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Effect of Cr(VI) Exposure on Sperm Quality: Human and Animal Studies

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The semen status of male workers occupationally exposed to hexavalent chromium(VI) was investigated. Sperm counts from exposed workers were 47.05±2.13×10⁶/ml and those from control group 88.96±3.40×10⁶/ml. Sperm motility decreased from 81.92±0.41% for the control group to 69.71±0.93% for the exposed workers. The levels of zinc, lactate dehydrogenase (LDH), and lactate dehydrogenase C4 isoenzyme (LDH-x) in seminal plasma for the exposed workers were 1.48±0.07 μmol/ml, 1.05±0.02×10³ U, and 0.47±0.01×10³ U, respectively, which were significantly lower than those of 5.72±0.15 μmol/ml, 1.49±0.02×10³ U, and 0.78±0.15×10³ U for the control group, respectively. Follicle stimulating hormone (FSH) (7.34±0.34×10⁻³ IU/ml) in serum from the exposed workers was significantly higher than that (2.41±0.08×10⁻³ IU/ml) from the control group. On the other hand, there were no significant differences in semen volume, semen liquefaction time, luteinizing hormone (LH) level in serum, and Cr concentration in both serum and seminal plasma between the exposed workers and the control group. Feeding Cr(VI) to rats significantly reduced the epididymal sperm counts from 87.40±3.85×10⁶/g epididymis in control group to 21.40±1.20×10⁶/g epididymis at a CrO_3 dose of 10 mg/kg body weight and to 17.48±1.04×10⁶/g epididymis at a CrO_3 dose of 20 mg/kg body weight. Exposure of rats to Cr(VI) also significantly increased the sperm abnormality from 2.75±0.06% in the control group to 6.68±0.32% in the exposed group at a CrO_3 dose of 10 mg/kg body and to 7.6±0.15% at a CrO_3 dose of 20 mg/kg body weight. In exposed rats, there was visible disruption in germ cell arrangement near the walls of the seminiferous tubules. The diameters of seminiferous tubules in exposed rats were smaller. These results suggest that occupational exposure to chromium(VI) leads to alteration of semen status and may affect the reproductive success of exposed workers. © **2001 British Occupational Hygiene Society. Published by Elsevier Science Ltd. All rights reserved**

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INTRODUCTION

Cr(VI)-containing compounds are major environmental contaminants with human exposure occurring through air, food, and water (Anon, 1990). Epidemiological studies have shown that workers exposed to Cr(VI) had a higher incidence of respiratory cancer than the general population. Cr(VI) compounds also induced tumors in experimental animals at the injection and implantation sites (Langard, 1990; Norseth, 1981; Tandon *et al.*, 1978; Tsapakos *et al.*, 1993). For example, intrabronchial implantation of Cr(VI) compounds in rats resulted in bronchial carcinomas, whereas intramuscular implantations and intrapleural and subcutaneous injection in rats and mice led to injection site sarcomas. Cr(VI) has been shown to induce chromosomal aberration, mutations, and transformation in cultured mammalian cells (De Flora *et al.*, 1990; De Flora and Wetterhahn, 1989; Sugiyama

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et al., 1992) and a variety of DNA lesions such as strand breaks, DNA protein cross-links, and DNA base modification (Shi *et al.*, 1992, 1994, 1999a,b; Standeve and Wetterhahn, 1991).

