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RESEARCH ARTICLE

EFFECTS OF HEXAVALENT CHROMIUM ON FEMALE REPRODUCTIVE FUNCTIONS

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ABSTRACT

Hexavalent chromium (CrVI) is an environmental contaminant which may be associated with reproductive abnormalities. In the present study, we examined the effect of CrVI on rat female reproductive function. Female Wistar rats received a daily intraperitoneal injection (ip) of potassium dichromate (1 or 2 mg/kg body weight) for fifteen consecutive days. The relative weight and histology of ovary and uterus were determined as well as follicule counting and duration of estrous cycle. Cr level in blood and ovary was estimated. Serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations were determined. Results indicated that after 15 days of CrVI treatment, a decrease of the relative weight of the ovary and uterus occurred with decreased follicle number, and extended estrous cycle. Moreover, a dose-dependent increase in blood and ovary Cr levels as well as an increase in FSH and LH serum levels were detected in treated rats. Histological analysis revealed morphological alterations in ovary and uterus at high dose of CrVI. The ovary showed atretic follicles and arrested follicle growth, while uterus revealed atypical and atrophic epithelium cells surrounding the lumen with decreased thickness of the myometrium. The results suggest that subacute treatment with potassium dichromate promotes reproductive system toxicity and affects ovary and uterus function of adult female rats.

INTRODUCTION

Chromium (Cr) is the 21st most abundant element in the earth crust and one of the eight metals in the top priority list for toxic substances (Abdalla *et al.,* 1987). It is widely used on a large scale in many different industries including metallurgical, electroplating, production of paints and pigments, tanning, textile, wood preservation, paper production, and photographic industries (Costa and Klein, 2001). Disposal of industrial wastes leads to severe environmental pollution. Cr can exist in several oxidation states ranging from −2 to +6, of which the trivalent (III) and hexavalent (VI) forms are of biological importance (Wise *et al.,* 2008). The two main hexavalent Cr forms dominant in the environment (CrO_7^- and $Cr_2O_4^-$) can readily cross cellular membranes with the help of nonspecific anion carriers, whereas trivalent form is poorly transported across membranes. The hexavalent form is usually linked with oxygen and is a strong oxidizing agent. It is widely known to cause allergic dermatitis, as well as toxic and carcinogenic effects in humans and animals (Domingo, 1994; Kawanishi *et al.,* 2002; Halasova *et al.,* 2009).

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High levels of Cr are reported to impair gestational development as evidenced by epidemiological studies in female workers exposed to this metal in the work environment (Shmitova, 1980; Greene *et al.,* 2010). Exposure to CrVI can induce complications during pregnancy and childbirth (Shmitova, 1980). Increased incidence of birth and developmental defects were reported among children living around tanneries of Cr and leather industries (Blacksmith, 2007). It was reported that CrVI induces developmental effects including post-implantation losses, resorptions, reduced fetal weight and malformations including reduced ossification (Abiaka *et al.,* 2001; Marouani *et al.,* 2011). Earlier studies point out the deleterious effects of CrVI on the structure and function of ovary (Banu *et al.,* 2008; Rao *et al.,* 2009). The ovary as the repository of oocytes, as well as the source of hormones controlling the uterus function, plays a major role in fertilization and initiation of a successful gestation. To our knowledge, limited studies were carried out to evaluate the toxicity of CrVI on female reproductive tract. Therefore, in the current study we have examined the effects of subacute treatment of CrVI on different reproductive parameters in adult female rats. The examined parameters included weight and histological analysis of ovary and uterus, duration of estrous cycle, follicle number as well as determination of serum concentrations of luteinizing hormone (LH) and follicle

stimulating hormone (FSH). In addition, Cr content in blood and ovary was studied.

