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Evaluation of *in vivo* reproductive toxicity of potassium chromate in male mice

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Abstract

To evaluate the effects of potassium chromate on mice sperm cells after a short term exposure, male ICR-CD1 mice were administered with 5 or 10 mg K_2CrO_4 /bw for four consecutive days. One group of mice was sacrificed at day 5, starting from the beginning of the experiment and another group was sacrificed at day 35. Testis and epididymis histology was evaluated by light microscopy and testicular cells populations were evaluated by flow cytometry (FCM). Spermatozoa were collected from the epididymis and sperm cell's morphology and several functional parameters (density, motility, viability, mitochondrial function, acrosome integrity) were evaluated. Furthermore, DNA fragmentation and chromatin status of sperm cells were assessed at both experimental periods.

Besides a reduction in seminiferous tubules diameter, exposure to potassium chromate did not induce further histopathological changes in mice testis or epididymis. These results were supported by the analysis of testicular cellular subpopulations by FCM. Concerning sperm cell's morphology, an increase in the percentage of multiple abnormalities and a decrease in the percentage of normal spermatozoa and was found at day 5 and 35, respectively. Although sperm cell's mitochondrial function or viability was not affected, sperm cell's motility was significantly reduced by potassium chromate exposure at both experimental periods. A decrease in acrosome integrity was found in mice injected with 10 mg K_2CrO_4 /bw after 35 days. Exposure to potassium chromate did not affect either DNA fragmentation or chromatin susceptibility to acid denaturation of sperm cells. In this work, we were able to show the effects of potassium chromate on spermatozoa physiological parameters such as motility, morphology and acrosome status and also demonstrate that the doses tested did not induce DNA damage to sperm cells after one spermatogenic cycle.

Key words: potassium chromate, function of sperm cells, acrosome, DNA damage of sperm cells, tubular diameter, testicular toxicity, flow cytometry, histopathology

Accepted manuscript

1. Introduction

Chromium can be found in the environment in soil, rocks, animals and plants (ATSDR, 2000). The three main oxidation forms of chromium, commonly found in the workplace and general environment are chromium(0), chromium(III) and chromium(VI) (Zhitkovich, 2005). Chromium(0) is the metal chromium, a steel-gray solid with a high melting point usually used for making steel and other alloys. Chromium(III) and chromium (VI) compounds are widely used industrially in stainless steel production, welding, electroplating, leather tanning, production of dyes and pigments and wood preservatives (ATSDR, 2000). Cr(III) occurs naturally in the environment and is an essential nutrient that plays an important role in glucose, proteins and fats metabolism by potentiating insulin action (ATSDR, 2000; Costa and Klein, 2006). Chromium(VI) occurs mostly due to anthropogenic origin and is considered a human carcinogen (e.g. Ding and Shi, 2002; Chiu et al., 2004; Costa and Klein, 2006).

Octahedral Cr(III) complexes can cross the cell membrane only very slowly by simple diffusion or phagocytosis (ATSDR, 2000). In opposite, Cr(VI) in the form of a chromate oxyanion crosses easily the cell membrane using a non specific anion carrier, the so called permease system, which transports a number of anions with tetrahedral configuration and negative charge such as SO_4^{2-} and PO_4^{2-} (Belagyi et al., 1999). Once inside the cell, Cr(VI) is rapidly reduced to Cr(III) by predominant cellular reductants as ascorbate, cysteine and glutathione leading to high intracellular accumulation of this element (Zhitkovich, 2005). During the reduction process other chromium species in intermediate oxidation states are generated, namely Cr(V) and Cr(IV). Cr(VI) does not react directly with nucleic acids, while Cr(V), Cr(IV) and Cr(III) appear to be highly reactive with DNA (Stearns et al., 1995). During the reduction process of Cr(VI), reactive oxygen species (ROS) are also generated in Fenton or Haber-Weiss reactions (Shi et al., 1999). As a result of chromium reduction and consequent intracellular

accumulation of Cr(III) several genetic lesions are originated both due to direct action of chromium or due to ROS (e.g. O'Brien et al., 2003). Cr(VI) reduction originates Cr-DNA adducts, Cr-DNA crosslinks, Cr-DNA-protein crosslinks, single-strand breaks, double strand breaks and base reduction, with formation of 8-hydroxydeoxyguanosine (8-OHdG) (O'Brien et al., 2003; Wise et al., 2008 for review).. These lesions may induce cell death by apoptosis or changes in the cell cycle (O'Brien et al., 2003; Wise et al., 2008 for review). These lesions, if left unrepaired or are misrepaired, can lead to growth arrest, cytotoxicity, and apoptosis, as well as mutations leading to neoplastic transformation and ultimately the formation of tumours (Wise et al., 2008).

Humans may be exposed to high levels of chromium in some occupational or environmental situations/conditions. Data available from epidemiological studies among workers exposed to chromium does not confirm by now the relationship between occupational exposure to this element and interference with reproductive function in men (Figà-Talamanca et al., 2001). Bonde and Ernst (1992) analysed the semen quality and sex hormones in welders exposed to hexavalent chromium and found an inconclusive relationship between the level of chromium in biological fluids (semen and urine) and the quality of semen or male sexual hormones (LH, FSH and testosterone). Hjollund et al. (1998) found no significant changes in semen quality of welding workers with metals (including chromium among others). However, when studying the fertility of workers of an Italian mint, Figà-Talamanca et al. (2000) did not exclude the hypothesis that exposure to fumes is associated with increase in Time To Pregnancy (TTP). Also, in a study performed with electroplating workers, Li and co-workers (2001) found that those individuals presented decreased sperm cell counts and motility. More recently, studies of Kumar et al. (2005) with chromium sulphate manufacturers showed a positive correlation between chromium concentration in blood and the percentage of abnormal sperm cells, however other endpoints such as semen volume, liquefaction time mean pH

value, spermatozoa viability, concentration and motility were not affected (Kumar et al., 2005).

Previous studies on the effects of chromium on rodent fertility are presented in Table 1. Some of these studies show the incidence of testicular toxicity due to chromium, namely Cr(VI) (Ernst, 1990; Murthy et al., 1991; Aruldas et al., 2005). Among the few available animal studies on the effects of chromium on sperm cell's function, some showed that spermatozoa abnormality usually increases in mice exposed to CrO₃ (that is Cr(VI)) (Acharya et al., 2004; Acharya et al., 2006) and rats (Bonde and Ernst, 1992). Another parameter usually affected by chromium is the concentration of sperm cells, which usually decreases after chromium exposure. This effect was observed in mice (Acharya et al., 2004; Acharya et al., 2006) and rats treated with CrO₃ (Li et al., 2001) and also in rabbits (Yousef et al., 2006) and Bonnet monkeys (Subramanian et al., 2006) exposed to potassium dichromate. Also, in previous studies, we showed the adverse effects of one instable chromium intermediate, [Cr(V)], on mice testis epithelium and also on sperm cell's function (Pereira et al., 2002; 2004; 2005).

Due to the scarce information about chromium effects on sperm cell's function, a more comprehensive analysis of functional and genetic parameters would be desirable. For that, in the present work, two concentrations of potassium chromate were tested in mice and the effects were assessed at two experimental time points after the end of treatment. This toxicity was evaluated using a battery of parameters including motility, vitality, count, acrosome integrity, DNA fragmentation and chromatin condensation. The effects of chromium toxicity on testis and epididymis were also assessed.

2. Materials and Methods

2.1 Chemicals and dyes

Propidium iodide (PI), rhodamine 123 (R123), glucose, HEPES, DMSO, Tris-HCl, EDTA, bovine serum albumin (BSA), Triton X-100, RNase and Coomassie blue were purchased by Sigma (St. Louis, MO, USA). LIVE/DEAD® Sperm Viability Kit (includes SYBR-14 and PI) for the sperm viability assay and Acridine orange (AO) was purchased by Molecular Probes (Eugene, OR, USA). K_2CrO_4 , NaCl, KCl, $MgCl_2 \cdot 6H_2O$, $NaH_2PO_4 \cdot 2H_2O$, $NaHCO_4$, $CaCl_2$ were purchase by Merck (Darmstadt, Germany). *In Situ* Cell Death Detection Kit, Fluorescein and DNase I were purchased by Roche Diagnostics GmbH (Mannheim, Germany).