Epidemiological studies have shown significant correlations between paternal exposure to chemical carcinogens and incidence of childhood cancers, including brain tumors (Savitz and Chen, 1990). This association has been established for parental occupational exposures to metals (Olsen and Chen, 1991; Tomatis, 1994). An increased risk of cancer has been observed among offspring of fathers employed in subsections of the iron and metal industries (Fabia *et al.*, 1974; Hemminki *et al.*, 1981; Lowengart *et al.*, 1987). Little or no information is available to evaluate the potential association between parental occupational exposure to Cr(VI) and risk for cancer in offspring. A recent study has shown that paternal exposure to Cr(III) is accompanied by an increased incidence of aging-associated neoplastic and non-neoplastic abnormalities (We *et al.*, 1999). It has been reported that stainless steel welders suffered an increased risk of reduced sperm quality (Mortensen, 1962). This increased risk is attributed to the smoke generated by welding of stainless steel. Welding fumes contain a number of heavy metals, of which chromium is found in high concentrations. Chromate has been shown to concentrate in the testes following intraperitoneal injection (Vittorio *et al.*, 1962). Seminiferous epithelial injuries were observed after intratesticular as well as subcutaneous injections of chromium chloride in mice (Kamboj and Kar, 1964). Possible incorporation of chromium into sperm has been suggested. In our recent studies (Shi *et al.*, 1999a,b), we have demonstrated that Cr(VI) can be reduced by cellular reductants to generate reactive chromium intermediates, such as Cr(V) and Cr(IV). These reductants include ascorbate, glutathione, and glutathione reductase. During the Cr(VI) reduction processes, molecular oxygen is reduced to superoxide radical ($O_2^{\bullet -}$), which then forms H_2O_2 by dismutation. Both Cr(V) and Cr(IV) are able to react with H_2O_2 to generate hydroxyl radical ($^{\bullet}OH$) through Fenton-like reactions. $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$ are collectively called reactive oxygen species (ROS). These species can cause various cellular injuries, including DNA damage, lipid peroxidation, and protein modification. It is likely that through reactions mediated by ROS, Cr(VI) may cause damage to the reproductive system and affect semen status.

The major goals of the present study were (a) to investigate the effect of occupational Cr(VI) exposure on semen status, such as seminal volume, sperm counts, and sperm motility, on the levels of LH, LDH, zinc, and Cr in seminal plasma, and on the levels of LH, FSH, and Cr(V) in serum; (b) to examine the effect of Cr(VI) on epididymal sperm number, the rate of sperm abnormalities, and seminiferous tubule morphology in rats exposed to Cr(VI).

MATERIALS AND METHODS

Human studies

Cr(VI) exposed workers. Twenty one workers exposed to Cr(VI) in electroplating factory in Henan, China for 1–15 yr were recruited for this study. The exposed workers were 30.24±4.56 yr old. The control group workers from the same factory were not exposed to any harmful chemicals and was 28.58±3.29 yr old. There was no statistically significant difference between exposed group and control group on average age. The protocol of the human studies was approved by the University board of Henan Medical University, China.

Collection of semen samples and preparation. The participants were given written and oral instructions on how to avoid contamination of semen samples. The semen samples were collected (preferably 5 days after sexual abstinence) into acid-washed glass test tubes. Aliquots of semen samples were analyzed immediately and remaining samples were centrifuged at 2000 rpm for 15 min at room temperature. The supernatants (seminal fluid) were stored at 4°C and analyzed within 4 weeks.

General studies of semen samples. Semen volumes were measured with graduated test tubes. For measurements of liquefaction time, the test tubes with semen samples were incubated in water baths at 37°C and the liquefaction time was recorded. For measurements of sperm motility, 0.1 ml liquefied semen sample and 0.1 ml 0.5% eosin were mixed and pipetted onto a glass plate. The sperm motility was observed after 30 min under the microscope. For sperm counts, 20 μl liquefied semen sample was added to a test tube containing 380 μl 5% $NaHCO_3$ solution. After mixing, the spermatozoa were counted under the microscope.

Collection of blood. All blood samples were taken by venipuncture (elbow). The samples were then transferred to a water bath at 37°C, incubated for 60 min, and were centrifuged 2000 rpm for 15 min at room temperature. The supernatant clear serum were stored at 4°C and analyzed within 4 weeks.

Chromium in serum and in seminal fluid. Cr concentrations were measured by a combination of a graphite furnace (Model GF-4A, Made in Japan)/an atomic absorbance spectrophotometer (AA-670, made in Japan).

