 0.04	3.99 ± 0.03	4.04 ± 0.12	4.07 ± 0.13
400-ppm chromium	3.91 ± 0.05	3.8 ± 0.08	3.867 ± 0.05	4.083 ± 0.04 [a]	4.413 ± 0.02 [a]	4.66 ± 0.02 [a]	4.73 ± 0.05 [a]
400-ppm chromium + vitamin C 2.0 g/L	3.91 ± 0.05	3.85 ± 0.02	3.783 ± 0.03	3.77 ± 0.08	3.95 ± 0.11	3.84 ± 0.03	3.79 ± 0.04

Each value is mean ± SEM, $n = 3$.
[a] Statistical difference compared to control at $P < 0.05$.

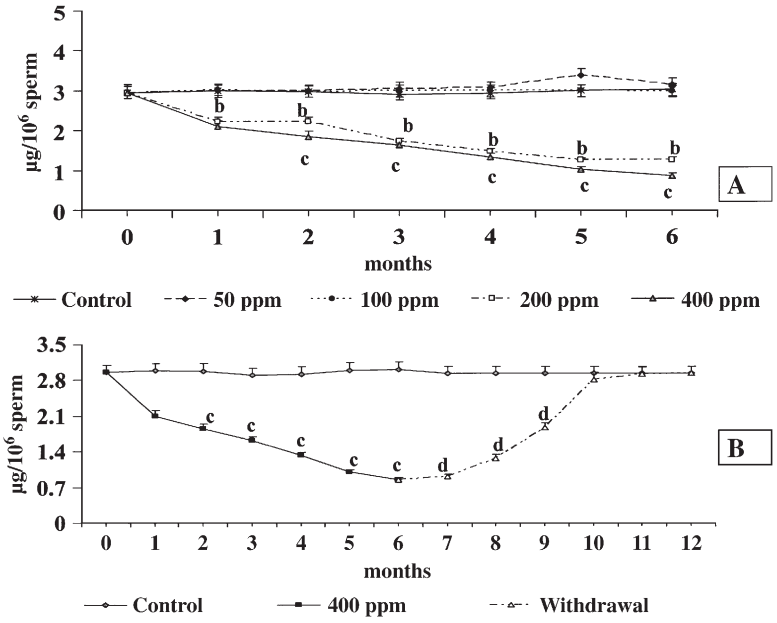

Fig. 8. Concentration of reduced glutathione in spermatozoa of monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Explanations are the same as given under Fig. 1.

among the three doses of vitamin C used, and therefore only data from the highest dose employed are shown (Table 2).

Glutathione reduced (GSH)

GSH concentration remained unchanged in both sperm and seminal plasma of monkeys exposed to 50- or 100-ppm chromium. On the other hand, higher concentrations of chromium (200, 400 ppm) diminished GSH content. Treatment with 200-ppm chromium in the drinking water decreased GSH concentration in seminal plasma (~25%) and sperm after 3 and 1 month (~25%) of exposure, respectively. However, 400-ppm chromium caused a diminution in seminal plasma GSH by the first month (~30%) and in sperm (~30%) by the end of second month (Figs. 7A, 8A). At the end of 6 months of chromium exposure, GSH concentration in 200- and 400-ppm-chromium-

