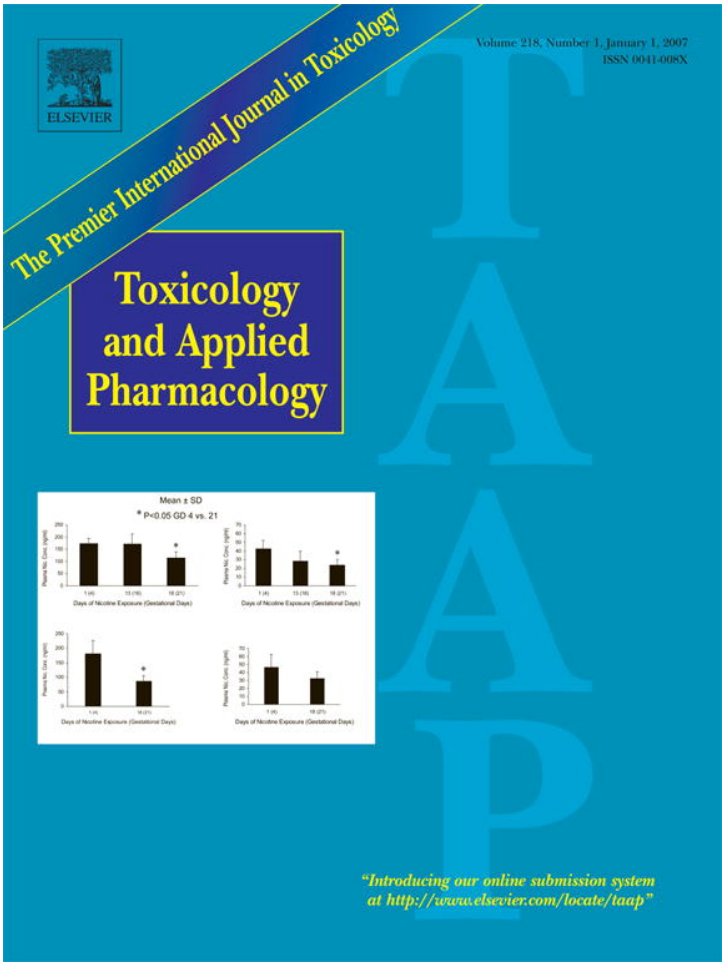

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In vivo and *in vitro* effects of chromium VI on anterior pituitary hormone release and cell viability

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Abstract

Hexavalent chromium (Cr VI) is a highly toxic metal and an environmental pollutant. Different studies indicate that Cr VI exposure adversely affects reproductive functions. This metal has been shown to affect several tissues and organs but Cr VI effects on pituitary gland have not been reported. Anterior pituitary hormones are central for the body homeostasis and have a fundamental role in reproductive physiology. The aim of this study was to evaluate the effect of Cr VI at the pituitary level both *in vivo* and *in vitro*. We showed that Cr VI accumulates in the pituitary and hypothalamus, and decreases serum prolactin levels *in vivo* but observed no effects on LH levels. In anterior pituitary cells in culture, the effect of Cr VI on hormone secretion followed the same differential pattern. Besides, lactotrophs were more sensitive to the toxicity of the metal. As a result of oxidative stress generation, Cr VI induced apoptosis evidenced by nuclear fragmentation and caspase 3 activation. Our results indicate that the anterior pituitary gland can be a target of Cr VI toxicity *in vivo* and *in vitro*, thus producing a negative impact on the hypothalamic–pituitary–gonadal axis and affecting the normal endocrine function.

Keywords: Hexavalent chromium; Anterior pituitary; Hormone; Cytotoxicity; Apoptosis

Introduction

Chromium (Cr) is a naturally occurring metal present principally in trivalent and hexavalent forms in trace amounts in the environment. While Cr III is an essential nutrient, Cr VI is highly toxic and a strong oxidizing agent (O'Brien et al., 2003; Zayed and Terry, 2003). Chromium VI compounds are extensively used in many industries such as tannery, welding, metal finishing, paint and pigment production, and wood preservation, so exposure to this metal is sometimes inevitable. Also, like all metals, Cr is non-biodegradable, persisting in the environment. Thus, it has become a widespread environmental contaminant (Costa, 2003; IARC, 1990; Zayed and Terry, 2003).

Occupational exposure to Cr VI compounds is associated with several adverse effects on health such as lung toxicity and bronchial asthma, and it also causes nephro- and hepatotoxicity (Bright et al., 1997; Costa, 1997; Dartsch et al., 1998; IARC, 1990). It has been shown to affect several other tissues and organs such as the brain but no reports have studied Cr accumulation and/or toxicity in the pituitary gland (Travacio et al., 2000). Related studies have demonstrated that Cr adversely affects reproductive functions (Elbetieha and Al-Hamood, 1997; Kumar, 2004). Deterioration of sperm morphology and motility has been observed among welders and mild steel welders (Bonde, 1990; Kumar, 2004). However, since occupational studies generally lack information concerning confounding factors, it is difficult to attribute these effects to a specific metal. Yet, animal studies have shown that Cr VI exposure affects ovarian and testicular physiology (Acharya et al., 2006; Ernst, 1990; Sutherland et al., 2000; Elbetieha and Al-Hamood, 1997; Murthy et al., 1996).

Although the metal seems to act directly on reproductive organs, it could also affect pituitary hormone secretion. Nevertheless, little is known about the effect of Cr on hormone release and there are no reports on the effect of Cr on anterior

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pituitary. This question is essential considering that altered pituitary hormone secretion may increase the vulnerability of reproductive organs to the metal and contribute to its toxic effect.