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) in serum. Serum FSH and LH were determined by radioimmunoassay. The measurements were carried out as described below. (a) Tube T (total count), non-specific tube (NSB), standard tubes A(zero), B, C, D, E, F, G, and sample tubes were prepared for the test. (b) Added 200 μl zero standard solution to the NSB and A tubes. Added 200 μl FSH

standardized solution to tubes B to G at FSH concentrations from A to G of 0, 0.8, 2.1, 4.1, 8.2, 41 ($\times 10^{-3}$ U/ml), respectively. (c) Added 200 µl serum to the sample tubes. (d) Added 100 µl FSH antiserum to all tubes except T and NBS tubes. (e) After mixing, incubated all the tubes in a water bath at 37°C for 120 min. (f) Added 100 µl [^{125}I]FSH and mixed. (g) Incubated the tubes in a water bath at 37°C for another 120 min. (h) Added 1 ml cold, sedimentation fluid to each tube. After mixing, the samples were centrifuged at 3000*g* for 15 min. (i) The supernatants were discarded and the sediments were counted for 1 min using a gamma counter. The results were quantitated using a standard curve.

Zinc in seminal fluid. The measurements were carried out as described below. (a) Added 0.5 ml water (blank), 0.5 ml zinc standard, and 0.5 ml diluted seminal plasma (diluted with distilled water 1:200) to a test tube. (b) Chromogen solution of 2.5 ml (prepared by mixing color reagent A and B in the ratio in 4:1) was added to each tube. (c) The mixture was mixed well and allowed to stand at room temperature for 5 min. The samples were measured within an hour at 560 nm using a blank as a reference.

LDH and LDH-x in seminal fluid. LDH and LDH-x activities in seminal fluid were determined after a 1:4 dilution by a colorimetric method. One unit of LDH activity equals to the amount of enzyme that catalyzes the reduction of 1.0 µmol of pyruvate to L-lactate in one minute at 37°C).

Animal studies

Reagents and animals. All reagents were purchased from Chemical Reagent Factory (Tanjin, China). Sixty-day-old male Wistar rats were purchased from Experimental Animal Center of Henan Medical University (Zhenzhou, China), and were acclimatized for 6 days. All animals were housed at 50±10% humidity and an ambient temperature of 20±1°C. The rats were divided into three groups ($N = 8-11$ per group). The animals were given 0 mg/kg(control), 10 mg/kg and 20 mg/kg CrO_3 per day by oral feeding daily for six days. The rats were sacrificed after 6 weeks and both testes and epididymides were removed immediately and analyzed.

Sperm counts of epididymis. The tail of epididymis was weighed and cut into pieces, which were transferred to a test tube containing 2 ml sperm counting liquid. After mixing, the spermatozoa were counted under the microscope.

Sperm abnormality in rats. Sperm suspension was first pipetted onto the glass plate, dried, fixed in methanol for 5 min and stained with 0.5% eosin. The morphologic characteristics of spermatozoa were evaluated under a light microscope. A minimum of 500 spermatozoa were observed per rat and the level of sperm abnormality was calculated.

Histological evaluation of testis in rats. After fixation in 10% formaldehyde, the testis was embedded in paraffin and sectioned. The slices were stained with eosin and hematoxylin and were evaluated under a light microscope and morphologic abnormalities and size dimension of seminiferous tubules were recorded.

RESULTS

Human studies

Effects of Cr(VI) exposure on seminal volume and liquefaction time are shown in Table 1. The seminal volume was 3.24±0.07 ml ($N = 21$) for the exposed group and 3.59±0.10 ml ($N = 22$) for the control group. There was no significant difference between exposed group and control group. The liquefaction time was 32.81±0.76 min ($N = 21$) for the exposed group and 30.90±0.86 min ($N = 22$) for the control group. The differences between these two groups was not significant.