2.2 Animals and treatment

Eight weeks old male ICR-CD1 mice were provided by Harlan Interfauna Ibérica SA, Barcelona, Spain. Animals were housed in a constant temperature ($22 \pm 2^\circ C$) and relative humidity (40-60%) *vivarium* on a light-dark 12h/12h cycle. Water and food were provided *ad libitum*. Mice were allowed to acclimate for one week before experimental use.

Animal experiments were conducted in accordance with institutional guidelines for ethics in animal experimentation (Rule number 86/609/CEE- 24/11/92).

Three groups of ten mice each were subcutaneously injected with 5 and 10 mg K_2CrO_4 /kg bw for four consecutive days. Control groups of ten mice each were injected with the saline vehicle (0.9 % NaCl). Mice were sacrificed by cervical dislocation 5 and 35 days after starting of treatment (n=5 per group and per period).

2.3 Chromium quantification

Chromium contents in mice testes were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). After mice dissection, the testis were removed and kept at -

20°C until digestion. Samples were digested in nitric acid (Suprapur grade) in a microwave digestion system (CEM 81D). ^{52}Cr contents in digested samples were then determined in a spectrometer ICP-MS Thermo X-Series (Thermo Fisher Scientific Inc., MA, USA). Detection limits were set at 0.3 $\mu\text{g/L}$. A certified reference material was digested and analysed together with samples (NCS ZC73016 “Chicken meat”; LGC Promochem, Barcelone). Chromium contents were expressed as $\mu\text{g/g}$ fresh weight.

2.4 Histological analysis

Left testes and epididymis were removed and fixed in Bouin’s solution. Samples were then dehydrated and embedded in paraffin wax. Sections of 5 μm thick were performed in a microtome Leitz 1512 (Leitz, Wetzlar, Germany) and stained with haematoxylin and eosin.

2.5 Measurement of seminiferous tubules diameter

Tubular diameters were measured using a new dedicated software named Snakes, as described by Guevara et al. (2003). Images were acquired with a camera Olympus Camedia C-5060 attached to a microscope Olympus BX41 (Olympus, Tokyo, Japan).

The detailed software tool specifications were described by Guevara et al. (2003). Briefly, after loading the image, some points in the tubule contour were picked up. Then, some morphological operations were performed to enhance the contrast and an edge detector was applied to obtain an approximation to tubule contours. Deformable models were then applied and the final contours were obtained. The results are presented in a Microsoft Excel worksheet.

The tubular diameters obtained with this new software were compared with the traditional calculations with an ocular micrometer calibrated with a stage micrometer for 10 times lens magnification. The round and slightly oblique tubules were measured by

the short axis of the tubular profile. Thirty two tubules were randomly selected from three different slides from three animals and measured.

With the aim of testing the statistics accuracy of the Snakes software (Guevara et al 2003) measurements with the traditional method were correlated with the measurements with the software tool using the Pearson's correlation coefficient. One way ANOVA was used to compare the tubular diameters from the different treatments.

2.6 FCM analyses of testicular cells

The procedure of obtaining testicular monocellular suspensions from paraffin embedded samples is described elsewhere (Oliveira et al., 2006). Briefly, two or more sections (40 μm thick) of five blocks from five animals were used. Sections were deparaffinized in xylol and rehydrated. Samples were then incubated with pepsin pH 1.5 at 37 °C and washed in PBS. Then, samples were treated with 50 $\mu\text{g}/\text{mL}$ of RNase in order to eliminate RNA and subsequently stained with 50 $\mu\text{g}/\text{mL}$ of propidium iodide (PI). Samples were analysed within a 15-min period. The relative fluorescence intensity of PI-stained nuclei was measured with a Coulter EPICS XL (Coulter Electronics, Hialeah, Florida, USA) flow cytometer equipped with an air-cooled argon-ion laser tuned at 15mW and operating at 488 nm. Integral fluorescence together with fluorescence pulse height and width emitted from nuclei was collected through a 645 dichroic long-pass filter and a 620 band-pass filter and converted on 1024 ADC channels. Prior to analysis, the instrument was checked for linearity with fluorescent check beads (Coulter Electronics) and the amplification was adjusted so that the peak corresponding to the haploid peak was positioned at channel 200. This setting was kept constant. The results were obtained in the form of three graphics: linear fluorescence light intensity (FL), forward angle light scatter (FS) versus side angle light scatter (SS) and FL pulse integral versus FL pulse height. This last cytogram was used to eliminate partial nuclei and other

debris, nuclei with associated cytoplasm and doublets (these events have a higher pulse area but the same pulse height as single nuclei).

2.7 Functional parameters of sperm cells

2.7.1 Density

Sperm cell's density was assessed as described by ESHRE Monographs (Kvist and Björndahl, 2002) with minor changes. Cauda epididymis were disrupted on Modified Tyrode's medium (MT6) containing 125 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂·6H₂O, 0.36 mM NaH₂PO₄·2H₂O, 5.56 mM glucose, 25 mM NaHCO₄, 1.80 mM CaCl₂, 100 units penicillin and 4 mg/ml BSA (Fraser, 1984) and were left for 30 min at 36°C to promote sperm cell's release into the media. Concentration of sperm cells was determined by counting the number of cells on a haemocytometer (Neubauer Improved Chamber). The number of cells in at least two of the corner larger squares (1 mm²) was counted, ignoring the cells on the upper and right boundaries of each square, and the mean value was calculated. The number of sperm cells/mL was calculated by multiplying the mean value by 10⁴.

2.7.3 Motility

An aliquot of sperm cells in MT6 medium was transferred onto a slide. Motility of sperm cells was determined by scoring the number of all progressive spermatozoa and then the non-progressive and the immotile spermatozoa in the same field. For each slide, at least 100 sperm cells were counted and duplicate slides were assessed for each semen sample. The number of sperm cells in each motility group was divided by the total number, and the average of the two slides was assayed according to Kvist and Björndahl (2002).

2.7.4 Viability

Cauda epididymis was disrupted in HEPES-buffered saline solution containing bovine serum albumin (BSA) (10 mM HEPES, 150 mM NaCl, 10% BSA, pH 7.4). A sperm cell

suspension of 200 μL was diluted with 1.8 mL of HEPES-buffered saline, and then 2 μL SYBR-14 (LIVE/DEAD® Sperm Viability Kit, reconstituted in DMSO) were added. The mixture was incubated for 8 min at 36°C, and after that 5 μL of PI were then added to a final concentration of 12.5 μM . The mixture was then incubated for further 8 min at 36°C. Samples were immediately analysed by flow cytometry, using a Coulter EPICS XL flow cytometer (Coulter Electronics, Hialeah, Florida, USA). The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm for excitation. Green fluorescence from SYBR-14 was detected in the FL1 sensor, through a 550-nm dichroic long-pass filter after a 525-nm band-pass filter, while the red fluorescence (PI) was collected in the FL3 sensor through a 645 dichroic long-pass filter and a 620 band-pass filter. Sperm cells were gated based on the forward scatter (FS) vs. side scatter (SS) properties. At least 10000 events were acquired for each sample at a flow rate of less than 200 cell/s. Live cells were PI- and SYBR-14+, and death cells are PI+ and SYBR-14-. Cells presenting a positive pattern for both SYBR-14 and PI were included in the live population. Percentage of live cells was calculated by the ratio of SYBR-14+/ total sperm cells gated in FS vs. SS cytograms.

2.7.5 Mitochondrial function

Evaluation of mitochondrial function of sperm cells was performed as previously described (Papaioannou et al., 1997). Briefly, 1 mg Rh123 /mL in methanol (-20°C) was diluted in water to a concentration of 0.1 mg/mL, before adding 10 μL to 400 μL of sperm cell suspension in MT6 medium. Samples were incubated at 36°C for 30 min, centrifuged at 500 *g* for 5 min and resuspended in fresh medium. Cells were counterstained with 0.1 mg PI /mL in MT6 medium, 5 min before flow cytometric analyses, as described above, with Rh123 being detected in the FL1 sensor. Cells with functionally active mitochondria are Rh123+. Percentage of sperm cells with functional

mitochondria were calculated as the ratio of Rh123+ versus total sperm cells, gated in FS vs SS cytograms.

2.7.6 Acrosome integrity

Acrosome integrity was determined according to Lu and Shur (1997). An aliquot of sperm cells in MT6 medium prepared as described in section 2.7.1 was fixed in 5% formaldehyde in PBS for 30 min at 23°C. Fixed spermatozoa were collected by centrifugation at 1000 g for 5 min and then washed once on 0.1 M ammonium acetate pH 9.0. An aliquot of this suspension was dried on a glass slide and stained for 2 min at 23°C with 0.22% Coomassie blue G250 in a solution containing 50% methanol and 10% glacial acetic acid. After staining, slides were rinsed with tap water, air-dried and mounted. The acrosome integrity was assessed by an intense staining on the anterior region of spermatozoon head under 1000x magnification (Figure 2).