At physiological pH, Cr VI exists as a chromate oxyanion and can readily cross cell membranes through the sulfate anion transport system (O'Brien et al., 2003). Once inside the cell, Cr VI is reduced to the ultimate reduced form Cr III. During this process, the reactive chromium intermediates Cr V and Cr IV, and diverse reactive oxygen species (ROS) are generated. ROS as well as the Cr intermediates, which could directly bind to cellular constituents, may explain the cytotoxicity of the metal (O'Brien et al., 2003; Harris and Shi, 2003; Bal and Kasprzak, 2002). It has been suggested that Cr VI exposure results in cell cycle arrest, neoplastic transformation or apoptosis induction depending on cell type, concentration of the metal and time of exposure (Pritchard et al., 2001; Waalkes et al., 2000; Carlisle et al., 2000). Different studies indicate the involvement of multiple pathways involved in Cr VI toxicity but the exact mechanisms have yet to be entirely clarified (Pulido and Parrish, 2003).

Concern has been expressed about Cr VI as an environmental pollutant due to the high levels found in nature as a result of anthropogenic activities (Costa, 2003; IARC, 1990; Zayed and Terry, 2003). Considering the diverse evidence suggesting an adverse effect of Cr VI on reproductive function and the role of anterior pituitary hormones in reproductive organs, the aim of this study was to investigate the effect of Cr VI on anterior pituitary gland. We performed *in vivo* studies to evaluate Cr accumulation in pituitary and serum hormone levels in rats receiving Cr VI in drinking water. In addition we ran *in vitro* studies to evaluate the effect of Cr VI on cell viability and hormone secretion in anterior pituitary cells in culture. We have now demonstrated, for the first time, that Cr VI accumulates in the pituitary gland. Cr VI affected prolactin secretion but had no effect on LH release, *in vivo* and *in vitro*. Cr VI was cytotoxic for anterior pituitary cells in culture, an effect due to apoptosis and oxidative stress generation.

Materials and methods

Drugs and reagents. Potassium dichromate ($K_2Cr_2O_7$) was purchased from Cicarelli, Argentina. *N*-acetyl-DEVD-paranitroanilide, Ac-DEVD-pNA and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were purchased from Alexis, San Diego, CA, USA. Reagents for cell culture were purchased from Gibco, Rockville, MD, USA; GenSA, Buenos Aires, Argentina and Sigma-Aldrich of Argentina, Buenos Aires, Argentina. Anti-gammaglobulin for radioimmunoassay was purchased from Dr. Enriori Lab., Buenos Aires, Argentina. All other drugs were obtained from Sigma-Aldrich of Argentina, Buenos Aires, Argentina.

Animals. Adult male Wistar rats (250–280 g), kept on a 12 h light–dark cycle with controlled temperature (20–22 °C), were used. Animals were maintained in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Food was supplied *ad libitum*.

Chromium treatment. Fifteen animals received 500 ppm of Cr VI (as potassium dichromate) in drinking water for 30 days (treated group). Fifteen animals receiving tap water were used as controls. Water consumption was measured every 2 days and animal weight was determined once a week. On day 30, animals were killed by decapitation and pituitary glands, hypothalami and livers were removed. Trunk blood was collected for prolactin and luteinizing hormone (LH) determination. According to the U.S. EPA, the LOAEL (lowest-observed-adverse-effect level) for Cr VI is 37 mg/kg-day. In the absence of other studies on the ability of the pituitary to accumulate Cr, we used a higher concentration than the LOAEL in order to ensure detection of any change in the gland.

Chromium content. Pituitaries, hypothalami and livers from control and Cr VI-treated animals were washed, blotted dry and weighed. Fifteen pituitaries of each treatment group were pooled. Hypothalami and livers were also pooled. Samples were mineralized with concentrated nitric acid using a microwave Milestone 120 ml. Total chromium content was determined by atomic absorption spectrophotometry (atomic absorption spectrophotometer, Varian 840 with Zeeman correction with GTA 100 graphite furnace and automatic seeder). Chromium levels were expressed as μg Cr/g tissue.

Cell culture. Animals were killed by decapitation and anterior pituitary glands removed. Five to eight pituitaries were pooled and cells were obtained from the glands by enzymatic (trypsin/DNase) and mechanical dispersion (extrusion through a Pasteur pipette) as described previously (Velardez et al., 2004). In all cases the cells were cultured for 3 days (37 °C, 5% CO_2 in air) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 μl/ml MEM amino acids, 2 mM glutamine, 5.6 μg/ml amphotericin B, and 25 μg/ml gentamicin (DMEM-S-10% FBS). Cell viability assessed by trypan blue exclusion was above 90%. For cell activity experiments, cells were seeded onto 96-well tissue culture plates (0.1×10^6 cells/well). For cytochemical studies, cells were seeded on glass coverslips onto 24-well tissue culture plates (0.1×10^6 cells/well). For caspase activity and flow cytometry studies, cells were seeded onto 24-well tissue culture plates (1×10^6 cells/well).

Cell treatment. To study the effect of Cr VI, cells were incubated for different time periods in DMEM-S-10% containing increasing concentrations of the metal.

To study the reversibility of the effect of Cr VI, cells were incubated with the metal for different time lapses in DMEM-S-10%. After each incubation period, the medium was replaced by fresh medium without Cr VI and the incubation proceeded to complete 48 h (total incubation time).

Cell activity assay. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to determine cell activity as described previously (Velardez et al., 2004). In brief, cells were washed twice with Krebs Ringer bicarbonate buffer and then incubated with 110 μl of a MTT solution (500 μg/ml) for 4 h at 37 °C. After incubation, 90 μl of the medium were removed, 100 μl of 0.04 M HCl in isopropanol were added to each well, and the plate was gently shaken for 3 min. Optical density was determined at 600 nm in an ELISA plate reader. Cell activity was considered as an index of cell viability (Newton, 1995).

Hormone determination. Prolactin was measured by a double antibody radioimmunoassay (Niswender et al., 1969) using reagents provided by Dr. A.F. Parlow (National Hormone and Pituitary Program, Torrance, CA, USA). Prolactin and LH RP-3 were used as reference preparations and NIDDK-anti-rPRL-S-9 and anti-rLH-11 as antiserum, respectively. Prolactin levels in control media were 48.73 ± 1.5 μg/ml and 69.02 ± 9.8 μg/ml at 48 and 72 h, respectively. LH levels in control media at 48 h was 3.05 ± 0.5 μg/ml. The sensitivity of the assay was 0.1 ng/ml for prolactin and 0.05 ng/ml for LH. The intra- and inter-assay coefficients of variation were under 10%.