Effects of Cr(VI) exposure on sperm counts and sperm motility are shown in Table 1. The sperm counts were $47.05\pm2.13\times10^6$/ml ($N = 21$) for the exposed group and $88.96\pm3.40\times10^6$/ml ($N = 22$) for the control group. The difference between these two groups was significant ($P<0.05$). The sperm motility was 69.71±0.93% ($N = 21$) for exposed workers, which was significantly lower than that of 81.92±0.41% ($N = 22$) for the control group ($P<0.05$).

Effects of Cr(VI) exposure on levels of LH, FSH and Cr in serum are shown in Table 2. The level of LH in serum was $6.33\pm0.16\times10^{-3}$ IU/ml ($N = 20$) for exposed workers and $6.85\pm0.30\times10^{-3}$ IU/ml ($N = 21$) for the control group. The difference was not significant. On the other hand, the FSH concentration of $7.34\pm0.34\times10^{-3}$ IU/ml ($N = 20$) in serum for the exposed group was significantly higher than that of $2.41\pm0.08\times10^{-3}$ IU/ml ($N = 20$) for the control group ($P<0.05$). The Cr concentration in serum was $1.40\pm0.01\times10^{-3}$ µmol/ml ($N = 20$) for the exposed group and $1.26\pm0.02\times10^{-3}$ µmol/ml ($N = 13$) for controls. The difference between the two groups was not significant.

Effects of Cr(VI) exposure on levels of zinc, LDH-x, LDH in seminal fluid are shown in Table 3. The zinc concentration in the seminal fluid of 1.48±0.07 µmol/ml ($N = 20$) for exposed workers was significantly lower than that of $5.72\pm0.15\times10^{-3}$ µmol/ml ($N = 19$) for controls ($P<0.05$). The Cr concentration in the seminal fluid was $7.55\pm0.06\times10^{-3}$ µmol/ml ($N = 18$) for the exposed group and $2.41\pm1.69\times10^{-3}$ µmol/ml ($N = 4$) for the control group. The difference was not significant. The concentrations of LDH-x and LDH were $0.47\pm0.01\times10^{-3}$ U ($N = 21$) and

Table 1. Parameter analysis from semen obtained from Cr(V) exposed workers (x±SE)

	Number of samples	Seminalvolume (ml)	Liquefactiontime (min)	Sperm counts(10^6/ml)	Spermmotility (%)
Exposed group	21	3.24±0.07	32.81±0.76	47.05±2.13*	69.71±0.93*
Control group	22	3.59±0.10	30.90±0.86	88.96±3.40	81.92±0.41

*$P<0.05$

Table 2. Concentrations of LH, FSH, and Cr in serum of workers occupationally exposed to Cr(VI) (x±SE)

	LH (IU/ml)	FSH (IU/ml)	Cr (serum μmol/ml)
Exposed group	$6.33\pm0.16\times10^{-3}$ $N=20$	$7.34\pm0.34\times10^{-3}$* $N=20$	$1.40\pm0.01\times10^{-3}$ $N=21$
Control group	$6.85\pm0.30\times10^{-3}$ $N=21$	$2.41\pm0.08\times10^{-3}$ $N=21$	$1.26\pm0.02\times10^{-3}$ $N=13$

*$P<0.01$

Table 3. Concentrations of zinc (μmol/ml), Cr (μmol/ml), LDH-x (U/ml), and LDH (U/ml) in seminal fluid of workers occupationally exposed to Cr(VI) (x±SE)

	Zinc(μmol/ml)	Cr (μmol/ml)	LDH-x (U/ml)	LDH (U/ml)
Exposed group	1.48±0.07* $N=20$	$7.55\pm0.06\times x10^{-3}$ $N=18$	$0.47\pm0.01\times10^{-3}$* $N=21$	$1.05\pm0.02\times10^{-3}$* $N=21$
Control group	5.72±0.15 $N=19$	$6.38\pm1.06\times10^{-3}$ $N=4$	$0.78\pm0.0.15\times10^{-3}$ $N=21$	$1.49\pm0.02\times10^{-3}$ $N=21$