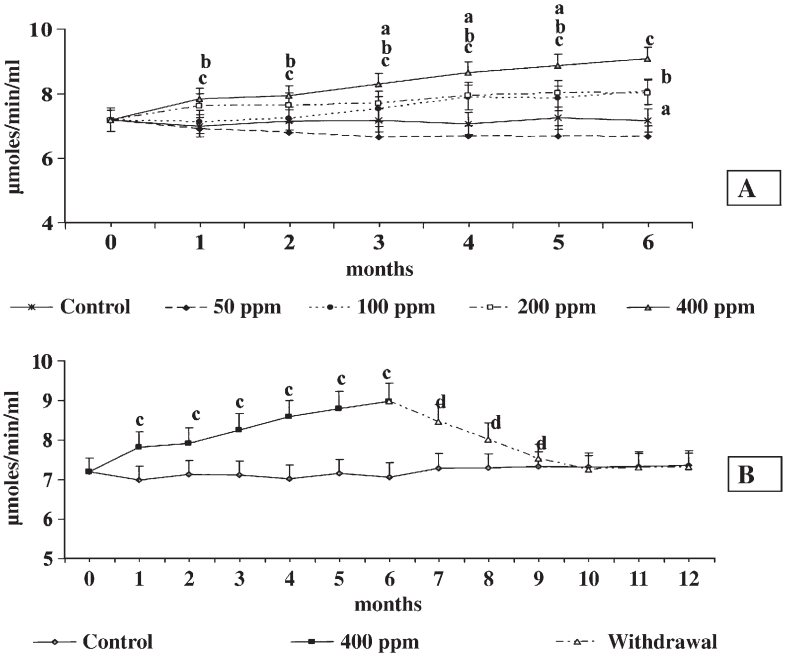

Fig. 9. Hydrogen peroxide production in the seminal plasma of monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Explanations are the same as given under Fig. 1.

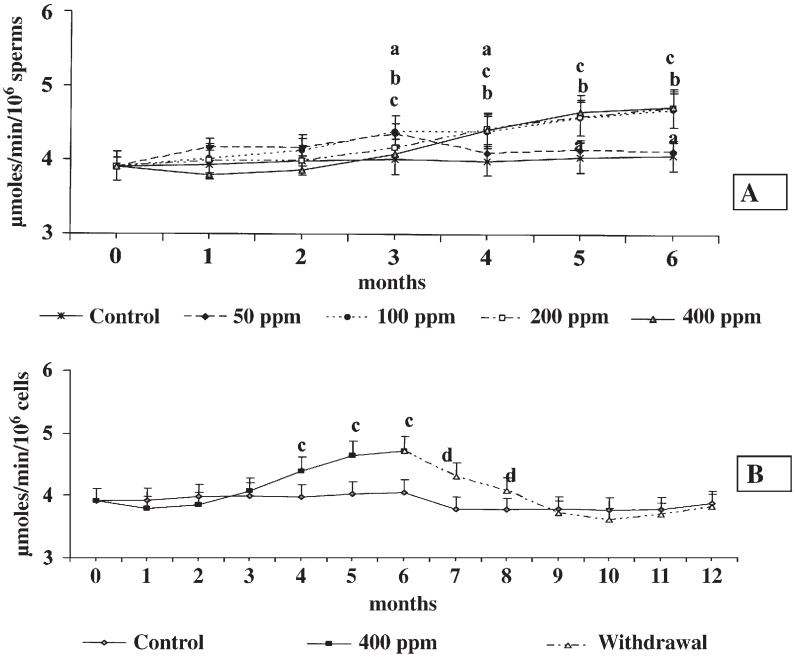

Fig. 10. Hydrogen peroxide production in spermatozoa of monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Explanations are the same as given under Fig. 1.

treated rats was reduced ~55% in the seminal plasma and ~55% and 70%, respectively, in sperm. Withdrawal of chromium treatment increased GSH concentration in both seminal plasma and sperm when compared to that of monkeys exposed to 400-ppm chromium and restored normality after 5 months in seminal plasma and 4 months in the sperm (Figs. 7B, 8B). Vitamin C supplementation along with chromium prevented the decrease in GSH concentration in seminal plasma and sperm, without any significant dose-dependent effect, and therefore only data from the highest dose employed are shown (Table 3).

Hydrogen peroxide (H_2O_2)

Exposure of animals to 50-ppm chromium failed to induce any change in H_2O_2 concentration in the seminal plasma or sperm, while 100-ppm chromium increased H_2O_2 production by the end of the third month in both seminal plasma (~6%) and

