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Short Communication

Hexavalent chromium induces testicular dysfunction in small Indian mongoose (Herpestes javanicus) inhabiting tanneries areas of Kasur District, Pakistan

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ABSTRACT

Hexavalent chromium (Cr-VI), widely used in tanning industries, is a potent toxic metal whose accumulation in the animal body can adversely affect the reproductive organs. District Kasur, Pakistan, is famous for having tanneries industry where Cr (VI) is directly discharged untreated into the adjoining water nullahs. Resultantly, wildlife species, reliant on these water sources, are exposed to chromium toxicity, which enters into the animal body through drinking water and food chain. The current study investigated toxic effects of Cr(VI) on testicular tissue of adult small Indian mongoose inhabiting the study area from February 2015 to January 2016. Average Cr-concentrations, in experimental area soil and water, and the blood and tissue samples of the species were found significantly higher compared to control. Average body and testicular weights of experimental animals were found reduced. Histological analysis revealed seminiferous tubules disorganized in experimental animals, depleted germ cells and hyperplasia of the Leydig cells. Sperm counts were found reduced. Serum testosterone and LH levels were found reduced while FSH levels increased in experimental animals. The study concludes that Cr being discharged from tanneries into the environment is up taken by small Indian mongoose leading to severe testicular tissue damage and potential impairment of reproductive function of the species.

Introduction:

Chromium (Cr) is a naturally occurring element that exists in a variety of oxidation states (−2 to +6). Among the ionic forms of Cr, hexavalent chromium [Cr(VI)], the most toxic form, can readily cross cellular membranes via nonspecific anion transporters1. After entering the cell, Cr(VI) is reduced to produce reactive intermediates, including Cr(V), Cr(IV), Cr(III) and reactive oxygen species (ROS). These species can cause DNA strand breaks, base modifications and lipid peroxidation, thereby disrupting cellular integrity and inducing toxic, as well as mutagenic effects. Cr(VI) is used in more than 50 different industries worldwide in a variety of applications, including pigment and textile production, leather tanneries, wood processing, chrome plating, metallurgical and chemical industries, stainless steel factories, welding, cement manufacturing factories, ceramic, glass and photographic industries, catalytic converter production for automobiles, heat resistance and as an anti-rust agent in cooling plants. The increased use by industries, coupled with improper disposal of Cr(VI) waste, has resulted in an increase in the levels of Cr(VI) in soil, water and air, leading to environmental pollution. It is estimated that approximately half a million workers in the United States and several million workers worldwide have been exposed to Cr(VI) (via inhalation and skin contact).

Environmental or occupational exposure to Cr(VI) results in an increased risk of asthma, nasal septum lesions, skin ulcerations and cancers of the respiratory system. Cr(VI) is also known to cause cytotoxic, genotoxic, immunotoxic and carcinogenic effects in both humans and laboratory animals, as well as allergic dermatitis and reproductive toxicity. In the welding industry, workers exposed to Cr(VI) have an increased risk of poor semen quality and sperm abnormalities that lead to infertility or cause developmental problems in children. An increase in spermatozoa with abnormalities and a decrease in sperm count have also been reported in Cr-treated/exposed mice, rats, rabbits and bonnet monkeys.

Although Cr(VI) is known to affect male reproductive health, there is limited scientific data concerning the toxicity and there are no appropriate in vitro models to clearly understand the possible cytotoxic effects, including oxidative stress and apoptosis. In the present study, we investigated the mechanism underlying the toxic effects of Cr(VI) in male somatic and spermatogonial stem cells (SSCs). Leydig cells are somatic cells adjacent to the seminiferous tubules that produce the primary androgen, testosterone, an important hormone for the maturation of sperm. Sertoli cells are located in the convoluted seminiferous tubules and are responsible

for supporting/promoting the development of germ cells. They also form the blood–testis barrier and provide physical support to SSCs, which are situated on the basement membrane of the seminiferous tubules, to form the stem cell niche. SSCs represent a self-renewing population of spermatogonia and support spermatogenesis by continuous division throughout the life of the male. Thus, damage to or dysfunction of the Leydig or Sertoli cells, and/or SSCs can have adverse effects on spermatogenesis and the production of sperm.