*$P<0.05$

Table 4. Effect of Cr(VI) on epididymal sperm number in rats (x±SE)

CrO_3 dose (mg/kg)	Number of rats	Sperm counts (sperm count $\times10^6$/g epididymis)
0	9	87.40±3.85
10	8	21.40±1.20*
20	11	17.48±1.04*

*$P<0.05$

$1.05\pm0.02\times10^{-3}$ U ($N=21$), respectively, for the exposed group and $0.78\pm0.15\times10^{-3}$ U ($N=20$) and $1.49\pm0.02\times10^{-3}$ U ($N=21$), respectively, for the control group. These differences were significant ($P<0.05$).

Animal studies

Effects of Cr(VI) on epididymal sperm count in rats are shown in Table 4. The epididymal sperm number was $87.40\pm3.85\times10^6$/g epididymis ($N=9$) for control rats. The sperm number decreased significantly in exposed groups depending on the dose of Cr(VI) exposure. At doses of 10 and 20 mg/kg, the sperm counts were $21.40\pm1.20\times10^6$/g epididymis ($N=8$) and $17.48\pm1.04\times10^6$/g epididymis ($N=9$), respectively. The differences between the exposed groups and control group were significant ($P<0.05$).

Effects of Cr(VI) on sperm abnormality in rats are shown in Table 5. The rates of abnormality of 6.68±0.32% and 7.6±0.15% for rats exposed to 10 and 20 mg/kg CrO_3, respectively, were significantly higher than that of 2.75±0.06% for the control group ($P<0.01$).

Effects of Cr(VI) on seminiferous tubules are shown in Fig. 1. Figure 1(A) shows the photomicrograph at 100× magnification of a testis section from a control rat and Fig. 1(B) at 400× magnification. Figure 1(C) shows the photomicrograph of a testis section from rat exposed to 10 mg/kg CrO_3 at 100× magnification and Fig. 1(D) at 400× magnification. It may be noted that the diameters of the seminiferous tubules were significantly decreased after Cr(VI) exposure [as indicated by arrows in Figs 1(A) and (C)]. In addition, the germ cell arrangement within the seminiferious tubules was disrupted by exposure to Cr(VI) [indicated by arrows in Fig 1(B) and 1(D)].

Table 5. Effect of Cr(VI) on sperm abnormality (x±SE)

CrO_3 dose (mg/kg)	Number of rats	level of sperm abnormality (%)
0	9	2.75±0.06
10	8	6.68±0.32*
20	11	7.6±0.15*