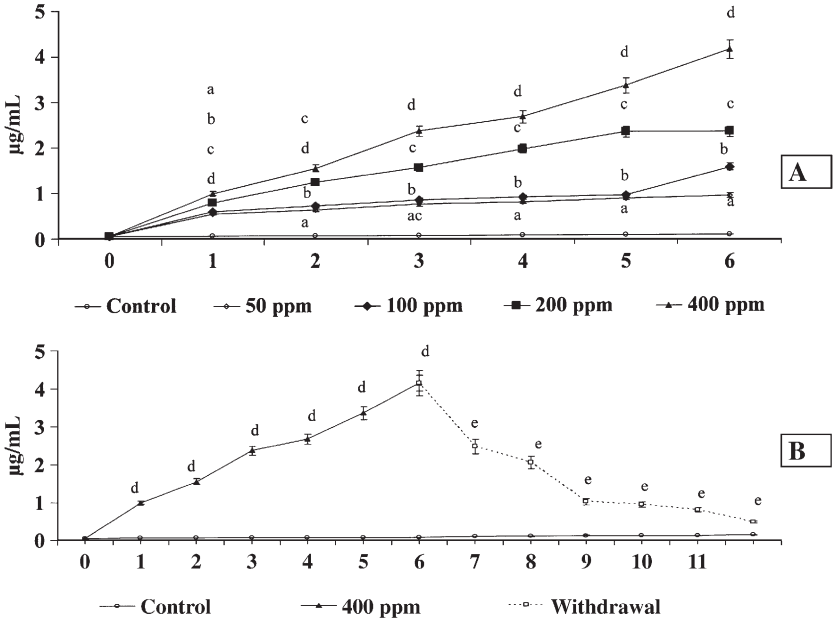

Fig. 11. Plasma chromium concentration in monkeys exposed to chromium for 6 months (A) and after withdrawal of chromium treatment (B). Animals were exposed to different concentrations of chromium, as potassium dichromate, through drinking water for 6 months. Each value is mean ± SEM. $n = 3$. a, b, c, d and e denote statistical significance at $P < 0.05$. a—basal vs. 50 ppm; b—basal vs. 100 ppm; c—basal vs. 200 ppm; d—basal vs. 400 ppm; e—400 ppm vs. withdrawal.

Table 4
Effect of vitamin C supplementation along with 400-ppm chromium through drinking water for 6 months on plasma chromium content

	Basal	1 month	2 months	3 months	4 months	5 months	6 months
Plasma chromium (μg/mL)							
Control	0.046 ± 0.002	0.051 ± 0.002	0.048 ± 0.001	0.048 ± 0.005	0.055 ± 0.006	0.049 ± 0.003	0.051 ± 0.002
400-ppm chromium	0.046 ± 0.002	0.987 ± 0.057 [a]	1.542 ± 0.098 [a]	2.35 ± 0.093 [a]	2.654 ± 0.078 [a]	3.331 ± 0.127 [a]	4.121 ± 0.333 [a]
Vitamin C 2.0 g/L	0.046 ± 0.002	0.248 ± 0.008 [b]	0.401 ± 0.009 [b]	0.447 ± 0.003 [a, b]	0.489 ± 0.006 [a, b]	0.554 ± 0.012 [a, b]	0.574 ± 0.004 [a, b]

Each value is mean ± SEM, $n = 3$.
[a] Statistical difference compared to control.
[b] Statistical difference compared to 400-ppm chromium treatment at $P < 0.05$.

sperm (~12%). On the other hand, both 200- and 400-ppm chromium increased the same in the sperm (~6%) by 3 months of exposure, whereas in seminal plasma the increase was observed after 1 month of exposure (~6 and 9%, respectively) (Figs. 9A, 10A). At the end of 6 months, H_2O_2 concentration in the seminal plasma of 100-, 200- and 400-ppm-chromium-treated monkeys was ~10, 10 and 25%, respectively, and ~20% in sperm from 100-, 200- and 400-ppm-chromium-treated monkeys. Restoration of normal level of H_2O_2 in sperm was attained after 3 months of the withdrawal period, whereas it was achieved after 4 months of the withdrawal period in the case of seminal plasma (Figs. 9B, 10B). Supplementation of vitamin C was able to prevent chromium-induced increase in H_2O_2 concentration in the seminal plasma and sperm. All three doses were equally effective in masking the effect of chromium, and therefore only data from the highest dose employed are shown (Table 3).