Nuclear morphology analysis. Cells were fixed in 4% formaldehyde for 30 min at 4 °C and mounted in anti-fade solution containing 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI), 23.3 mg/ml 1,4 diazabicyclooctane (DABCO), 20 mM Tris–HCl (pH 8) in glycerol. Nuclear morphology was observed and quantified in an Olympus-BX50 microscope. Data of at least 500 nuclei per triplicate obtained from random fields and from three independent experiments were expressed as number of apoptotic nuclei/total number of nuclei × 100.

Immunocytochemical studies. Cells were fixed in 4% formaldehyde for 30 min at 4 °C, permeabilized with 0.1% Triton X-100 in PBS for 10 min at 4 °C,

and incubated in blocking solution (5% normal serum, 0.1% Triton X-100) for 30 min at room temperature. The cells were incubated with primary prolactin (1:4500) and LH (1:1200) antibodies for 1 h at room temperature, and after three washes, secondary antibody conjugated to fluorescein isothiocyanate (1:75) was added. Cells were mounted in anti-fade solution containing DAPI and DABCO and nuclear morphology was observed. Data of at least 400 nuclei obtained from random fields were expressed as number of immunostained cells with apoptotic nuclei × 100/number of immunostained cells with normal nuclei.

DNA content. To measure nuclear fragmentation, cells were washed with calcium- and magnesium-free Krebs buffer, resuspended by trypsinization in the same buffer and centrifuged at 1000×*g* for 10 min. Cells were resuspended in 2 ml ethanol 70% and incubated for 2 h at 4 °C. Cells were centrifuged and incubated for 15 min at 37 °C with 0.2 ml of PBS containing 10 μg/ml RNAse and 50 μg/ml propidium iodide (exλ: 488 nm, emλ: 585 nm, FL2). DNA content was analyzed in a Becton Dickinson FACScalibur flow cytometer (San Jose, CA). At least 10000 events were measured for each treatment. Further analysis of flow cytometric data was done with WinMDI 2.8 software.

Caspase activity assay. Cells were washed 3 times with chilled PBS, then incubated with 75 μl of lysis buffer (50 mM Tris HCl pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 μM digitonin and 500 μM phenylmethylsulfonyl fluoride, PMSF) for 30 min at 37 °C. Thereafter, the content from three wells was pooled and centrifuged at 20,000×*g* for 20 min (4 °C). The supernatant was mixed (1:1) with reaction buffer (100 mM HEPES, 1 mM EDTA, 10 mM dithiothreitol, 0.5 mM PMSF, 10% glycerol). Reaction began with addition of 5 μl (5 mg/ml) of the colorimetric agent Ac-DEVD-pNA (caspase 3 substrate) and caspase activity was measured by absorbance at 405 nm 1 h after incubating the mix at 37 °C. Caspase activity was expressed as (absorbance/mg of protein in treated sample)/(absorbance/mg of protein in control sample) × 100.

Measurement of ROS and mitochondrial membrane potential. To measure ROS production, cells were loaded with 10 μM DHR123 20 min before the end of the treatment. To measure mitochondrial membrane potential (MMP), cells were loaded with 10 nM $DiOC_6$ (a MMP sensitive fluorescence probe) 20 min before the end of the treatment Once finished, cells were resuspended by trypsinization in calcium- and magnesium-free Krebs buffer (10^6 cells/ml) and analyzed in a Becton Dickinson FACScalibur flow cytometer (exλ: 488 nm, emλ: 535 nm, FL1). Immediately before the measurement, 10 μg/ml propidium iodide (PI) (exλ: 488 nm, emλ: 585 nm, FL2) was added to each FACS tube in order to detect cells with disrupted plasma membrane, 10^4 cells were measured per treatment. Data were analyzed using WinMDI 2.8 software. In the control, plasma membrane integrity after trypsinization was always above 95%. Unviable cells (PI positive) were not used in the analysis. Increase $DiOC_6$ intensity represents an increase in normal MMP and vice versa.

Protein determination. Protein content was assayed by the Bradford method (BioRad, Buenos Aires, Argentina) using bovine serum albumin as standard.

Statistical analysis. The results were expressed as mean ± S.E.M. and evaluated by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison *post hoc* test. Student's *t* test, with Welch correction if required, was also used depending on the experimental design. Differences between groups were considered significant if $p < 0.05$. Results were confirmed by at least three independent experiments.

Results

Cr accumulates in the pituitary gland and affects prolactin release

To evaluate the effect of Cr VI on the pituitary gland, we first studied whether Cr accumulates in the gland and to what extend. Hypothalamic levels were also measured. Since the liver is a known target of the metal, liver levels were measured as control. Rats receiving 500 ppm of Cr VI in drinking water for a period of 30 days showed a marked increase (4.8 fold increase) of Cr levels in pituitary. Cr levels in hypothalamus also rose (1.6 times). The accumulation of Cr in liver was higher than in the hypothalamic–pituitary axis (Table 1). Serum hormone determination showed reduced prolactin levels in Cr treated rats (41.14 ± 8.60% of control, $p < 0.001$ vs. control, Student's *t* test) while LH levels were unmodified (99.97 ± 2.24% of control). At the end of the treatment, animals exposed to Cr VI presented a 30.5% reduction in water consumption and a 11.6% reduction in body weight than control animals. Based on body weight and water intake Cr VI treated rats consumed 73.05 mg Cr/kg-day.