*$P<0.01$

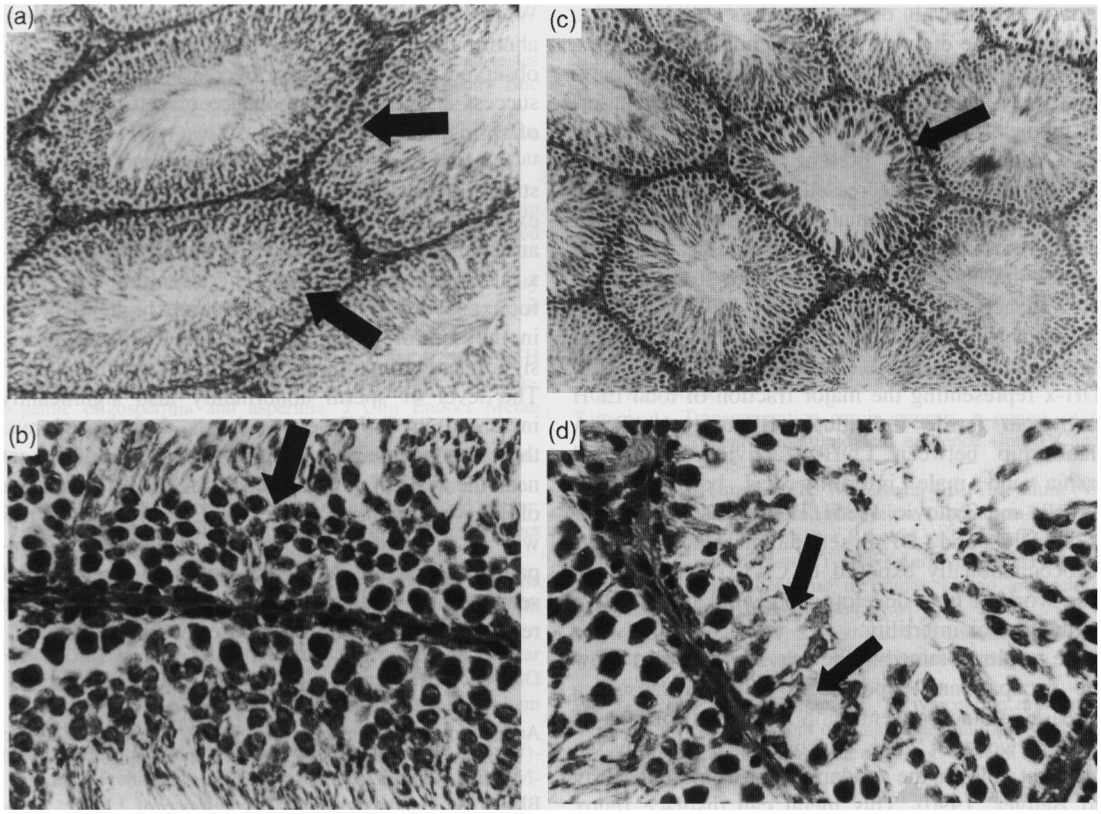

Fig. 1. (a) Photomicrograph (100× magnification) of a testis section from a control rat. (b) Same as (a) but at 400× magnification. (c) Photomicrograph (100× magnification) of a testis section from a rat exposed to CrO_3 at a dose of 10 mg/kg. (d) Same as (c) but at 400× magnification. The arrows in (a) and (b) indicate that Cr(VI) exposure resulted in a decrease in the diameter of seminiferous tubules. The arrows in (c) and (d) indicate that the germ cell arrangement near the seminiferious tubules was disrupted by Cr(VI) exposure.

DISCUSSION

The results obtained from the present study show that occupational exposure to Cr(VI) reduced sperm count and motility, zinc, LDH-x and LDH concentrations in seminal plasma, and increased the FSH concentration in serum. Exposure of rats to Cr(VI) reduced the epididymal germ cell number and the diameter of seminiferous tubules and increased the level of sperm abnormality.

As mentioned in the Introduction, Cr(VI)-containing compounds are established carcinogens. In the last several decades, studies in this area were focused mostly on the mechanism of Cr(VI)-induced carcinogenesis. Only limited studies have addressed Cr(VI)-induced reproductive dysfunction. In general, most of these studies have been focused on the effects of occupational reproductive outcome in occupationally exposed females due to the fact that reproductive endpoints can be more easily evaluated in women than in men. The present study shows that occupational exposure to Cr(VI) reduced sperm counts almost by half. In addition, occupational exposure to Cr(VI) also decreased the sperm motility. The reduction in sperm counts and decrease in sperm motility may lead to a decrease in male fertility. It may be noted that sperm counts and motility may be used as biological indicators for occupational Cr(VI) exposure. At present, only limited efforts have been directed toward using semen as an indicator of chemical exposure. It may be possible that alteration in sperm parameters may be used as biomarkers for occupational exposure to Cr(VI).