Plasma chromium concentration

Plasma chromium showed an increase from the first month of exposure. The increase was ~12-fold in 50- and 100-ppm-chromium-treated monkeys, and ~17- and 20-fold respectively, in 200- and 400-ppm-chromium-treated groups (Fig. 11A). After 6 months of chromium exposure, the plasma chromium increased ~20-, 34-, 50- and 90-fold, respectively, in 50-, 100-, 200- and 400-ppm-chromium-treated monkeys. Withdrawal of chromium treatment decreased plasma chromium from the second month onwards, but normal level of plasma chromium was not restored even after 6 months of chromium withdrawal (Fig. 11B). In vitamin C co-administered monkeys, a significant decrease in chromium concentration was observed from the second month onwards. However, the level remained higher than that of untreated controls (Table 4). As there was no dose-dependent difference, only data from the highest dose employed are shown.

Discussion

Data on plasma chromium concentration, which revealed a dose-dependent increase, attest the uptake and accumulation of chromium in monkeys exposed to chromium through drinking water and validate the route of exposure employed in the present study. The results also reveal that simultaneous vitamin C supplementation decreases the bioaccumulation of chromium as evidenced from the reduced chromium concentration in the plasma of animals given vitamin C together with chromium. Chromium concentration in the seminal plasma/sperm could have given a better picture about the status of chromium accumulation within the reproductive tract organs of the exposed animals. However, this could not be estimated in the present study due to practical difficulties.

The reduction in sperm count and forward motility observed in monkeys exposed to chromium clearly indicates the male reproductive toxicity of chronic chromium exposure. The reproductive toxicity of chromium has been underplayed, despite a few experimental studies since welders occupationally exposed to chromium did not show any change in the semen quality. Recently, Hjollund et al. (1998) have concluded that the absence of any effect due to chromium exposure on semen quality in welders may not be applicable to long-term exposure. The present long-term study in a non-human primate attests the reproductive toxicity of chronic chromium exposure. Data from the present investigation also reveal enhanced oxidative stress in the seminal plasma/sperm from monkeys chronically exposed to chromium and support the hypothesis that oxidative stress mediates reproductive toxicity of chromium.

Oxidative stress due to an imbalance between pro- and antioxidant levels may initiate several metabolic and functional dysregulations, eventually leading to cell death (Aitken, 1989). Oxidative stress can be induced either by enhanced production of ROS or by suppression of the antioxidant defense system (Gutteridge and Halliwell, 1994). In the semen of chromium-treated monkeys of the present study, oxidative stress might have developed through both mechanisms. This could be evidenced from the suppressed activities of SOD, catalase and decreased GSH concentration concomitant with increased H_2O_2 production in seminal plasma and sperm from these monkeys. SOD is the antioxidant enzyme, which was severely affected under chromium exposure in this study. This could be due to the direct effect of chromium as chromium has been shown to have the ability to directly bind to SOD (Yamakura and Suzuki, 1980).

The increase in the H_2O_2 production under chromium treatment may have caused an enhanced lipid peroxidation in sperm membrane as reported in the case of intestinal epithelial cells, brain, liver and kidney tissues (Sengupta et al., 1990; Rungby and Ernst, 1992; Bagchi et al., 1995, 1997; Travacio et al., 2000). We have recently shown an elevated oxidative stress in the testis of chromium-treated monkeys caused by an imbalance between the pro-oxidant and antioxidant systems of the testicular tissue (Aruldhas et al., 2005). In another study, we

have also shown increased lipid peroxidation in the testis of adult rats exposed to different doses of chromium for 1 month (Subramanian, 2001). On the other hand, GSH supplementation has been shown to have a protective action against seminal plasma lipid peroxidation (Brezezinska-Slebodzinska et al., 1995), and GSH treatment has been implicated in the treatment of male infertility (Irvine, 1996). Hence, the observed reduction in the GSH concentration in seminal fluid and sperm of monkeys exposed to chromium treatment might have lead to enhanced oxidative stress in the semen.