Cr VI affects anterior pituitary cell viability in a time-dependent way

Since we were able to demonstrate that Cr accumulates in the pituitary gland, we decided to investigate whether this metal could affect anterior pituitary gland. With this aim, we studied the effect of Cr VI on anterior pituitary cells in culture. Cells were incubated with increasing concentrations of the metal for 48 h and 72 h and cell activity was examined, as an index of cell viability, by MTT assay (Fig. 1A). The lowest concentration tested, 0.1 μM, had no effect on cell viability at any of the periods evaluated while 1 μM did not affect cell viability at 48 h but decreased it at 72 h ($p < 0.05$). 10 μM Cr VI considerably reduced cell activity (65% vs. control, $p < 0.001$) after 48 h of incubation. We simultaneously examined hormone release into the medium and observed reduced prolactin levels even though cell viability was not affected. The lowest concentration, 0.1 μM, decreased prolactin level at 72 h ($p < 0.001$) but not at 48 h, while 1 and 10 μM significantly reduced prolactin levels at both times (Fig. 1B). Since 10 μM Cr VI clearly demonstrated the cytotoxic effect of the metal at 48 h, this concentration was chosen for all other experiments.

To examine the effect of Cr VI over time, cells were incubated with the metal for different periods (Fig. 2). No significant effect of Cr VI on cell activity was observed during the initial 18 h of exposure but it was markedly reduced by 24 h ($p < 0.001$).

Cr VI toxicity is an early irreversible effect

To determine the point in time at which the effect of Cr VI on anterior pituitary cells becomes irreversible, cells were incubated with the metal for different periods followed by

Table 1
In vivo Cr VI accumulation

Tissue	Cr content (μg Cr/g of tissue)	
	Control	Cr treated
Pituitary	0.133	0.64
Hypothalamus	0.036	0.059
Liver	0.05	4.36

Fifteen animals receiving 500 ppm of Cr VI as potassium dichromate (treated rats) for 30 days and 15 animals receiving tap water (control) were used. Total chromium content was measured in pituitaries, hypothalami and livers pooled from these animals by atomic absorption spectrophotometry.

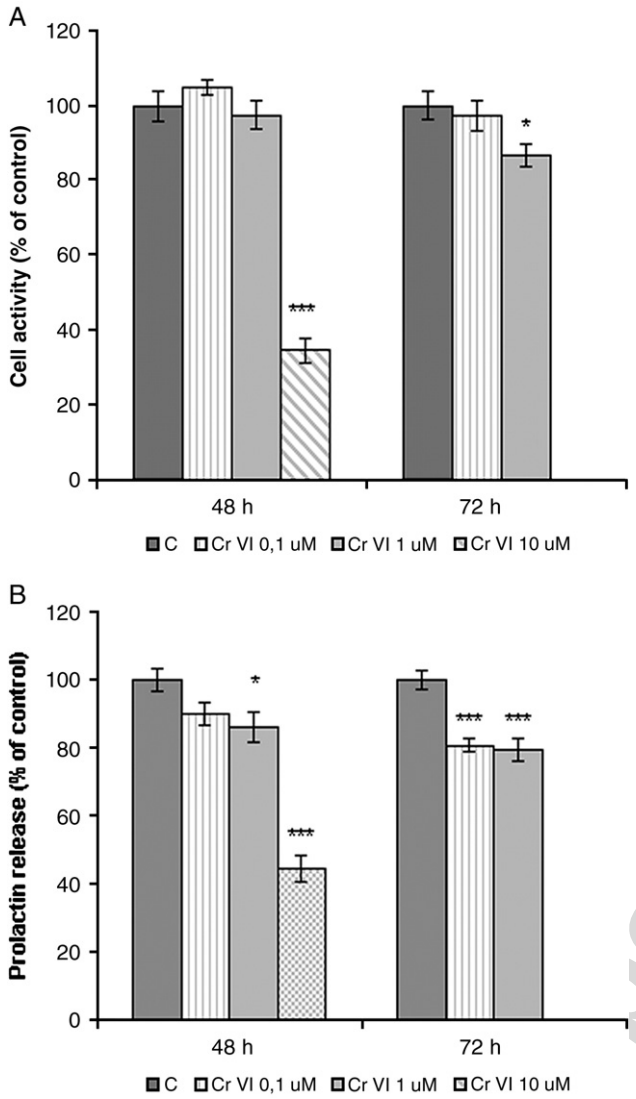

Fig. 1. Cr VI reduces the viability of anterior pituitary cells in culture (A) and decreases prolactin release (B). Anterior pituitary cells were incubated with increasing concentrations of Cr VI for 48 and 72 h. Cell activity, considered an index of cell viability, was measured by MTT assay. Hormone release into the medium was determined by RIA. Bars represent means ± S.E.M., $n=8$. *$p<0.05$, ***$p<0.001$ vs. control without Cr VI (ANOVA followed by Student–Newman–Keuls test). Results are representative of at least three independent experiments.

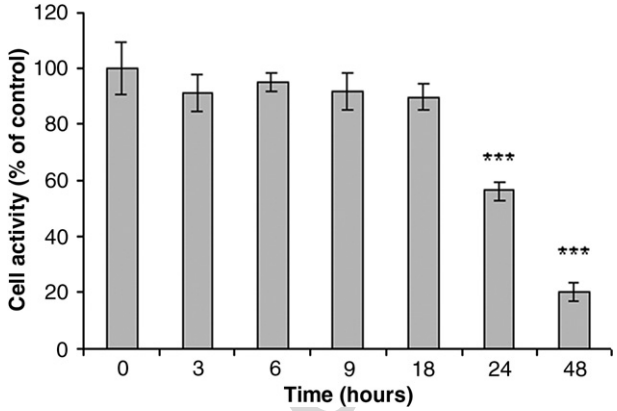

Fig. 2. Cr VI effect over time. Anterior pituitary cells were incubated with 10 μM Cr VI for different times. Cell activity, considered an index of cell viability, was measured by MTT assay. Bars represent means ± S.E.M., $n=8$. ***$p<0.001$ vs. respective control without Cr VI (ANOVA followed by Student–Newman–Keuls test). Results are representative of at least three independent experiments.

incubation with fresh medium without Cr VI to complete a total incubation time of 48 h (Fig. 3A). In these experimental conditions 3 h of Cr VI exposure were enough to reduce cell viability ($p<0.001$), an effect that increased over time. Simultaneously, we examined the effect of Cr VI on hormone secretion. In accordance with our *in vivo* observation, Cr VI significantly reduced prolactin release into the medium without modifying LH release (Fig. 3B).