The present study also shows that occupational exposure to Cr(VI) increased FSH level in the serum. Previous studies in virile oligoaspermics have shown a correlation between high serum FSH (but not LH) and low sperm counts (Rosen and Weintraub, 1971). It has been reported that the serum FSH concentration is highest in situation where damage to the tubules is the most profound, and where aspermia is related to problems of delivery rather than of production (Rosen and Weintraub, 1971). Elevation of FSH can reflect a decreased sperm density, while LH or testosterone concentrations will not change until testicular damage is almost complete (Lipschultz *et al.*, 1980). The histopathological examination of testis from Cr(VI) exposed rats performed in the present study shows that there was visible disruption in germ cell arrange-

ment near the wall of seminiferous tubules and a decrease in the diameter of the seminiferous tubules. It appears that Cr(VI)-induced decrease in sperm counts may be partly due to damage to the seminiferous tubules.

As shown in the present study, occupational exposure to Cr(VI) decreased the concentrations of LDH-x and LDH in seminal plasma. LDH-x is an isoenzyme of lactic dehydrogenase specific in mature testicles and sperm and is specific to study seminal fluid (Blanco and Zinkham, 1963). LDH activity in the seminal fluid has been reported to be the highest among all corporal fluids (Tsutsumi *et al.*, 1984) with LDH-x representing the major fraction of total LDH activity in fertile individuals (Blanco, 1980). A relationship between LDH-x activity in seminal plasma and male infertility has been reported (Gavella and Cvitovic, 1985). The decrease in the levels of LDH-x and LDH in seminal plasma from workers occupationally exposed to Cr(VI) suggests that these workers may develop a potential reproductive dysfunction or infertility.

The results obtained in the present study also show that occupational exposure to Cr(VI) caused a decrease in zinc concentration in seminal plasma of exposed workers. Zinc is considered to play an important role in the oxidant defense system (Bray and Bettger, 1990). This metal can displace redox active metals, for example, Fe and Cu, from membrane binding sites. Zinc has been shown to reduce $Fe^{2+}-$ induced lipid peroxidation (Girotti *et al.*, 1985). Therefore, zinc may function as an indirect antioxidant. It can induce the synthesis of metallothionein. The thiol groups of this protein can scavenge $^{\bullet}OH$ and singlet oxygen ($^1O_2$) (Sato and Bremner, 1993). Zinc is also an important component in intracellular and extracellular CuZn superoxide dismutase. Change in zinc status can influence the balance between ROS production and antioxidant defense. It has been demonstrated that a zinc deficient diet can result in higher rates of oxidative damage to testes lipids, proteins and DNA (Oteiza *et al.*, 1999, 1995, 1997). It has been reported that zinc deficiency impairs the maturation of spermatid cells (Reeves and Rossow, 1993). While further studies are required to understand the effect of zinc on semen status, it is likely that a decrease in zinc concentration in seminal plasma may reduce the overall antioxidant levels against Cr(VI)-induced ROS, resulting in oxidative injuries to germ cells.

The results obtained from the present study also show that feeding Cr(VI) to rats significantly increased the level of sperm abnormality. In vitro studies have demonstrated that Cr(VI) can cause a variety of cellular injuries, including DNA strand breaks, lipid peroxidation, and protein modification through Cr(VI)-mediated free radical reactions (Shi *et al.*, 1999a,b). It is likely that sperm abnormality is partly due to the Cr(VI)-induced sperm injuries. While the mechanism of Cr(VI)-induced sperm abnormality needs to be further investigated, the observed abnormality may decrease the reproductive success and increase the chance of getting cancer in offspring.

In conclusion, the results obtained from the present study demonstrate that workers occupationally exposed to Cr(VI) exhibited reduced the sperm counts and motility and lower levels of zinc, LDH and LDH-x in seminal plasma. The FSH concentration in serum for exposed workers was significantly higher than that in the control group. Rats fed on Cr(VI) exhibited significant reduction in epididymal sperm number. The level of sperm abnormality also significantly increased in Cr(VI) exposed rats. In exposed rats, there was visible disruption in germ cell arrangement near the wall of seminiferous tubules. The diameter of the seminiferous tubules in Cr(VI) exposed rats was also reduced. The results suggest that occupational exposure to Cr(VI) leads to alteration of semen status and sperm quality that may lower the reproductive potential of exposed workers.

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