Mammalian spermatogonial membrane is rich in highly unsaturated fatty acids and is sensitive to oxygen-induced damage mediated by lipid peroxidation (Tramer et al., 1998). It was reported five decades ago that radical/reactive oxygen species (ROS) could be toxic to sperm (MacLeod, 1943; Iwasaki and Gagnon, 1992). Human spermatozoa are known to be susceptible to lipid peroxidation because of the high concentration of unsaturated fatty acids (Jones et al., 1979). In human sperm, ROS generation induces lipid peroxidation by initiating a chain reaction within the membrane followed by propagating the damage throughout the cell (Aitken and Fisher, 1994). Generation of ROS and peroxidation of sperm membrane can bring about mid-piece abnormalities and impair motility, sperm capacitation and sperm–oocyte fusion (Aitken, 1997; de Lamarinde et al., 1997; Kim and Parthasarathy, 1998). Lipid peroxidation has been suggested as the cause of abnormal acrosome reaction and loss of membrane fluidity culminating in the loss of fertilizing potential of spermatozoa (Verma and Kanwar, 1999). High concentrations of H_2O_2 have been shown to affect sperm capacitation through modifying oxidative process (Oehninger et al., 1995).

On the other hand, spermatozoa are protected from the deleterious effects of ROS and oxidative stress by various enzymatic and non-enzymatic antioxidants in the seminal plasma and sperm per se (Tramer et al., 1998). A recent in vitro study has shown that the addition of seminal plasma to sperm subjected to exogenous ROS production (by incubating them with H_2O_2, in the presence of ferrous sulfate and ADP) produced a significant decrease in DNA strand breaks and lipid peroxidation induced by ROS (Potts et al., 2000). Alkan et al. (1997) reported an increase in the concentrations of ROS in the seminal plasma of infertile men and suggested that decreased seminal plasma antioxidant activity and increased reactive oxygen species could be responsible for idiopathic male infertility.

A significant reduction in the activities of seminal plasma SOD and catalase has been reported in infertile men (Sanocka et al., 1996). Recently, a significant decrease in GSH content in the spermatozoa of infertile men has been reported (Oschsendorf et al., 1998). Oxidative stress in the seminal plasma of infertile men, due to subnormal antioxidant status, as measured by the formation of thiobarbituric reactive substances (TBRS), has also been reported (Lewis et al., 1995; Pasqualotto et al., 2000). Thus, the role of ROS in male infertility is proved beyond doubt and excessive generation of ROS by spermatozoa has been identified as one of the few defined etiologies for male infertility (Sharma and Agarwal, 1996; Sikka, 1996; Pasqualotto et al., 2000). Therefore, it is logical to conclude that chromium-induced reduction in sperm count and motility is the result of oxidative stress in the testis and semen.

The decreased sperm count noticed in this study in chromium-treated monkeys may be attributed to subnormal spermatogenesis. Testicular histology of these animals showed adverse effect of chronic chromium treatment with loss of germ cells from the seminiferous epithelium and accumulation of cell debris in the lumen (Aruldhas et al., 2005). It takes about 10 weeks for a spermatozoon produced in the testis of a monkey to be ready for ejaculation from the vas deferens (Harper, 1994; Sharpe, 1994). We have also observed impaired pro-oxidant and antioxidant balance in the testis of these monkeys treated with various doses of chromium (Aruldhas et al., 2005). Data from the present study reveal that it takes a minimum of 2 months to visualize the adverse effect of the maximum effective dose of chromium on sperm count. This study also shows that the adverse effect of exposure to chromium containing water on semen quality is reversible after chromium is replaced from the drinking water.

It has been reported that lead-induced oxygen toxicity in rat sperm can be prevented by vitamin C administration (Hsu et al., 1998). Our study reveals that it is also possible to preempt the reproductive toxicity of chromium by simultaneous supplementation of vitamin C, in spite of chromium accumulation in the system. Vitamin C is a known cellular reductant that reacts with hexavalent and converts it into trivalent chromium. Thus, enhanced neutralization of hexavalent chromium by vitamin C may be the underlying mechanism behind the protective effect of ascorbic acid. The decrease in total chromium content in the blood plasma may be due to decreased bioaccumulation of chromium, under vitamin C co-administration.

Taken together, the present study provides evidence for the reproductive toxicity of chronic chromium exposure, the intensity of which may vary depending upon the dose and duration of chromium exposure. Chromium-induced oxidative stress in the semen may be the cumulative effect of increased ROS production and decreased antioxidant defense system in the reproductive tract organs like testis and epididymis. Chromium-induced reproductive toxicity is reversible, and the toxicity may be prevented by simultaneous supplementation of antioxidant vitamins.

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