Quantitative Changes in Rat Seminiferous Epithelium After Chronic Administration of Low Doses of Cadmium and Zinc: A Stereological Study

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Abstract: The present study deals with the stereological analysis of the changes in the seminiferous epithelium of rat testis, mediated either by cadmium alone or by cadmium plus zinc, chronically administered at low oral doses. Stereological estimates of both labeling indices of proliferative cell nuclear antigen and TUNEL-stained nuclei of spermatogonia, and of ubiquitin, ubiquitin carboxyl-terminal hydrolase L1, caspase-3, and metallothionein cytoplasmic immunoreactivities from seminiferous epithelium were performed in rats non-treated (controls), $CdCl_2$-exposed animals, and $CdCl_2$ plus $ZnCl_2$ -treated rats. The following conclusions can be drawn: a) The intake of low, oral doses of cadmium chloride over a long period of time induces quantitative changes in apoptosis of the seminiferous epithelium of the rats, without noticeable morphologic or proliferative alterations. b) The increase of mono-ubiquitin levels mediated by cadmium is caused by over expression of ubiquitin carboxyl-terminal hydrolase L1. c) Zinc exposure was able to decrease ubiquitin carboxyl-terminal hydrolase L1 and ubiquitin, but not sufficiently to reverse the apoptotic rate of the spermatogonia at the control level. d) It seems that metallothionein is not induced by the cadmium treatment alone. However, the results indicate that either cadmium in combination with zinc or zinc itself induces this enzyme.

Keywords: Cadmium, cell proliferation, apoptosis, ubiquitin, UCHL1, rat testis.

INTRODUCTION

The increasing use of cadmium in industry also increases the risk of human exposure to this metal [1, 2]. Exposure may be either occupational (mainly by inhalation) or non-occupational (by cadmium contamination of drinking water or food). Toxicological studies state that ingestion of a daily amount of 110 μg of cadmium was associated with adverse effects on human health, and that the maximum amount of cadmium tolerated was 1 μg per kg of body weight per day [3]. Since a tobacco plant can concentrate cadmium from contaminated soils, a cigarette contains from 1 to 2 μg of cadmium. Cadmium levels in blood and semen of smokers are higher than those in non-smokers [4]. On the other hand, chronic occupational exposure in men has been related to lung and prostate cancers [5, 6].

Cadmium exposure may affect several organs such as the lung, kidney, liver, and prostate, but the rodent testis is highly sensitive to the action of this metal. However, the testicular damage mediated by cadmium can be modified by several factors. Zinc, in particular, has been shown to exert a protective effect on the testis [7]. A number of authors studied the cadmium effect on testicular tissue, administering the metal at a variety of doses and means of exposure [8, 9]. Information about the effect caused by cadmium administered in conditions similar to those in the human population at risk of cadmium contamination, i.e., oral, chronic, and at low doses, is scarce.

As has been demonstrated [10, 11], one of the first detectable quantitative changes in reproductive organs after cadmium exposure was related to the balance between cell proliferation and cell death (apoptosis). Because of the similarity in the chemical characteristics of zinc and cadmium, it was suggested that these metals share a common transport pathway [12], and zinc treatments should reduce or abolish the adverse effects of cadmium.

In the present study, we will estimate the germ cell proliferation by quantification of proliferative cell nuclear antigen (PCNA) in spermatogonia. The apoptosis will be quantified measuring the ratio of apoptotic nuclei to total

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nuclei from spermatogonia, and estimating the volume fraction of cytoplasmic immunoexpression of the apoptotic enzyme caspase-3 [13, 14] in the seminiferous tubules.

Changes in ubiquitin (UB) in relation to the exposure to cadmium and/or zinc will be investigated as UB is a protein implicated in extra-lysosomal proteolysis and importantly associated to apoptosis [15, 16]. Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) will be also studied, because it is an enzyme essential for maintaining ubiquitination activity by releasing UB from its substrata [17]. UCHL1 can be detected in mouse testicular germ cells, mainly spermatogonia and Sertoli cells [18], and changes in this enzyme were detected in some testicular injuries, such as acute testicular ischemia-reperfusion [19], however its role in cadmium exposure has been not studied.

On the other hand, it would also be interesting to correlate the changes detected in the testis exposed to cadmium or cadmium plus zinc with quantitative estimates of metallothionein (MT), which is known as a protein containing a detoxification function for heavy metals. It is a low- molecular-weight protein, rich in cysteine, which binds and detoxifies metals such as Zn, Cu, and Cd, interacting with cysteine residues [19]. Metallothionein was observed in spermatogenic and Sertoli cells of seminiferous tubules in the testis without heavy metal exposure. In the cadmium-treated testis, MT was observed in Sertoli and interstitial cells. However, only a few studies have reported the quantification of MT after chronic treatment at low doses of Cd and/or Zn [19].

The aims of this study are: a) To confirm the hypothesis that quantitative morphologic changes related to proliferation and apoptosis can be detected before histologic alterations are observed in the seminiferous epithelium of rat testis mediated by cadmium administered at low oral doses; b) To evidence that zinc exposure can modify these changes; and c) To show the quantitative changes experimented by MT immunoreactivity in this model of chronic Cd/Zn exposure.