Cr VI induces apoptosis of anterior pituitary cells: differential cell type effect

Cr VI induces apoptosis in many cell types (Carlisle et al., 2000; Pulido and Parrish, 2003; Ye et al., 1999). Morphologically, apoptotic cells are characterized by membrane blebbing, cell shrinkage, nuclear condensation and chromatin aggregation. Apoptotic events finally result in DNA fragmentation (Kiechle and Zhang, 2002). To investigate whether the decrease in cell viability observed in cells exposed to the metal was due to apoptosis, we first examined nuclear morphology. Cells were incubated with the metal for different times and then fixed and stained with the nuclear dye DAPI. Cr VI exposure increased the number of cells presenting condensed and fragmented nuclei, which are typical apoptotic features (Fig. 4). Apoptotic morphology was observed 12 h following exposure and increased after that time ($p<0.001$). No significant changes in nuclear morphology were observed in the previous time lapses (data not shown). Furthermore, cells treated with Cr VI presented fragmented DNA as evaluated by propidium iodide staining and flow cytometry (Fig. 5). The percentage of cells undergoing DNA fragmentation was recognized by their subdiploid DNA content. Cells showing a hypodiploid DNA content increased after incubation with the metal while cells exhibiting diploid DNA content were reduced.

In order to study whether the observed effect on hormone release was due to differential death of a specific cell type, we examined the nuclear morphology of lactotrophs and gonadotrophs by immunocytochemistry with specific antibodies against prolactin and LH. Both cell types presented an increased in the percentage of apoptotic cells when treated with Cr VI for 24 h ($p<0.01$), however, the percentage of apoptotic lactotrophs was significantly higher than apoptotic gonadotrophs ($p<0.001$) (Fig. 6).

Cr VI causes caspase 3 activation

Caspase 3 is an effector caspase with a central role in the execution of programmed cell death (Stennicke and Salvesen, 2000). In order to determine whether Cr VI induces caspase 3 activation in our system, we measured its activity by a colorimetric assay, 6, 12 and 24 h after Cr VI treatment. Cr VI caused a significant increase in caspase 3 activity at all times tested (Table 2).

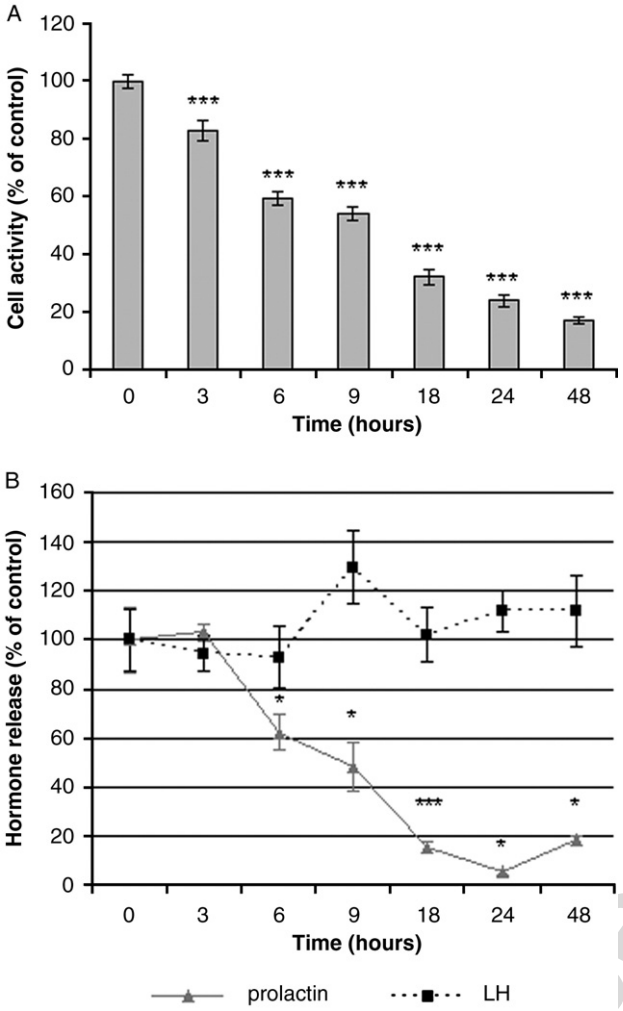

Fig. 3. Cr VI effect becomes irreversible after 3 h of treatment (A). Cr VI reduces prolactin (▲) and does not modify LH release (■) (B). Anterior pituitary cells were incubated with 10 μM Cr VI for the periods indicated and then incubated with medium without Cr VI until complete 48 h. Cell activity, considered an index of cell viability, was measured by MTT assay. Hormone release into the medium was determined by RIA. Values represent means±S.E.M., $n=8$. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. respective control without Cr VI (Student's t test with Welch correction when required). Results are representative of at least three independent experiments.

Cr VI causes an early hyperpolarization of the mitochondrial membrane

Mitochondria play an important role in the apoptotic process (Green and Reed, 1998). Both hyperpolarization and depolarization of mitochondrial membrane have been reported to occur in association with apoptosis (Green and Reed, 1998; Poppe et al., 2001; Pritchard et al., 2001; Sanchez-Alcazar et al., 2000; Ye et al., 1999). To determine whether Cr VI-induced apoptosis involves changes in mitochondrial membrane potential (MMP), cells were exposed to the metal for different times and then the MMP was determined by flow cytometry using the potentiometric probe $DiOC_6$. Increased MMP was observed at 1 and 3 h after Cr VI exposure (Fig. 7). This increase was prevented by co-treatment with the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), indicating that the increase in $DiOC_6$ fluorescence is due to an increase MMP (relative fluorescence, % of control, Cr 1 h: 208%, CCCP: 31%, Cr 1 h+CCCP: 89%). After this early hyperpolarization, MMP returned to control levels, decreasing only 24 h after Cr VI treatment (Fig. 7), when most of the cells were already compromised in the apoptotic process (Fig. 2).