MATERIALS AND METHODS

Animals and reagents

2 month-old Wistar female rats weighing 130-150g, purchased from Siphat-Tunis were used in this study. The rats were housed under controlled conditions of temperature (22 ± 1°C) with a constant day/night cycle (light from 8:00 to 20:00). Food and water were provided *ad libitum*. CrVI as potassium dichromate ($K_2Cr_2O_7$) was purchased from Merck (Darmstadt, Germany). The rats were randomized into three experimental groups of approximately similar weight (n=14) as follows: (1) animals received an ip injection of potassium dichromate diluted in sterile distilled water at dose of 1mg/kg body weight (b.wt), (2) animals were administered 2mg of potassium dichromate/kg b.wt, (3) control group received equal volumes of vehicle. Dilution of CrVI was so made that the volume of each injection was maintained to 0.1ml/100g of rat. The doses and the duration of CrVI treatment were selected from a preliminary experiment carried out in the laboratory. Absorption of Cr depends on its valency state. Hexavalent Cr can rapidly enter the cell than its trivalent form. CrVI is less readily by oral route than by the other routes. CrVI is reduced to CrIII by gastric juices inside the stomach which significantly decreases its absorption capacity by oral route. Dermal exposure to CrVI has been demonstrated to produce irritant and allergic contact dermatitis (USEPA, 1998). Therefore, ip route was selected for CrVI administration in the present study to enhance the absorption capacity. The treatment schedule was started during the estrous phase. The body weight of rats was determined daily through the experiment. After 15 days of treatment, animals were killed by decapitation; the left ovary and the uteri were dissected and weighed. Animals were cared for in compliance with the Tunisian code of practice for the Care and Use of Animals for Scientific Purposes. The experimental protocols were approved by the Faculty Ethics Committee (Faculté des Sciences de Bizerte, Tunisia).

Chromium estimation

The ovary was grinded in a mixture of $HNO_3/HClO_4$ (1:1). The residue was dissolved with 25 ml of nitric acid solution (0.2 ml HNO_3 in 100 ml ultrapure water). The solution aliquots were used to estimate Cr using an atomic absorption spectrometer (Perkin-Elmer 306) at 357.9 nm wavelength. The values are expressed as µg/g fresh weight. Blood Cr was directly quantified after dilution with distilled deionized water (Davidson and Secrest, 1972). The value is expressed as µg/ml of blood. The laboratory glass wares used for ovary and blood collection and processing were soaked overnight in analytical grade nitric acid and washed three times with deionized water.

Study of duration of estrous cycle

Vaginal smears of rats from each group were taken once every morning and viewed under microscope to assess the length of different phase of the estrous cycle (Goldman *et al.,* 2007).

Ovarian follicle number

The number of growing follicle stages at various stages of development were counted from sections of ovary fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin and stained with haematoxylin and eosin. Follicle counts were performed by examining every 12th section of each ovary. Classification of various stage of follicle development was performed as described by Devine and co-workers (2002).

Histological analysis

The ovary and uterus were fixed overnight at room temperature by direct immersion in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The samples were dehydrated with ethanol and toluene series and embedded in paraffin. Serial sections (4µm) were mounted on gelatin- coated glass slides cut and stained with haematoxylin and eosin.

Enzyme- linked immunosorbent assay and radioimmunoassay

The serum FSH and LH were determined in the same female rats used for the examination of relative weight of ovary and uterus. After decapitation, trunk blood was collected, centrifuged (500×g, 10 min, 4°C) and serum stored at -80°C until analysis. FSH and LH concentrations were determined in duplicate using an enzyme immunoassay system (enzyme-linked immunosorbent assay, ELISA) with commercial kits (BiotrakTM from Amersham Biosciences, UK). Serum hormone levels are expressed as ng/ml. The cross reactivity of rLH to rFSH was <0.016%. The cross reactivity of rFSH to rLH was <0.17%. The sensitivity of rLH and rFSH assay was respectively 0.1 ng/ml and 8.66ng/ml.

Statistical analysis

Data were analyzed using Stat View 512+ software (Abacus Concept, Inc. Calabasas, CA, USA**).** Overall differences in mean values between control and treatment groups were measured using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls as post hoc test. The results were expressed as means ± SEM and differences were considered significant at $p<0.05$.

RESULTS

Fifteen day-CrVI treatment caused a significant decrease in ovary relative weight (Figure 1). This decrease reached 21.4 and 30% of controls for 1 and 2mg of Cr/kg b.wt, respectively. A decrease of relative weight was also observed in uterus at the high dose. This decrease was about 29% compared to the control group (Figure 2). Cr accumulation in blood and ovary of the control and treated groups are shown in figure 3. A higher concentration of Cr was found in blood (0.07±0.01 and 0.09±0.01µg/ml) and ovary (0.096±0.015 and 0.144±0.014µg/g) respectively for 1 and 2mg/kg of CrVI. In control rats, values are (0.03±0.006µg/ml in blood and 0.044±0.003µg/g in ovary). Our results indicate also that the length of estrous cycle did not show any significant change in treated group with 1mg of Cr/kg while it increased significantly with the highest dosed group (Cr 2mg/kg) in comparison to controls group (Table 1).