2.8. Morphology of sperm cells

Morphology of sperm cells was assessed as described by Kvist and Björndahl (2002) with minor changes. A drop of sperm cell suspension in MT6 medium was smeared on a slide and air dried. Samples were fixed in 95% ethanol, and stained with haematoxylin-eosin. Spermatozoa morphology was evaluated on bright field microscopy and spermatozoa were classified according to the Wyrobeck and Bruce criteria (1975). The incidence of abnormalities in head, neck/mid-piece and tail were assessed and at least 200 sperm cells were observed under 1000x magnification.

2.9. DNA fragmentation and chromatin status of sperm cells

2.9.1 TUNEL assay

The presence of DNA strand breaks in spermatozoa was evaluated by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL), using

In Situ Cell Death Detection Kit, Fluorescein with slight modifications to the manufacturer's protocol.

Sperm cells were collected as described in section 2.7.1 and after that samples were transferred to conical tubes to allow the sedimentation of larger pieces. Samples were then filtered through a 200 μm nylon mesh to microtubes containing glycerol to a final concentration of 10% (v/v) and kept in an ultra-cold freezer (-80°C) until FCM analysis. Samples were thawed in crushed ice, centrifuged (500 g for 6 min) and washed twice in phosphate buffered saline (PBS) /1% bovine albumin serum (BSA) pH 7.4.

One hundred μL of 1% freshly prepared paraformaldehyde was added to an aliquot of 100 μL of sample and cells were fixed in a rotative shaker for 1 h at room temperature. After that, cells were washed twice in PBS with 1% BSA to remove the fixative and then were permeabilized with 100 μL 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After washing once with PBS with 1% BSA, the supernatant was removed and the labelling reaction was performed by incubation with 50 μL of labelling solution (supplied with the *In situ* Cell Death Detection Kit) containing TdT for 1 h at 37°C in the dark. For each sample, a negative control was prepared by omitting TdT from the reaction mixture. After labelling, two subsequent washes in PBS/1% BSA were performed and the sample was resuspended in the same buffer to a volume of 500–700 μL , counterstained with PI to a final concentration of 0.5 $\mu\text{g}/\text{mL}$, and measured by flow cytometry. At least 10,000 events were analysed for each sample at a flow rate less than 200 cell/s, after gating the debris component in the SS vs. FL3 characteristics of sperm cells.

The following controls were used: as positive control, fixed and permeabilized sperm cells were incubated with 5 U DNase I for 45 min at 37°C before the labelling reaction, in order to induce DNA fragmentation; as negative control, fixed and sperm cells were prepared as above but were incubated with labelling solution without the TdT enzyme.

2.9.2 Sperm Chromatin Structure Assay (SCSA)

The SCSA provides an index of spermatozoa with abnormal chromatin structure as assessed by the susceptibility of sperm cell's DNA to acid-induced denaturation *in situ* (Evenson and Jost, 2000). This assay is based on the properties of acridine orange (AO) that fluoresces green (515-530 nm) when intercalated into native double stranded DNA, and red (>630 nm) when bound to denatured single stranded DNA.

SCSA assay was performed according to the procedure described by Traina et al. (2005). Briefly, the epididymis were removed and minced in a Petri dish containing 2 mL of TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA, pH 7.4) at 4°C. Samples were transferred to conical tubes to allow the sedimentation of larger pieces and then filtered through a 200 µm nylon mesh to microtubes containing glycerol (10% v/v) and kept in an ultra-cold freezer (-80°C), until FCM analysis. Samples were thawed in crushed ice and diluted in TNE buffer until a concentration of 1-2 x 10⁶/mL. Then, 200 µL of acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1 % Triton X-100, pH 1.2) were added to 100 µL of sample. After 30 s sperm cells were stained with 600 µL of AO staining solution containing 6 µg AO per ml of buffer (0.15 M NaCl, 0.1 M citric acid monohydrate, 0.2 M Na₂HPO₄, 1 mM EDTA, pH 6.0). After 3 min of staining, samples were analysed by FCM. At least 10,000 events were accumulated for each sample at a flow rate of less than 200 cell/s. Raw data were exported as Static Dimension data files LMD files (Listmode File Format) and analysed using FlowJo (Tree Star Inc., Ashland, Oregon, USA), as described by Banks et al. (2005). The extent of DNA denaturation was quantified by calculating the %DFI (DNA Fragmentation Index) that is based on the calculation of the ratio red/(red + green) fluorescence. The %DFI indicates the percentage of cells with a larger ratio of red/green fluorescence, indicating abnormal chromatin structure. Also, the standard deviation of DFI (SD-DFI) was computed and it shows the homogeneity of DFI of the spermatozoa sample. In addition, the High DNA

Stainability (%HDS) was calculated representing the percentage of immature sperm cells with incomplete chromatin condensation.

2.10 Statistical analysis

Results are expressed as mean \pm standard error of the mean (SE). Statistical comparisons were performed using SigmaStat for Windows (Version 3.1, SPSS Inc., USA). Comparisons between control and potassium chromate treated groups were performed using one-way analysis of variance (ANOVA), followed by the Tuckey or Student-Newman-Keuls test. Pearson's correlation coefficient (r) was calculated in order to evaluate correlations between the different parameters analysed. The level of statistic significance was set at $p \leq 0.05$.

3. Results

3.1 Chromium quantification

Figure 1 shows chromium accumulation in control and potassium chromate injected mice. Control animals presented levels of 0,15 $\mu\text{g Cr/g fw}$. Chromate injected mice presented higher levels of chromium ($p < 0.001$) on their testis (445% increase for mice injected with 5 mg $\text{K}_2\text{CrO}_4/\text{kg bw}$ and 689% increase for mice injected with 10 mg $\text{K}_2\text{CrO}_4/\text{kg bw}$, $y = 0,5079x - 0,3104$, $r^2 = 0,9719$).

3.2 Histological analysis and tubular diameter

Testis and epididymis histology were not affected by exposure to 5 and 10 mg $\text{K}_2\text{CrO}_4/\text{kg bw}$ for four consecutive days in none of the periods tested.

Image processing for seminiferous tubules diameter measurement with Snakes software is presented in Fig. 2. The tubular contour and both minor and major tubular axes are signalled. The diameter values were presented in a Microsoft Excel worksheet.

Before applying to toxicological studies, the results obtained with both manual measurements with the ocular micrometer and snakes software were compared and a positive correlation was obtained ($r=0.97$, $p<0.0001$) (Fig. 3). Seminiferous tubules of both control and potassium chromate injected mice were then measured with Snakes software. Tubular diameter was significantly reduced by exposure to 10 mg K_2CrO_4/kg bw potassium chromate, 5 days after starting the exposure. After 35 days, tubular diameter was significantly reduced by both chromate doses (Fig. 4).

3.3 FCM analysis of testicular cells

Based on DNA content, testicular cells can be categorized in five distinct subpopulations and four main peaks. The sub-haploid peak (HC) consists of elongated spermatids and the haploid peak (1C) refers to round spermatids. The discrimination of the haploid nuclei in two peaks is due to different staining of elongated and round spermatids, since the former present a highly condensed chromatin and consequently PI intercalation with DNA is lower. Somatic cells, spermatogonia and secondary spermatocytes are included in the diploid peak (2C). Cells in the G2 phase of the cell cycle and primary spermatocytes are included in the tetraploid peak (4C). The region between the diploid and the tetraploid peaks corresponds to cells that are actively synthesising DNA (S phase).

The profiles of the different testicular subpopulations are presented in Table 2. Spermatids (both round and elongated) are the most abundant cell type. This pattern is maintained in all conditions tested, demonstrating that the administration of 5 and 10 mg of potassium chromate/kg bw for four consecutive days did not induce changes on testicular germ cells subpopulations after 5 or 35 days (Table 2).

3.4 Functional parameters of sperm cells

For controls, a significant increase in the density of sperm cells was found between the two time points considered ($p=0.04$). At day 5 of the experiment, control animals presented 6.7 million sperm cells/mL and at day 35 they showed 9.8 million sperm cells/mL. However the parameter spermatozoa density was not significantly affected by the potassium chromate doses tested (Table 3) at both 5 and 35 days periods.