Cr VI increases ROS production

Cr VI is known to generate ROS through a redox process (Harris and Shi, 2003). In order to investigate whether Cr VI modifies ROS levels, we measured the changes in the fluorescence emitted by the oxidation of the broad spectrum probe DHR by flow cytometry. In anterior pituitary cells in culture, Cr VI induced an increase in ROS for the first 2 h (Relative Fluorescence, % of control, Cr :100%, Cr 1 h: 114.5%, Cr 2 h: 121.5%, Cr 3 h: 101.2%, Cr 6 h: 90.1%).

Antioxidants reverse Cr VI cytotoxicity

To study the role of ROS in Cr VI-induced apoptosis, we examined the effects of an antioxidant, NAC, on Cr VI-induced cytotoxicity by an MTT assay. Cells were pre-incubated with NAC for an hour, then incubated with the metal plus NAC for 9 h, and finally with medium without the metal to complete 48 h. These experimental conditions were chosen because cells treated with Cr VI for 9 h exhibited a 50% reduction of cell viability measured after 48 h ($p<0.001$). Treatment with the antioxidant almost completely prevented the Cr VI effect on cell viability ($p<0.05$ vs. control without Cr VI, $p<0.001$ vs. Cr VI alone) (Fig. 8).

Discussion

In the present study we show for the first time that Cr VI accumulates in the pituitary gland affecting its function. Cr VI is known to be readily taken up by the gastrointestinal tract because it resembles phosphates and sulfates; it can thus

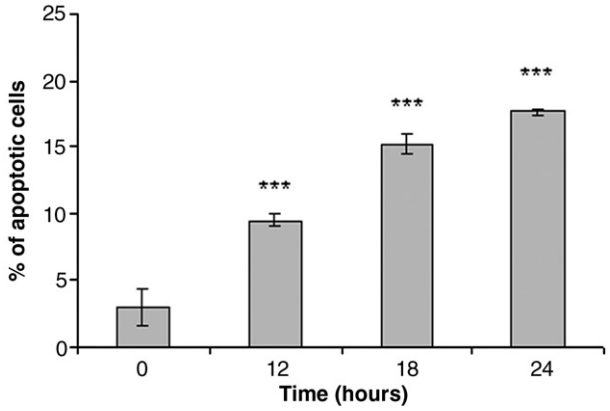

Fig. 4. Cr VI induces apoptosis of anterior pituitary cells. Anterior pituitary cells were incubated with 10 μM Cr VI for different times. Nuclear morphology was studied by DAPI staining. Bars represent means±S.E.M., $n=6$. ***$p<0.001$ vs. control without Cr VI (ANOVA followed by Student–Newman–Keuls test). Results are representative of at least three independent experiments.

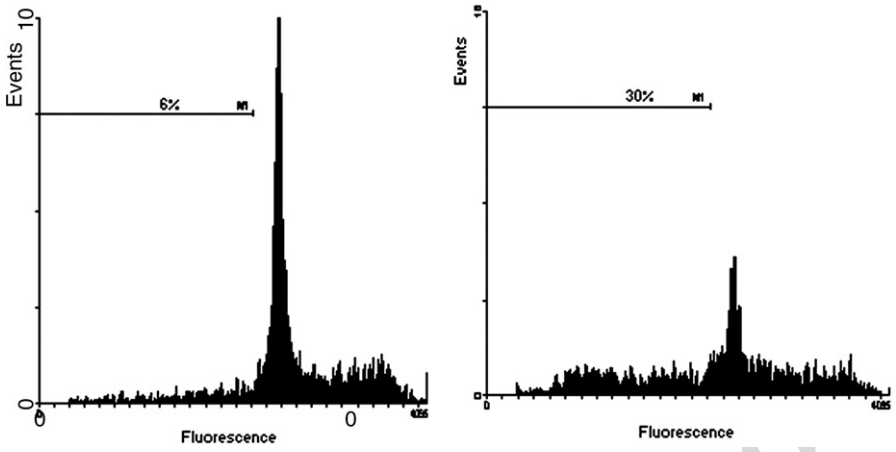

Fig. 5. Cr VI induces DNA fragmentation. Anterior pituitary cells were incubated with (right picture) or without (left picture) 10 μM Cr VI for 18 h and then with medium without Cr VI to complete 48 h. The DNA content was evaluated by propidium iodide staining and flow cytometry. Values represent the percentage of cells with subdiploid DNA content. Results are representative of at least three independent experiments.

penetrate many tissues and organs (Costa, 1997, 2003; O'Brien et al., 2003). Different studies have shown high Cr levels in liver, bone, lungs, kidney, testis and total brain (Costa, 1997; Ernst, 1990; Travacio et al., 2000) but till the moment there have been no measurements of Cr accumulation in pituitary and hypothalamus. We showed that rats receiving Cr VI in drinking water for a period of 30 days presented a marked increase of pituitary Cr content (4.8 fold increase vs. control). This increase was higher than that observed at the hypothalamic level. Several reports indicate that other heavy metals such as cadmium, lead and mercury cause a disturbance in the hypothalamic–pituitary–gonadal axis and affect hormonal status (Gerhard et al., 1998; Lafuente et al., 2001; Schantz and Widholm, 2001). Here, we demonstrated that *in vivo* administration of Cr VI to rats causes a decrease in serum prolactin levels while LH levels remained unmodified. Many studies indicate that Cr VI adversely affects reproductive functions (Acharya et al., 2006; Elbetieha and Al-Hamood, 1997; Ernst, 1990; Murthy et al., 1996). These effects seem to be a consequence of a direct action of the metal on reproductive organs. In our study, Cr VI did not modify LH release, one of the main hormones related to gonadal function. Nevertheless, considering its broad effects, the decrease of prolactin secretion might contribute to the toxicity of the metal not only in reproductive organs but in other organs and physiological processes as well.