Moreover, CrVI treatment significantly reduced the follicle number in dose-dependant fashion (Table 2). This decrease was of 50 and 68% in primordial follicles, of 43 and 61% in primary follicles, of 77 and 79% in secondary follicles and of 78 and 89% in antral follicles, respectively with 1 and 2mg of Cr/kg b.wt. Further, CrVI treatment significantly ($p<0.05$) increased serum LH and FSH levels in a dose-dependent manner (Table 3). The increase was of 27 and 36% in serum LH levels and of 67.5 and 151% in serum FSH levels, respectively with 1 and 2mg of Cr/kg b.wt. Ovary histopathological analyzed indicated that ovaries of the control rats showed normal histology in the form of various stages of follicles in the cortex (Figure 4, Photo A). The primary follicles were surrounded by a single layer of flattened follicular cells encircling an immature ovum (primary oocyte) with a large nucleus. The mature Graafian follicle were visible with an antral cavity and a stratified layer of follicular granulosa around the cavity *(*Figure 4, Photo A*)*. However, ovary from CrVI treated rats with the high dose of CrVI (2mg/kg) revealed atretic follicles and arrested follicle growth throughout the stromal spaces. CrVI treatment with the dose of 1mg/kg had less pronounced effect (data not shown). Uteri from the control rats showed normal endometrial glands and the lumen was lined with simple cuboidal epithelium cells (Figure 4, Photo A and B). Exposure to 1mg of Cr/kg did not produce any changes in endometrium uterine histology (Figure 4, Photo C and D). However, in animals treated with 2mg of Cr/kg the lumen was lined with atypical and atrophic epithelium cells (Figure 4, Photo E and F). Furthermore, in the treated groups, decreased dose-dependant thickness was noted significantly in the myometrium uterine compared to control (Figure 5).

DISCUSSION

The major physiological function of the female reproductive system is to produce ovum necessary for healthy progeny. Ovarian steroid hormones play a vital role in the production of ovum and other functions associated with reproductive behavior. The hormones secreted by the hypothalamus and pituitary also regulate regular cyclical changes in the ovary and endometrium. CrVI is the established endocrine disruptor reported to disturb the reproductive process in females (Banu *et al.,* 2008; Marouani *et al.,* 2011). The purpose of this study was to investigate the toxic effects of CrVI in two major female reproductive organs (ovary and uterus). Our results showed that exposure of rats to 1 and 2mg of Cr/kg b.wt during 15 consecutive days decreased significantly the ovarian and uterine relative weights. Our data is concomitant with that of Al-Hamood and co-workers (1998) who also reported decreased ovarian and uterine weights in trivalent Cr exposed mice. Samuel co-workers (2011) also found a decrease in uterus weight in CrVI-treated rats. The reduction in ovarian weight might be due to hormonal imbalance and reduction in total proteins levels caused by Cr (Rao *et al.,* 2009). Recently, it has been reported that CrVI decreased the semen levels of testosterone, estradiol and progesterone in rats (Samuel *et al.,* 2011). Estrogens are known to regulate growth and cell division in uterus (Newbold *et al.,* 1994). Therefore, the observed decrease in uterus weight may be due to the decreased blood level of estrogens. Our results revealed that serum and ovary Cr levels increased in a dose-dependent manner in treated rats, confirming the absorption of this metal after ip administration. Besides, the present study showed that different stages of ovarian follicular maturation are sensitive to CrVI as evident from the reduction in their number. In addition, histological changes were observed in the ovary of treated animals, including atretic follicles and arrested follicle growth throughout the stromal spaces. CrVI-exposed rats exhibited an extended estrous cycle compared to control. Our findings were in accord with previous studies showing that CrVI exposure leads to higher accumulation of Cr in uterus and ovary and revealed delayed sexual maturation with extended estrous cycle compared to control rats (Murthy *et al.,* 1996; Banu *et al.,* 2008; Samuel *et al.,* 2011; 2014). The process of follicular development for all mammalian species is believed to be multifactorial ensuring as appropriate number of ripe follicles to be available for ovulation at the appropriate time. Any effect of xenobiotics such CrVI on these stages of follicular maturation and differentiation reflects an effect on the ovarian physiology, thereby impairing the normal reproductive competency in affected females. During the long period of meiotic arrest, the primary oocytes remain vulnerable to genetic or epigenetic damage, which may destroy the oocytes or impair their ability to develop after fertilization (Murthy *et al.,* 1996). The ovary cannot replace the oocytes destroyed by CrVI.