As far as sperm cell's motility is concerned, three categories were considered, namely, progressive, non-progressive and immotile spermatozoa. Both potassium chromate doses (5 and 10 mg /kg bw) induced changes on sperm cell's motility. In mice sacrificed on day 5 of the experiment, a significant decrease in the percentage of sperm cells with progressive motility was found for both treatment doses (5 and 10 mg K_2CrO_4 /kg bw), and conversely an increase in the percentage of immotile sperm cells for the highest chromium dose (Table 3). Mice sacrificed 35 days after starting the experiment showed increased percentages of immotile spermatozoa in those animals injected with the highest chromium dose.

Sperm cell's viability, assayed by the staining with SYBR-14/PI, and mitochondrial function were not significantly affected by any chromium dose in none of the testing periods (Table 3).

Figure 5 shows acrosome staining with Coomassie blue. At day 5 of the experiment, acrosome integrity was not affected in mice injected both with 5 or 10 mg K_2CrO_4 /kg bw. Contrarily to mice sacrificed at day 35, that presented a reduction in the percentage of intact acrosomes being, however, only statistically significant the dose of 10 mg K_2CrO_4 (Fig. 6).

3.5. Spermatozoa morphology

Figure 7 shows considered morphological classification of epididymal sperm cells. Briefly, five types of spermatozoa morphological classifications have been considered:

normal morphology, abnormal head, abnormal midpiece, abnormal tail and multiple abnormalities (head and tail). Mice injected with 5 and 10 mg K_2CrO_4 for four consecutive days and sacrificed at day 5 of the experiment presented an increase in percentage of sperm cells with multiple abnormalities ($p<0.001$) (Fig. 8A). After 35 days a significant decrease ($p<0.05$) in percentage of normal sperm cells was found (Fig. 8B).

3.5 DNA fragmentation and chromatin status of sperm cells

3.5.1 TUNEL

Concerning sperm cells's DNA fragmentation assessed by TUNEL assay, it was detected a tendency to an increase in the percentage of sperm cells with fragmented DNA in chromate injected mice, however in none of the experimental periods the differences reached statistical significance ($p>0.05$) (Table 4). Control samples showed 1.91% and 2.19% cells with DNA fragmentation at days 5 and 35 of the experiment, respectively.

3.5.2 SCSA

Considering SCSA, chromium administration did not induce any change on chromatin condensation, since none of the parameters assayed (%DFI, Mean DFI, SD DFI, %HDS) were significantly affected ($p>0.05$) (Table 4). Control samples presented 2.21%DFI and 1.65% DFI at days 5 and 35 of the experiment, respectively.

4 Discussion

The purpose of this study was to evaluate the immediate effects of an exposure to two levels of chromium(VI) one day after a short period of exposure (4 days) on testis and sperm cell's function and also to study the effects after one entire spermatogenic cycle

(35 days). A similar approach has been conducted previously by Ernst and Bonde (1992) on their experimental studies on rats exposed to tri-and hexavalent chromium.

Testis and epididymis histology was not affected by 4 days exposure to 5 and 10 mg potassium chromate/kg bw for neither at day 5 nor 35 of sacrifice. However previous studies refer histological change in the testis after chromium exposure (Table 1). Ernst (1990) referred seminiferous epithelium degeneration in rats exposed to 1, 2 and 4 mg of Cr(VI)/kg bw for 5 days. In another study, Chandra and co-workers (2007) detected degenerative changes in seminiferous epithelium and Leydig cells in rats administered with 0.6 mg potassium dichromate/kg bw for 26 days. In rats exposed to chromium trioxide, Li and co-workers (2001) detected changes in seminiferous epithelium cell organization. In mice exposed to chromium(V) as [CrV-BT]²⁻ changes in blood-testis barrier permeability were detected (Pereira et al., 2002), as well as alterations in testis histoarchitecture with disorganization, degeneration, necrosis and vacuolization of germ cells and also in epididymis epithelium cells (Pereira et al., 2004; 2005).

None of potassium chromate doses tested in these assays induced changes on relative percentages of germ cells evaluated by FCM. This result is in agreement with the histological observations.

In the present work, a decrease in the seminiferous tubules diameter was found in mice injected with 10 mg K₂CrO₄/kg bw and sacrificed after 5 days, as well as in mice injected with both chromate doses and sacrificed after 35 days. Reduction of seminiferous tubule diameter seems to be a current response to Cr(VI) toxicity, since it has been detected in monkeys (Aruldas et al., 2005) and rats (Chandra et al., 2007) exposed to potassium dichromate and in rats exposed to chromium trioxide (Li et al., 2001).

Mice administered with 5 and 10 mg K₂CrO₄/kg bw presented higher levels of chromium on their testis than on respective controls. Murthy and co-workers (1991) also found

higher levels of chromium on testis of rats administered with 2 mg potassium dichromate/kg bw for 15 days.

In the present work, concentration of sperm cells was not affected by exposure to chromium in none of the experimental periods. In the first period this effect could be expected since the time delay from the beginning of exposure until sacrifice was short and could be not enough to induce effects on sperm cell numbers. On the second period, changes on sperm cell numbers could be expected since this element in this particular oxidation state could affect spermatogenesis and thus altering spermatozoa production, as has been previously referred (Li et al., 2001; Acharya et al., 2004; 2006). However, no statistically significant changes in the concentration of sperm cells were found, suggesting that only higher doses and/or longer periods of chromium(VI) exposure may induce changes.

Despite density of sperm cells was not affected, an increase on the percentage of multiple abnormalities after 5 days was observed in both doses of chromium, and a decrease in the percentage of normal sperm cells on the day 35 was also found. Changes on spermatozoa morphology due to chromium have been previously described in both mice (Acharya et al. 2004; 2006) and rats (Li et al., 2001). Changes on spermatozoa motility were observed in both periods, however a high degree of change was found after 5 days with a decrease in the percentage of spermatozoa with progressive motility in both chromium concentrations, and, simultaneously, an increase in the percentage of immotile spermatozoa. Also, there was a strong negative correlation between the sum of progressive and non-progressive sperm cells and chromium dose ($r=-1$; $p=0.00518$) meaning that decrease in spermatozoa motility was chromium dose dependent. A decrease in spermatozoa motility as a result of chromate exposure has been previously reported in rats exposed to sodium chromate (Ernst and Bonde, 1992) and in rats

(Murthy et al., 1991), rabbits (Yousef et al., 2006) and Bonnet monkeys exposed to potassium dichromate (Subramanian et al., 2006).

Although spermatozoa motility was affected, other parameters such as viability or mitochondrial function were not significantly affected ($p>0.05$) on this period, but there was a tendency to a decrease with chromium dose. For mitochondrial function a very strong correlation with chromium dose was found ($r=-0.999$; $p=0.0255$). Furthermore, a positive correlation between mitochondrial function and the sum of progressive and non-progressive spermatozoa was denoted ($r=1$; $p=0.00518$). In the second period of sacrifice (35 days), an increase in the percentage of immotile sperm cells in mice administered with 10 mg K_2CrO_4/kg bw was found. As stated for the period of 5 days, this effect was not accompanied by statistical significant changes in spermatozoa mitochondrial function or viability, in spite of a tendency to a decrease with chromium dose for both parameters. For mitochondrial function a negative correlation with chromium dose ($r=0.997$; $p=0.05$) was also observed. Mitochondrial function and spermatozoa viability were also positive correlated ($r=0.997$; $p=0.049$). Spermatozoa viability and mitochondrial function were also positively correlated with the sum of progressive and non-progressive sperm cells ($r=1$; $p=0.0191$ and $r=0.999$; $p=0.0314$, respectively). Reactive oxygen species (ROS) generated during Cr(VI) reduction may induce lipid oxidative damage and may indirectly interfere with spermatozoa motility (Baumer et al., 2000; Wathes et al., 2007). ROS may also reduce spermatozoa motility by reducing phosphorylation of axonemal proteins, essential for spermatozoa movement (de Lamirande and Gagnon, 1992). A decrease in ATP levels due to ROS may also occur without reduction of mitochondrial potential (Armstrong et al., 1999). This may explain the decrease in spermatozoa motility induced by potassium chromate without a corresponding change on mitochondrial function. Furthermore, recently Oliveira et al.

(2009) also found a decrease in spermatozoa motility in mice exposed to lead chloride without any detectable change in viability or mitochondrial function.