The objectives of the present in vitro study were to:

1. determine the cytotoxic effects of Cr(VI) on mouse TM3 cells and mouse SSCs;
2. evaluate the effects of Cr(VI) on oxidative stress;
3. assess the effects of Cr(VI) on apoptotic signaling mechanisms;
4. understand the role of Cr(VI) in cell proliferation/self-renewal mechanisms of SSCs; and (v) explore the effects of Cr(VI) on the physiological functions of TM3 and TM4 cells.

This study aimed to determine the in vitro cytotoxic effects of Cr(VI) on mouse Leydig (TM3) cells, Sertoli (TM4) cells and SSCs that have critical roles in spermatogenesis. We examined the effects of Cr(VI) on oxidative stress and apoptosis-related signaling mechanisms in TM3, TM4 and SSCs, cell proliferation/self-renewal mechanisms of SSCs and the physiological functions of TM3 and TM4 cells. For this purpose, cells were treated with Cr(VI) at doses of 0, 3.125, 6.25, 12.5, 25, or 50 μM for 24 h; and then analyzed biochemically and by flow cytometry, fluorescence microscopy, qRT-PCR and immunoblotting. To the best of our knowledge, this study is the first to report that the dose-dependent cytotoxic effects of Cr(VI) exposure in both male somatic cells and SSCs are mediated through apoptosis. Since somatic cells and SSCs play an important role in the process of spermatogenesis, damage to these cells has adverse effects on the production of healthy sperm cells. We investigated whether oxidative stress and mitochondrial dysfunction played a role in our current experimental model and observed that, after 4 h, exposure to Cr(VI) increased the production of ROS, which was maintained up to 24 h. In contrast, 24 h exposure to Cr(VI) decreased MMP in a dose-dependent manner. Treatment with a ROS inhibitor, NAC, abrogated the effects of Cr(VI). This demonstrates that oxidative stress and subsequent mitochondrial damage play a crucial role in Cr(VI)-induced cytotoxicity. We showed that the transcriptional expression of antioxidant enzymes that play a significant role in scavenging free radicals, including Cat, Sod1, Sod2, Gpx1 and Gsta4 decreased with increasing doses of Cr(VI). Thus, the oxidative stress in the somatic cells and SSCs observed after exposure to Cr(VI) appears to be due to poor scavenging of free radicals by the antioxidant enzymes. These results are supported by those of previous reports demonstrating that Cr(VI)-induced oxidative stress via the suppression of antioxidant enzymes plays a major role in male infertility. The over-expression of Gsta1 (seen with lower doses of Cr(VI) in SSCs and all doses in somatic cells) might protect the cells against Cr(VI)-induced oxidative stress. The induction of the mitochondria-dependent (intrinsic) pathway of apoptosis in the male somatic cells observed after Cr(VI) exposure, was confirmed by the decreased BCL2/BAX protein ratio, increased expression of cytosolic CYCS and cleavage of CASP9, CASP3 and PARP proteins. Cell viability depends on the balance between survival and pro-apoptotic signaling, regulated by the AKT1, MAPK and P53 pathways. AKT1 and ERK1/2 signaling pathways are associated with cell proliferation and survival, while ROS produced by several toxicants, including Cr(VI), serve as second messengers to activate pro-apoptotic kinases, such as JNK1/2 and P38. Cr(VI)-induced DNA damage and oxidative stress also promoted the activation of P53 leading to intrinsic apoptosis. We showed that, in male somatic cells, exposure to Cr(VI) increased the phosphorylation of JNK1/2 and P38 in a dose-dependent manner, while the phosphorylation of ERK1/2 decreased (down-regulation was higher in TM4 cells). The expression levels of P-AKT 1 were not altered up to the IC50 dose level of Cr(VI). At a concentration of 25 μM, however, Cr(VI), decreased the expression of P-AKT1, by only 20% in TM3 cells, but a 40% reduction was observed in TM4 cells. Cr(VI) also increased P-P53 expression in a dose-dependent manner (up-regulation was more pronounced in TM3 cells). These cell-specific differences in the regulation of P-AKT1, P-ERK1/2 and P-P53 proteins are probably at least partially responsible for the differential sensitivity of TM3 and TM4 cells to Cr(VI). The increased phosphorylation of P53 and cleavage of CASP9, CASP3 and PARP proteins showed that Cr(VI) induced intrinsic apoptosis in SSCs at concentrations up to 12.5 μM, but a higher concentration (25 μM) decreased apoptosis. We therefore investigated other factors, apart from apoptosis, that could also be involved in Cr(VI)-induced cytotoxicity. In SSCs, GDNF plays two major roles, regulating the self-renewal/maintenance via AKT1–Mycn signaling pathways and the proliferation/differentiation via ERK1/2–Fos pathways. We demonstrated that Cr(VI) not only affected the self-renewal/maintenance pathway but also the proliferation/differentiation pathways in SSCs in a dose-dependent manner. Our results demonstrated that Cr(VI) (i) at 6.25 μM (IC50), induced cytotoxicity mainly through P53-dependent apoptosis; (ii) at 12.5 μM, increased apoptosis and disrupted GDNF signaling pathway; and (iii) at 25 μM, decreased apoptosis (like 6.25 μM) and further disrupted GDNF signaling pathway (compared to 12.5 μM). In conclusion, the loss of cell viability observed at higher doses of Cr(VI), involves the apoptosis pathways and impairment of the GDNF signaling pathway. Cr(VI) toxicity has been reported to disrupt steroidogenesis and decrease serum testosterone levels and 3-HSD enzyme activity in male rats. We also observed that Cr(VI)-treatment significantly decreased the testosterone release from the TM3 cells into the culture media. Besides, Cr(VI) exposure in TM3 cells significantly decreased transcriptional expression of Cyp11a1 (a rate-limiting enzyme in steroidogenesis that converts cholesterol to pregnenolone in mitochondria) and Hsd3b1 (that converts pregnenolone to progesterone in the endoplasmic reticulum), without significant alteration in the levels of Hsd17b3. In contrast, Cr(VI) increased the expression of Cyp17a1 and Star, possibly through a negative feedback compensatory mechanism counterbalancing the Cr(VI)-induced inhibition of Cyp11a1 and Hsd3b1. Steroidogenesis is a complex multi-enzyme process wherein steroid hormones are produced from cholesterol. A number of researchers have shown that impairment of steroidogenesis by environmental toxicants does not require the inhibition of all the steroidogenic enzyme genes. In the present study, therefore, Cr(VI) impaired steroidogenesis, at least to some extent, by inhibiting Cyp11a1 and Hsd3b1 in TM3 cells. Cr(VI) also decreased the expression of Cyp19a1, which is required for the conversion of androgens into estrogens. In TM4 cells, Cr(VI) decreased the transcriptional expression of the tight-junction signaling molecules, Tjp1, Vim and Ocln, which play a key role in forming the blood-testis barrier. These results are supported