In order to get a better understanding of the role of Cr VI on the pituitary, we carried out experiments with anterior pituitary cells in culture. As seen in the *in vivo* experiments, there was a differential effect of Cr VI on hormone release. Cr VI treatment reduced prolactin secretion without affecting LH secretion. This difference may be the result of a higher sensitivity of lactotrophs to the cytotoxic effect of Cr VI, as observed in the immunocytochemical study. Reduced cell activity was observed earlier than the decrease in prolactin release (Fig. 3), so it is possible that Cr VI effect on hormone release may be a result of cell damage rather than a direct inhibition.

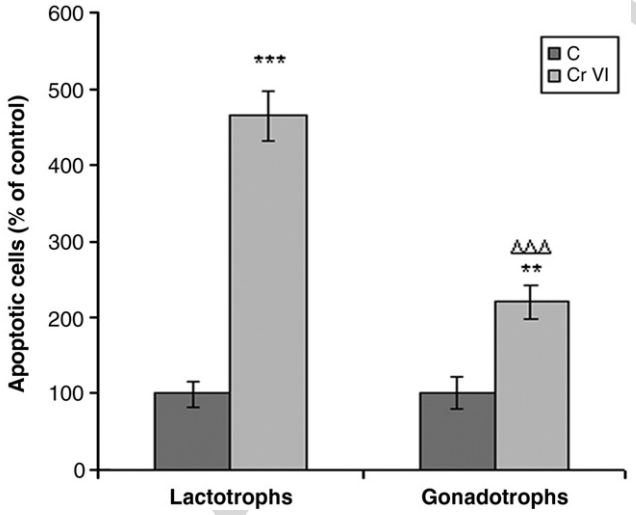

Fig. 6. Cr VI differentially affects lactotrophs and gonadotrophs. Anterior pituitary cells were treated with 10 μM Cr VI for 24 h. Then, the cells were incubated with primary prolactin and LH antibodies and with secondary antibody conjugated to fluorescein isothiocyanate. Nuclear morphology was simultaneously studied by DAPI staining. Data were calculated as number of immunostained cells with apoptotic nuclei × 100/number of immunostained cells with normal nuclei, and expressed as percentage of control ± S.E.M., $n=4$ $^{**}p<0.01$, $^{***}p<0.001$ vs. respective control without Cr VI; $^{\Delta\Delta\Delta}p<0.001$ vs. lactotrophs (ANOVA followed by Student–Newman–Keuls test). Results are representative of at least three independent experiments.

Table 2
Cr VI promotes caspase 3 activation

Caspase 3 activity/mg of protein (% of control)			
Control	Cr VI		
	6 h	12 h	24 h
100 ± 21.8	155.9 ± 5.2**	249.3 ± 47.8***	389.1 ± 31.4***

Anterior pituitary cells were incubated with 10 μM Cr VI for the times indicated. Caspase 3 activity was analyzed by the hydrolysis of the specific colorimetric substrate Ac-DEVD-pNA on cell lysates and the absorbance was measured at 405 nm. Data are expressed as percentage of control ± S.E.M., $n=6$. $^{**}p<0.01$, $^{***}p<0.001$ vs. control (ANOVA followed by Student–Newman–Keuls test).

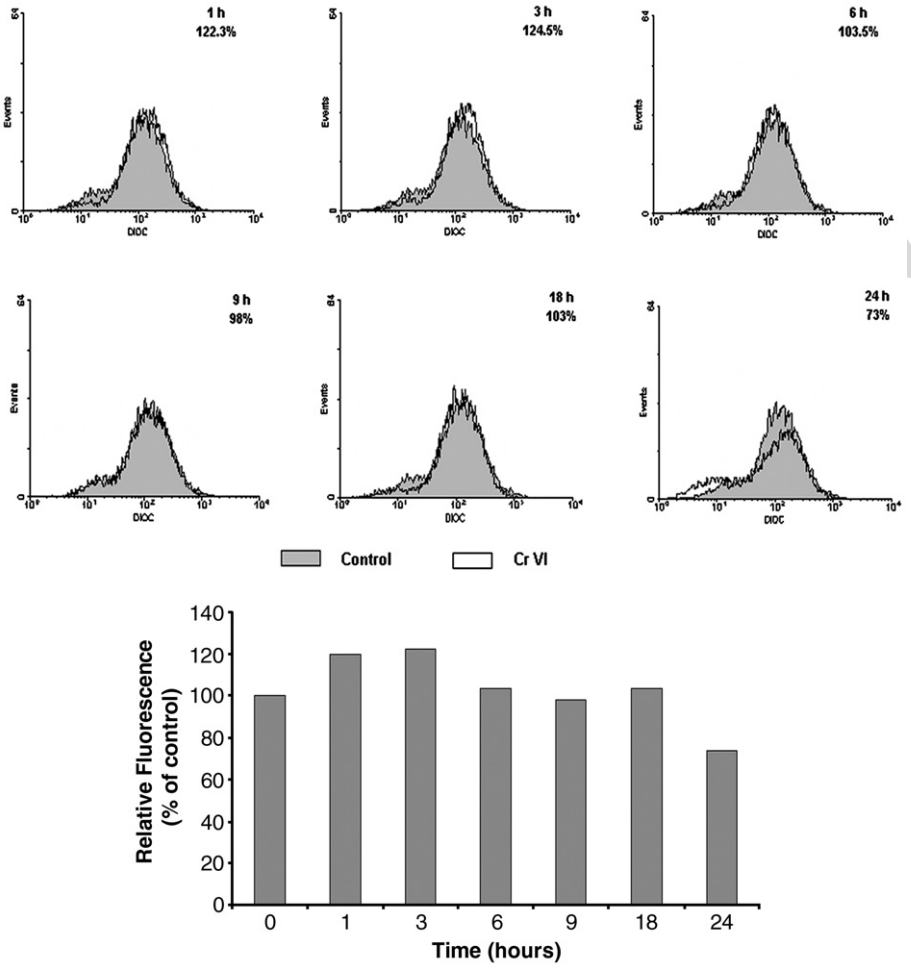

Fig. 7. Cr VI causes an early hyperpolarization of the mitochondrial membrane. Anterior pituitary cells were incubated with or without 10 μM Cr VI for 1, 3, 6, 9, 18 and 24 h. Changes in the mitochondrial membrane potential were measured by cell staining with $DiOC_6$ and flow cytometry. Time of Cr VI treatment and mean fluorescence intensity expressed as % of control, are shown above each graph. Results are representative of at least three independent experiments.