Concerning acrosome integrity, a decrease in the percentage of intact acrosomes was found after 35 days on sperm cells of mice administered with 10 mg K_2CrO_4/kg bw, indicating that a premature acrosome reaction has occurred only for the higher chromium dose. This effect has been previously shown in mice injected with a 8 μmol chromium(V) compound, namely $[CrV-BT]^{2-}$ (Pereira et al., 2005).

Previous studies showed that in the process of intracellular chromium(VI) reduction DNA fragmentation occurs (Stearns et al., 1983; Tsapakos et al., 1983). In the present work, the effects of potassium chromate $[Cr(VI)]$ on mice spermatozoa DNA fragmentation were evaluated. Sperm cells from mice exposed to potassium chromate showed higher levels of DNA fragmentation detected by TUNEL assay, more visible after 35 days. However, none of these differences reached the level of statistical significance ($p>0.05$). Nevertheless, at day 35 the percentage of cells with fragmented DNA were highly correlated with chromium dose ($r=1$; $p=0.0106$). DNA fragmentation is also correlated with the presence of multiple abnormalities in sperm cells ($r=0.998$; $p=0.0351$). At day 5 a highly negative correlation between percentage of TUNEL positive cells and cells with normal morphology was found ($r=-1.0$; $p=0.00527$).

Normal spermatozoa development leads to a chromatin structure in which DNA is resistant to denaturation, contrarily to DNA with abnormal chromatin structure that is susceptible to acid DNA denaturation. The susceptibility of chromatin to acid denaturation was measured by SCSA. As showed by the TUNEL assay, K_2CrO_4 exposure did not induce DNA damage assayed by SCSA. At day 5 a negative correlation was found between %HDS and percentage of progressive sperm cells ($r=-1.0$; $p=0.0183$). The association between motility and the size of HDS fraction has been reported elsewhere (Giwerzman et al., 2003). HDS fraction corresponds to spermatozoa

nuclei that are not fully condensed allowing thus higher accessibility to acridine orange. In the epididymis sperm cells acquire capacity for progressive motility and it is also in the epididymis that spermatozoa chromatin is stabilized due to oxidation of cystein free thiol groups providing a more rigid structure.

In conclusion, in this work we were able to show the effects of potassium chromate on morphology and physiological parameters of sperm cells, such as motility and acrosome status. This paper also demonstrates that the doses tested did not induce DNA damage to sperm cells either after exposure or after one full spermatogenic cycle.

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References

- Acharya UR, Mishra M, Mishra I, Tripathy RR. Potential role of vitamins in chromium induced spermatogenesis in Swiss mice. *Environ Toxicol Pharmacol* 2004; 15: 53-59
- Acharya UR, Mishra M, Tripathy RR, Mishra I. Testicular dysfunction and antioxidative defense system of Swiss mice after chromic acid exposure. *Reprod Toxicol* 2006; 22:87-91
- Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological profile for chromium. Atlanta: U.S. Department of Health and Human Services; 2000
- Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ, Sikka SC. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic Biol Med*. 1999;26:869-80.
- Banks S, King SA, Irvine DS, Saunders PT. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. *Reproduction* 2005; 29:505-514.
- Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation *J Androl*. 2000;21:895-902.
- Belagyi J, Pas M, Raspor P, Pesti M, Pali T. Effect of hexavalent chromium on eukaryotic plasma membrane studied by EPR spectroscopy. *Biochim Biophys Acta* 1999;1421:175-82
- Bonde JP, Ernst E. Sex hormones and semen quality in welders exposed to hexavalent chromium. *Hum Exp Toxicol*. 1992;11: 259-63.
- Chandra AK, Chatterjee A, Ghosh R, Sarkar M, Chaube SK. Chromium induced testicular impairment in relation to adrenocortical activities in adult albino rats. *Reprod Toxicol*. 2007; 24:388-96.
- Costa M, Klein CB. Toxicity and carcinogenicity of chromium compounds in humans. *Crit Rev Toxicol*. 2006;36:155-63.

Chiu A, Katz AJ, Beaubier J, Chiu N, Shi X. Genetic and cellular mechanisms in chromium and nickel carcinogenesis considering epidemiologic findings. *Mol Cell Biochem* 2004; 255:181-94

de Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. *J Androl*. 1992;13:368-78.

Ding M, Shi X. Molecular mechanisms of Cr(VI)-induced carcinogenesis *Mol Cell Biochem* 2002; 234: 235:293-300

Ernst E, Bonde JP. Sex hormones and epididymal sperm parameters in rats following sub-chronic treatment with hexavalent chromium. *Hum Exp Toxicol*. 1992; 11: 255-8.

Ernst E. Testicular toxicity following short-term exposure to tri- and hexavalent chromium: an experimental study in the rat. *Toxicol Lett* 1990; 51: 269-75.

ESHRE Monographs: Manual on Basic semen Analysis. Sperm Morphology pp:13-17, 2002

Evenson D, Jost L. Sperm chromatin structure assay is useful for fertility assessment. *Methods in Cell Sci*. 2000; 22:169-189.

Figà-Talamanca I, Petrelli G, Tropeano R, Papa G, Boccia G. Fertility of male workers of the italian mint. *Reprod Toxicol* 2000; 14:325-30.

Figà-Talamanca I, Traina ME, Urbani E. Occupational exposures to metals, solvents and pesticides: recent evidence on male reproductive effects and biological markers. *Occup Med* 2001; 51: 174-88.

Fraser LR: Mouse sperm capacitation *in vitro* involves loss of a surface-associated inhibitory component. *J Reprod Fert*. 1984; 72: 373-384.

Giwercman A, Richthoff J, Hjollund H, Bonde JP, Jepson K, Frohm B, Spano M. Correlation between sperm motility and sperm chromatin structure assay parameters. *Fertil Steril* 2003; 80: 1404-12

Hjollund NH, Bonde JP, Jensen TK, Ernst E, Henriksen TB, Kolstad HA, *et al*. Semen quality and sex hormones with reference to metal welding. *Reprod Toxicol*. 1998; 12: 91-95.

- Kumar S, Sathwara NG, Gautam AK, Agarwal K, Shah B, Kulkarni PK, *et al.* Semen quality of industrial workers occupationally exposed to chromium J Occup Health. 2005 Sep;47(5):424-30.
- Li H, Chen Q, Li S, Yao W, Li L, Shi X, *et al.* Effect of Cr(VI) exposure on sperm quality: human and animal studies. Ann Occup Hyg 2001; 45: 505-11.
- Lu Q, Shur BD. Sperm from β 1,4-galactosyltransferase-null mice are refractory to ZP3-induced acrosome reactions and penetrate the zona pellucida poorly. Development. 1997; 124: 4121-31.
- O'Brien TJ, Ceryak S, Patierno SR. Complexities of chromium carcinogenesis: role of cellular response, repair and recovery mechanisms. Mutat Res. 2003; 533: 3-36.
- Oliveira H, Spanò M, Santos C, Pereira ML. Lead chloride affects sperm motility and acrosome reaction in mice. Cell Biol Toxicol 2009
<http://dx.doi.org/10.1007/s10565-008-9088-4>.
- Papaioannou KZ, Murphy RP, Monks RS, Hynes N, Ryan MP, Boland MP, Roche JF. Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cytometry. Theriogenology. 1997; 48:299-312.
- Pereira ML, das Neves RP, Oliveira H, Santos TM, de Jesus JP. Effect of Cr(V) on reproductive organ morphology and sperm parameters: an experimental study in mice. Environ Health 2005; 27: 4-9.
- Pereira ML, Santos TM, das Neves RP, Costa FG, de Jesus JP. Cr(V) involvement in the toxicity pathway of testicular damage. Asian J Androl. 2002; 4: 153-5.
- Pereira ML, Santos TM, Garcia e Costa F, de Jesus JP. Functional changes of mice Sertoli cells induced by Cr(V). Cell Biol Toxicol. 2004; 20:285-91
- Shi X, Chiu A, Chen CT, Halliwell B, Castranova V, Vallyathan V. Reduction of chromium(VI) and its relationship to carcinogenesis. J Toxicol Environ Health B. 1999; 2: 87-104
- Subramanian S, Rajendiran G, Sekhar P, Gowri C, Govindarajulu P, Aruldas MM. Reproductive toxicity of chromium in adult bonnet monkeys (*Macaca radiata* Geoffrey).