by those of the previous report where the authors showed that Cr(VI) altered the Sertoli cell barrier, which might affect the blood-testis barrier. Cr(VI) also decreased the transcriptional expression of the cell receptors, Fshr and Ar, which play a key role in the maturation and normal functioning of Sertoli cells and those of some secreted molecules, including Gdnf, Etv5 and Fgf2, which are essential for the maintenance of SSCs within the stem cell niche. Our results suggest that Cr(VI)-toxicity may also affect the maintenance of SSCs in vivo. Thus, Cr(VI)-induced impairment of the physiological functions of TM3 and TM4 cells could directly affect normal spermatogenesis.

Conclusion:

Cr(VI) induced mitochondria-dependent apoptosis in male somatic cells and SSCs, possibly through the induction of oxidative stress and DNA damage. The cell-specific pattern of regulation of P-AKT1, P-ERK1/2 and P-P53 proteins is probably responsible, at least in part, for the differential sensitivity of TM3 and TM4 cells to Cr(VI). Cr(VI) also disrupted the differentiation and self-renewal mechanisms of SSCs and impaired the physiological functions of TM3 and TM4 cells. To the best of our knowledge, our study is the first to uncover the underlying signaling mechanisms of Cr(VI)-induced cytotoxicity and apoptosis in male somatic cells and SSCs. Our experimental findings could provide a basis for the development of improved antagonists of Cr(VI) to combat Cr(VI)-induced reproductive toxicity.