Complete destruction of oocytes, at any time during the reproductive life span, will lead to infertility although all the reproductive organs will have a normal development and appearance (Murthy *et al.,* 1996). Another cause for the depletion in the number of follicles at all stages might be due to the damage caused, either directly or indirectly, by CrVI treatment to the follicle cells. The follicle cells ensure appropriate development of the oocytes by nursing and nourishing them. These cells are transformed into the cumulus oophorus mass and produce pyruvic acid and lactic acid essential for nutrition of oocytes under the influence of gonadotropins. If the surge in gonadotropins is blocked, follicles fail to form more than a single layer of granulosa cells (Baker, 1988). Recent reports showed that CrVI decreased the ovarian granulosa cell proliferation through cell cycle arrest and decreased cyclin-dependent kinases (CDKs) and increased the granulose cell apoptosis (Banu *et al.,* 2011; Samuel *et al.,* 2011). It may either be a direct effect of the metal on the ovarian tissue or mediated by an effect on the gonadotropins, necessary for normal follicular development and ovulation (Murthy *et al.,* 1996; Samuel *et al.,* 2014). This postulation also gets support by the lengthening of estrous cycle in the highest CrVI-dosed rats in the present study.

Pituitary and hypothalamus are considered to be a classical target organ for reproductive toxicants. During the antral follicular development, follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) are present on granulose and thecal cells, respectively. During follicular phase, estradiol synthesis increases in response to FSH and LH levels. In the present study, CrVI significantly increased FSH and LH levels in a dose-dependent manner. Our result is in accordance with previous studies (Banu *et al.,* 2008; Rodriguez *et al.,* 2008; Samuel *et al.,* 2014). CrVI - exposure may delay the development of FSHR and LHR, which is expected to be assisted by inhibitory action of aromatase activity in follicles.

In the present study, the accumulation of CrVI may have delayed synthesis of FSHR leading to elevate the FSH and LH levels. Increased levels of FSH, LH in rats exposed to CrVI could also be due to decreased estradiol levels along with the direct effect of CrVI on pituitary lactotrophs and somatotrophs. Previous studies revealed the decreased expression of steroidogenic genes in ovarian cells exposed to CrVI (Banu *et al.,* 2008). Thus, the CrVI induced impairment of steroidogenic mechanisms may be the important factor behind the extended estrous cycle. In the present study, we showed that high dose of CrVI caused histological alterations in uterus including atypical and atrophic epithelium cells surrounding the lumen of endometrium and decreased thickness in the myometrium uterine. We have reported earlier that the subacute treatment with CrVI causes oxidative stress in uterus inducing epithelial and stromal cells apoptosis by activation of Bax and p53 proteins (Marouani *et al.,* 2013). Hexavalent Cr has shown to be involved in Fenton-like oxidative cycling generating reactive oxygen species (ROS) (Shi *et al.,* 1999). Increased ROS production in uterus may be responsible of the deleterious effects observed in the uterus cells. In addition, estrogen deficiency is shown to be associated with oxidative stress and induced apoptosis in the luminal and glandular epithelia of rat uterus by activation of Bax protein (Morishita *et al.,* 2003; Muthusami *et al.,* 2005). Thus, the damage observed in uterus could be the result of increased ROS level in uterus and/or of decreased steroid hormone secretion particularly estrogen. In conclusion, the results obtained from the present study demonstrate that the subacute treatment with CrVI affects the ovary and uterus and causes delayed follicular development and extended estrous cycle with impaired FSH and LH blood levels.

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