Reversible oxidative stress in the semen. *Toxicol Appl Pharmacol.* 2006 Sep 15; 215: 237-49

Traina ME, Guarino M, Urbani E, Saso L, Eleuteri P, Cordelli E, Rescia M, Leter G, Spanò M. Lonidamine transiently affects spermatogenesis in pubertal CD1 mice. *Contraception.* 2005; 72:262– 267

Wathes DC, Abayasekara DR, Aitken RJ. Polyunsaturated fatty acids in male and female reproduction. *Biol Reprod.* 2007;77:190-201.

Wise SS, Holmes AL, Wise JP Sr. Hexavalent chromium-induced DNA damage and repair mechanisms. *Rev Environ Health.* 2008;23:39-57.

Wyrobek AJ, Bruce WR. Chemical induction of sperm abnormalities in mice. *Proc Natl Acad Sci U S A.* 1975; 72: 4425-9.

Yousef MI, El-Demerdash FM, Kamil KI, Elswad FA. Ameliorating effect of folic acid on chromium(VI)-induced changes in reproductive performance and seminal plasma biochemistry in male rabbits. *Reprod Toxicol.* 2006 21:322-8

Zhitkovich A. Importance of chromium-DNA adducts in mutagenicity and toxicity of chromium(VI). *Chem Res Toxicol.* 2005; 18:3-11.

Table 1 Studies of chromium effects on rodent sperm cells.

Strain	Reference	Age at start	Dose	Duration of exposure	Effects
Schedule					
Mice:					
<i>ICR-CD1</i>	Pereira et al. (2005)	60 days old	[Cr(VI)-BT] ²⁻ 8 µmol of Cr/mouse, during 5 days	Sacrifice after 24 h	Decrease in acrosome integrity; Degenerative changes on Sertoli cells and spermatid sloughing toward the lumen; Germ cell necrosis and vacuolization.
<i>Swiss Albino</i>	Acharya et al. (2004)	9-10 weeks	10mg CrO ₃ /kg ip	Sacrifice after 5-8 weeks post-injection	Decrease sperm cell concentration; Increase sperm cell abnormality.
	Acharya et al. (2006)	10 weeks	1mg CrO ₃ /kg bw i.p.	Sacrifice after 5- 8 weeks post-injection	Increase in lipid peroxidation; Increase in sperm cell abnormality; Decrease in sperm cell counts; Decrease in ascorbic acid, Decrease catalase, peroxidase and SOD activities.
Rats:	Ernst (1990)	100-120 days old	1, 2 and 4 mg Cr(III) or Cr(VI)/kg bw ip for 5 consecutive days	Sacrifice 7 and 60 days after last administration	Less weight gain in animals treated with Cr(III) and Cr(VI); Absence of histopathological changes in rats sacrificed after 7 days; In rats sacrificed after 60 days: -Decrease in testis weight in rats exposed to Cr(VI); -Seminiferous tubules atrophy, -Loss of germinative epithelium; -Changes on testis organization; -Leydig cells atrophy.

Table 1 Continued

Strain	Reference	Age at start	Dose Schedule	Duration of exposure	Effects
Rats:					
<i>Male Wistar</i>	Ernst and Bonde (1992)	100-120 days old	0.5 mg Na ₂ CrO ₄ /kg bw 5 days a week for 8 weeks	One experimental group was sacrificed at the end of exposure and another was sacrificed after 8 weeks (without exposure)	The group sacrificed after treatment showed: -Decrease in sperm cell motility; -No changes in morphology of sperm cells; The group sacrificed after 8 weeks showed: -No differences on motility of sperm cells.
	Li et al. (2001)	60 days	0, 10 and 20 mg CrO ₃ /kg /day Give orally for 6 days	Sacrifice after 6 weeks	Decrease in epididymal numbers of sperm cells; Increase in abnormality of sperm cells; Decrease in seminiferous tubules diameter; Changes in seminiferous epithelium.
<i>Sprague Dawley</i>	Chandra et al (2007)	200 g	0.2; 0.4 and 0.6 mg K ₂ Cr ₂ O ₇ /kg bw /day for 13 or 26 days	Sacrifice 24 hours after last administration	Less increase in body weight of rats treated with K ₂ Cr ₂ O ₇ ; Decrease in testis weight in rats exposed to 0,6 mg K ₂ Cr ₂ O ₇ (26 days); Reduction of seminiferous tubules diameter in rats exposed to 0,4 mg (26 days) and 0,6 mg K ₂ Cr ₂ O ₇ (13 and 26 days); Sloughing of seminiferous epithelium in rats exposed to 0,6 mg (26 days); Spermatocyte disintegration, tubular necrosis and Leydig cell degeneration in rats exposed to 0,4 and 0,6 mg K ₂ Cr ₂ O ₇ ; Increase in lipid peroxidation in testis

Table 2 Percentage of germ cells isolated from testis of control and exposed to 5 and 10 mg of K_2CrO_4/kg bw mice. Percentages were estimated as number of nuclei in each ploidy level/total number of nuclei $\times 100$. Mean values \pm SE.

	5d			35d		
	Control	5 mg	10 mg	Control	5 mg	10 mg
HC	31.3 \pm 0.7	31.7 \pm 1.1	30.8 \pm 2.5	34.4 \pm 1.1	33.5 \pm 1.3	32.8 \pm 0.9
1C	42.0 \pm 0.5	40.6 \pm 0.5	41.4 \pm 1.1	40.0 \pm 1.3	41.3 \pm 0.9	41.1 \pm 1.8
2C	10.2 \pm 0.1	10.6 \pm 0.5	11.5 \pm 1.4	11.5 \pm 0.9	10.8 \pm 0.4	11.0 \pm 0.6
S phase	4.8 \pm 0.3	4.9 \pm 0.1	5.2 \pm 0.3	5.1 \pm 0.2	5.2 \pm 0.3	5.9 \pm 0.7
4C	11.7 \pm 0.6	12.1 \pm 0.7	11.1 \pm 0.4	8.9 \pm 0.3	9.2 \pm 0.5	9.2 \pm 0.5

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Table 3 Effects of K_2CrO_4 on sperm cell's density, motility, viability and mitochondrial function, 5 and 35 days after start of exposure. Mean values \pm SE * $p < 0.05$; ** $p < 0.001$.

	5d			35d		
	Control	5 mg	10 mg	Control	5 mg	10 mg
Density ($\times 10^6$)						
Progressive	6.7 \pm 1.19	5.6 \pm 0.81	5.18 \pm 0.98	9.8 \pm 0.76	8.9 \pm 1.52	11.1 \pm 2.50
Non-progressive	40.8 \pm 1.64	28.2 \pm 2.19**	24.0 \pm 2.17**	37.0 \pm 2.42	38.2 \pm 2.80	34.7 \pm 1.12
Motility (%)						
Non-progressive	6.7 \pm 0.78	12.5 \pm 1.93	8.5 \pm 1.78	15.7 \pm 1.64	10.0 \pm 1.83*	10.6 \pm 0.8*
Immotile	52.5 \pm 1.93	59.3 \pm 3.04	67.5 \pm 2.43**	47.2 \pm 2.10	51.8 \pm 2.94	54.7 \pm 1.5*
Viability (%)						
PI⁺SYBR⁺/PI⁺SYBR⁺	37.3 \pm 5.40	34.1 \pm 6.23	34.3 \pm 4.64	37.2 \pm 2.14	34.3 \pm 1.6	32.6 \pm 2.32
Mitochondrial Function (%)						
PI⁺Rh⁺/PI⁺Rh⁺	15.9 \pm 1.09	15.3 \pm 1.38	14.7 \pm 0.5	18.8 \pm 3.11	16.20 \pm 1.91	14.2 \pm 0.9

Table 4 Effects of K₂CrO₄ on sperm cell's DNA fragmentation assessed by the TUNEL assay and chromatin status assessed by SCSA 5 and 35 days after start of exposure. Mean values ± SE.