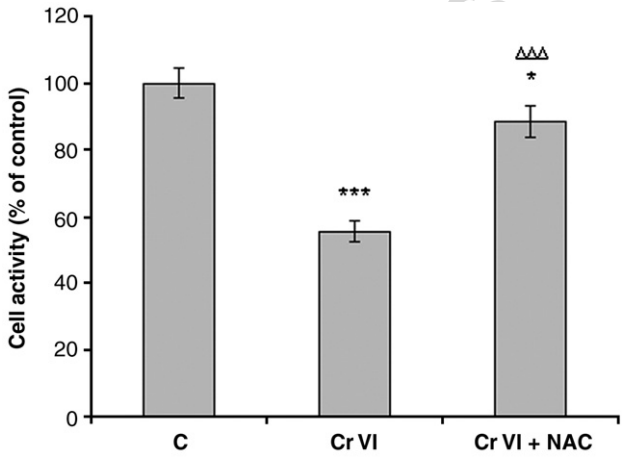

Fig. 8. Cr VI cytotoxicity can be reverse by NAC, an antioxidant. Anterior pituitary cells were incubated with 10 μM Cr VI for 9 h and with or without 1 mM NAC. After that time, fresh medium without the metal was added to complete 48 h of incubation. Cell activity, considered an index of cell viability, was measured by MTT assay. Bars represent means ± S.E.M., $n=8$. *$p<0.05$, ***$p<0.001$ vs. control without Cr VI, $^{\Delta\Delta\Delta}p<0.001$ vs. Cr VI (ANOVA followed by Student–Newman–Keuls test). Results are representative of at least three independent experiments.

Cr VI cytotoxicity, an effect due to apoptosis, was time-dependent. Apoptosis is a genetically regulated cellular suicide mechanism, characterized by DNA fragmentation, chromatin condensation and segregation, membrane blebbing, cell shrinkage and disassembly into membrane-enclosed vesicles (apoptotic bodies) (Kiechle and Zhang, 2002). In our study cells displayed the characteristic nuclear apoptotic morphology, DNA fragmentation and activation of caspase 3. Analysis of cell morphology indicate that Cr VI treated cells underwent nuclear condensation and fragmentation after 12 h of exposure. However, cells seem to be involved in the apoptotic process from earlier times, since 3 h of exposition to the metal was enough to decrease cell activity assayed after 48 h of incubation.

Research into the mechanisms of chromium-induced apoptosis has revealed multiple possibilities, but has yet to determine the exact pathways. Mitochondria have an important role in the apoptotic pathway in many cell types (Bernardi et al., 1999; Green and Reed, 1998). Outer membrane permeabilization causes the release of proteins involved in the apoptotic cascade such as cytochrome *c*. Once into the cytosol, cytochrome *c* forms a complex which in turn activates effector caspases such as caspase 3. Caspases are central effectors that cleave key

intracellular targets leading to cell death (Stennicke and Salvesen, 2000). Several reports indicate that the mitochondria may be involved in the apoptotic process induced by Cr VI (Hayashi et al., 2004; Pritchard et al., 2000, 2001;Ye et al., 1999). In our system, Cr VI induced an early hyperpolarization of mitochondrial membrane. The increase of $DiOC_6$ fluorescence was due to the proton gradient existing across the mitochondrial inner membrane since it was prevented by CCCP, a protonophore that allow the passage of protons across the mitochondrial inner membrane collapsing the proton gradient. Disruption of MMP was observed only after 24 h of incubation with the metal, when cell viability reached almost 50% and cells were irrevocably dying. Cr VI also increased ROS production early in time. Similar results have been reported for different cell types (Harris and Shi, 2003; Pulido and Parrish, 2003; Wang et al., 2000; Ye et al., 1999). Increased mitochondrial activity (seen as an increase in MMP) could be the source of this early increase in ROS, though it is known that Cr VI itself can also generate ROS. Recent reports suggest that MMP increase occurs early in the apoptotic process (Poppe et al., 2001; Sanchez-Alcazar et al., 2000). Moreover, some studies demonstrate that cytochrome *c* can be released from mitochondria and activate caspase 3, when hyperpolarization, depolarization or even no changes in MMP take place (Miyato et al., 2001; Poppe et al., 2001; Ricci et al., 2003; Sanchez-Alcazar et al., 2000). In our system, both ROS production and increased MMP precede caspase 3 activation and antioxidant treatment almost completely reverses the cytotoxicity of Cr VI. Therefore, increased ROS production may be a key event involved in the activation of the caspase cascade through the generation of oxidative stress. ROS may also act as a second messenger and activate different pathways leading to cell death. In certain cell types, ROS have been reported to be involved in the activation of p53, which is responsible for Cr VI-induced cell death (Carlisle et al., 2000; Wang et al., 2000; Wang and Shi, 2001; Ye et al., 1999).

Our study demonstrated for the first time that Cr VI accumulates in the pituitary and affects prolactin release. *In vivo* Cr VI administration decreases serum prolactin levels while it has no effect on LH levels. In anterior pituitary cells in culture, Cr effect on hormone secretion follows the same differential pattern, lactotrophs being more vulnerable to the cytotoxic effect of the metal. Cr VI causes apoptosis of anterior pituitary cells, as confirmed by morphological studies and DNA fragmentation. Apoptosis induced by Cr VI involves generation of oxidative stress and caspase 3 activation. Our results demonstrate that the pituitary can be a target of Cr VI toxicity. The accumulation of Cr in the hypothalamic–pituitary axis suggests that the metal may have a negative impact on the normal endocrine function. The study of Cr VI effect over the pituitary physiology is important considering the dearth of knowledge on this subject and the adverse effects of the metal on reproductive function.

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