	<i>5d</i>			<i>35 d</i>		
	Control	5 mg	10 mg	Control	5 mg	10 mg
DFI (%)	2.21 ± 0.27	2.26 ± 0.39	2.82 ± 0.48	1.65 ± 0.25	2.15 ± 0.22	1.92 ± 0.31
Mean DFI	0.24 ± 0.003	0.23 ± 0.002	0.24 ± 0.002	0.23 ± 0.005	0.24 ± 0.002	0.23 ± 0.004
SD DFI	8.77 ± 0.69	8.26 ± 0.92	8.83 ± 0.88	6.75 ± 0.34	6.84 ± 0.72	6.87 ± 0.48
HDS (%)	3.27 ± 0.49	3.75 ± 0.42	3.93 ± 0.54	3.03 ± 0.18	3.23 ± 0.17	3.51 ± 0.33
TUNEL (%)	1.91 ± 0.35	2.23 ± 0.40	2.24 ± 0.20	2.19 ± 0.15	2.83 ± 0.89	3.51 ± 0.88

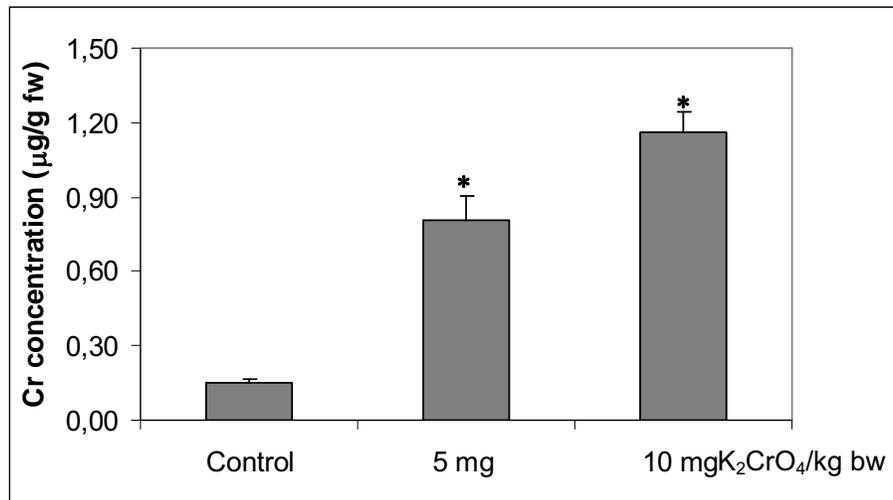


Figure 1

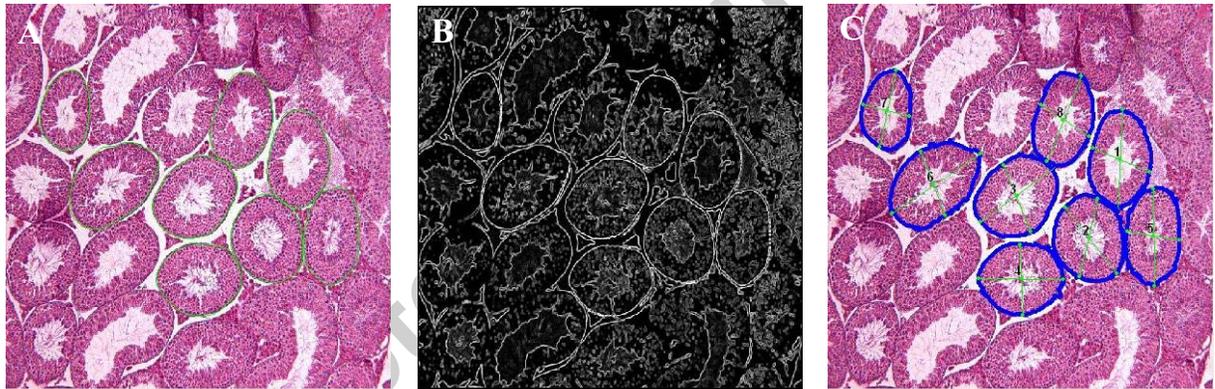


Figure 2

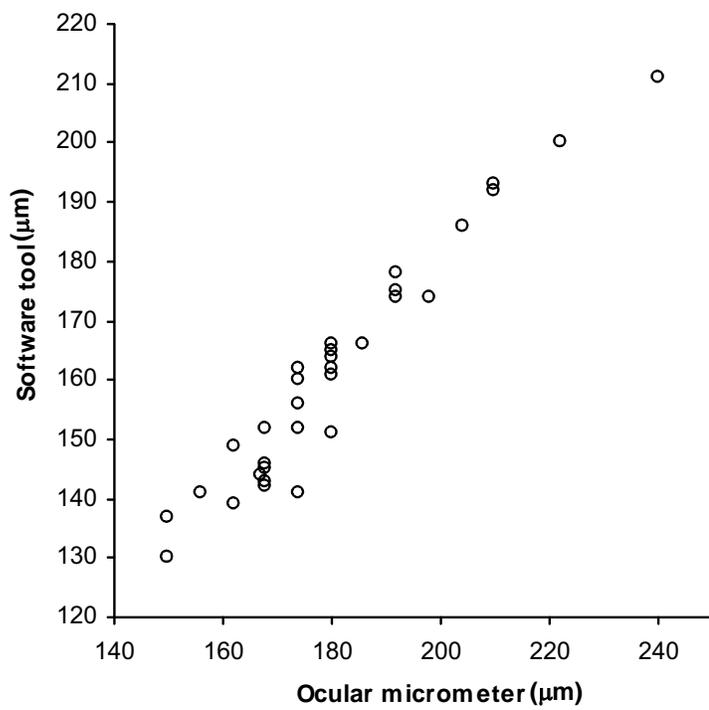


Figure 3

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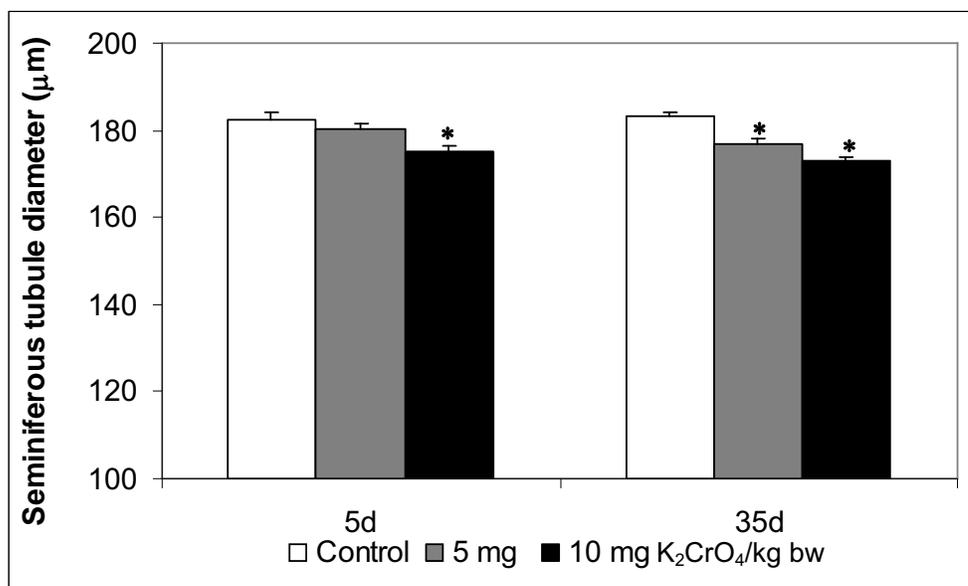


Figure 4

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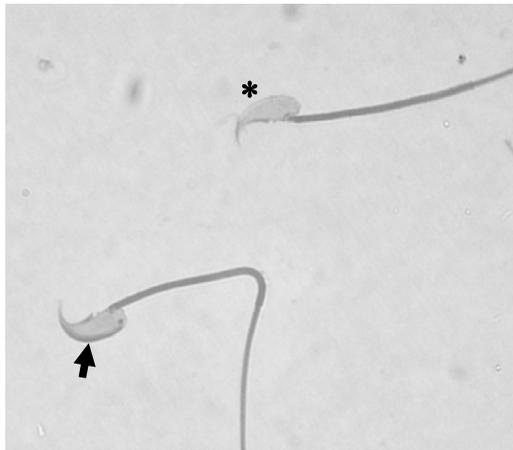


Figure 5

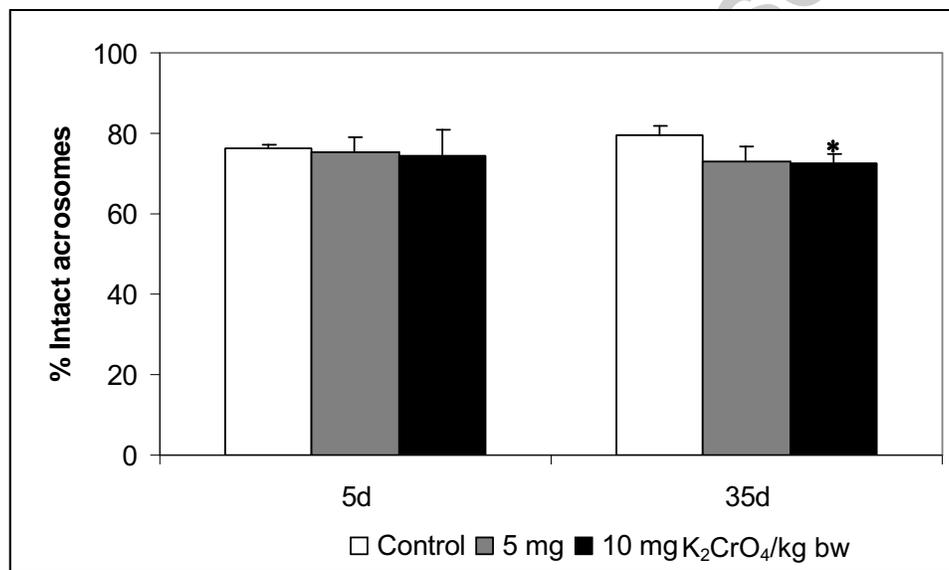


Figure 6

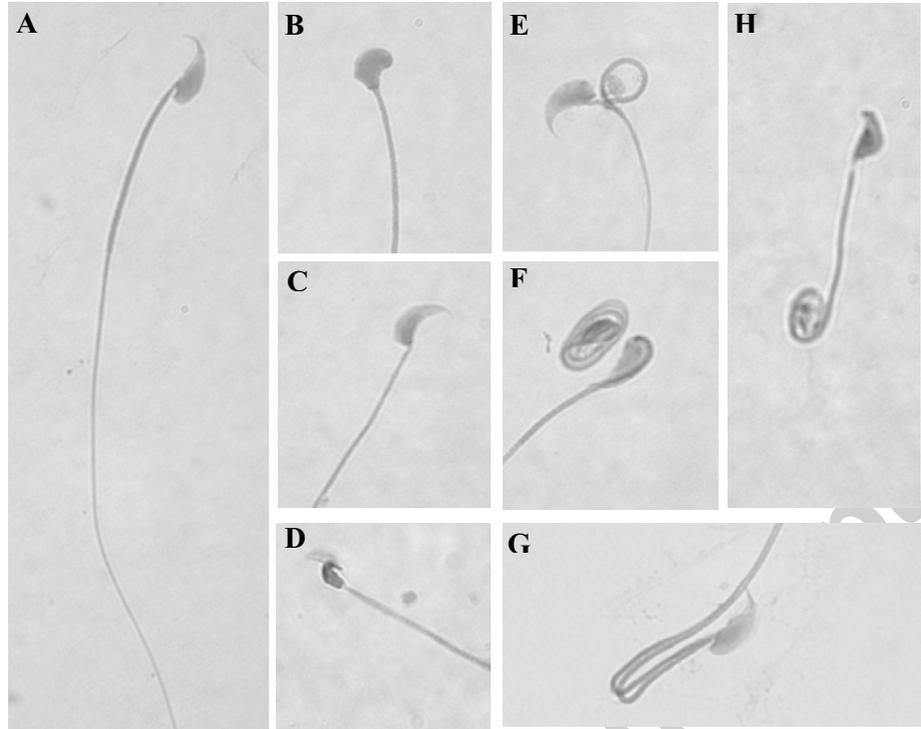


Figure 7

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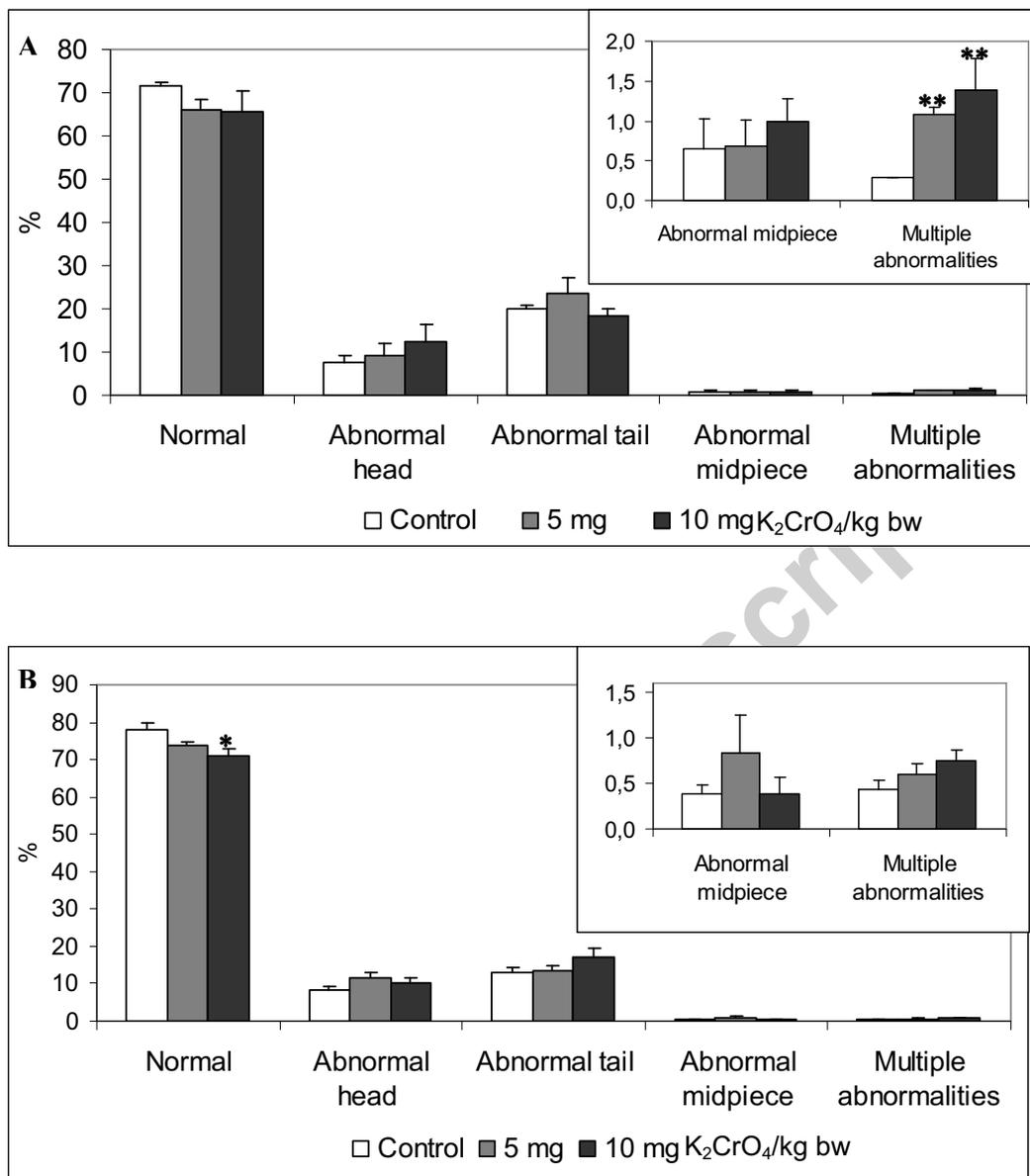


Figure 8

Legend to figures

Figure 1 Effects of potassium chromate on chromium accumulation in mice testis.

Figure 2 Snake deformation: (A) initial snakes, (B) pre-processed image and (C) final snakes.

Figure 3 Pearson's correlation between the measurements obtained with the software tool and with the ocular micrometer.

Figure 4 Effects of potassium chromate on seminiferous tubules diameter. Mean values \pm SE. * $p < 0.001$

Figure 5 Mice sperm cell with intact acrosome intensely stained with Coomassie blue (\rightarrow) is easily distinguished from a sperm cell with reacted acrosome (*).

Figure 6 Effects of potassium chromate on sperm cell's acrosome integrity. Mean values \pm SE. * $p < 0.05$

Figure 7 Morphological classification of epididymal sperm cells from control and potassium chromate exposed mice. A) Normal sperm cell, B-D) Abnormal head, E) Abnormal midpiece, F, G) Abnormal tail, H) Multiple abnormalities (head and tail).

Figure 8 Effects of potassium chromate on sperm cell's morphology at day 5 (A) and 35 (B) of the experiment. Mean values \pm SE. * $p < 0.05$; ** $p < 0.001$