MATERIALS AND METHODS

Animals

Thirty adult male Wistar rats (200 ± 30 grs body weight, 90 days old at the beginning of the experiment) were used for immunohistochemical and stereological studies. The animals were fed with Panlab Lab Chow (Panlab, Barcelona, Spain) and water ad libitum. Animal protocols are in compliance with the guidelines for the care and use of research animals adopted for the Society of Reproduction. During this work, all animal studies were conducted in accordance with the European Community's Council ruling of 24 November 1986 (86/609/EEC) [20] and Spanish and local directives ruled by "Real Decreto 1201/2005".

The rats were classified into 3 groups, according to treatment (10 rats per group, i.e., 20 testes per group). Cadmium chloride (Panreac, Madrid, Spain) was added to the drinking water of the first group at a concentration of 80 ppm. The second group received zinc chloride (Panreac, Madrid, Spain) at a concentration of 95 ppm, plus cadmium chloride (80 ppm) in the drinking water. These doses were established according to those indicated in other studies on the effectiveness of Zn in preventing testicular dysfunction caused by dietary Cd. The Cd/Zn exposure was continued for the 12 months of the study [11]. The third group was used as a control and received drinking water that was shown to be free of these metals.

All the rats were euthanized by exsanguination after CO_2 narcosis 12 months after the beginning of the experiment. All the organic remains from the animals and the residua of drinking water were adequately processed according to the guidelines in relation to the safety in the use of heavy metals established by the communitarian normative of the European Union. Immediately after death, both testes from each rat were excised and their fresh volume (V testis) measured by water displacement. All the specimens were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4, for 24 hr. After fixation, the testes were cut into 5 mm-wide slices, the section plane perpendicular to the sagittal axis of the gonad. Thereafter, the slices were embedded in paraffin and the paraffin blocks were then serially sectioned. Five μm-thick sections (for routine hematoxylin-eosine staining), alternating with 10 μm-thick sections (for immunohistochemistry and stereological cell counting), were obtained from each block.

Immunohistochemical and Apoptosis Detection Methods

In the three groups, at least five selected slides per animal and per antigen were immunostained. The selection of the slides was performed by systematic random sampling [21]. This is important in order to assure that the encounter between the counting probe and the profiles of seminiferous tubules allows the same chance of being estimated in all the stages of the spermatogenic wave, as it is well known that in the rat, there is a distinct ordering of cell associations along the length of the seminiferous tubule [22]. Deparaffinized and rehydrated tissue sections were treated for 30 min with hydrogen peroxide 0.3% in PBS pH 7.4, to block endogenous peroxidase. Mouse monoclonal and rabbit polyclonal antibodies were used as primary antibodies. To detect PCNA, caspase-3, and UCHL1 immunoreactivities, sections were incubated with a monoclonal anti-PCNA antibody (Biomeda, Foster City, CA, USA) diluted at 1:200; a monoclonal anti-caspase-3 antibody (Cell Signaling, Beverly, MS, USA) diluted at 1:50; and a monoclonal anti-UCHL1 antibody (Biomeda) diluted at 1:50, respectively. To detect ubiquitin (UB) and metallothionein (MT) immunoreactivities, the sections were incubated with a polyclonal anti-UB antibody (Dako, Glostrup, Denmark) diluted at 1:200, and with a polyclonal anti-MT antibody (Zymed, San Francisco, CA, USA) diluted at 1:100, respectively. Pretreatment of sections by heat in citrate buffer pH 6.0, using a pressure cooker [11], was performed to enhance all the immunostainings. The details of the immunohistochemical technique are shown elsewhere [23].

Apoptosis was studied using a technique for detecting DNA fragmentation: terminal deoxynucleotide transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL technique) (Roche, Mannheim, Germany) [24]. This method is described in detail in another study [23] .

After immunoreactions and the TUNEL method, sections were counterstained with Harris hematoxylin. All slides were dehydrated in ethanol, and mounted in a synthetic resin (Depex, Serva, Heidelberg, Germany). The specificity of the

immunohistochemical procedures was checked by incubation of sections with nonimmune serum instead of the primary antibody, and the specificity of the TUNEL method was also checked by incubation in the TdT mixture without labeled nucleotides.

Quantitative Methods

Estimates of the number of total germ cells and germ cells immunoreactive to PCNA and positive to TUNEL were calculated, using the technique of the optical disector, an unbiased stereological method [21, 25]. Measurements were carried out using an Olympus microscope fitted with a motorized stage and equipped with an X100 oil immersion lens (numerical aperture of 1.4) at a final magnification of X1,200, employing the stereologic software CAST-GRID [26].

In each selected field from PCNA-immunostained sections, the area of the seminiferous tubules was scanned and the numerical densities (N_V) of spermatogonia (N_V G), spermatocytes (N_V CYT), immature spermatids (N_V IM-SP), mature spermatids (N_V M-SP), and Sertoli cells (N_V SC) were estimated. The N_V of germ cells immunoreactive to PCNA was simultaneously estimated. In TUNEL-stained sections, positive nuclei from spermatogonia (N_V apoptotic cells) were evaluated. In all the cases, the unit of counting was the nucleus, eligible according to the Sterio rule [27].

The N_V is determined by the formula: $\sum Q^-_D / \sum (Vdis^+) \cdot Fr$, where: Q^-_D = number of eligible nuclei, $Vdis^+$ = volume of disectors in which the upper-right corner hits seminiferous epithelium, and Fr = 1.15 (shrinkage factor, resulting from the processing of the tissue) [28].

To estimate the reference space, i.e., seminiferous tubule (V tubular), the frames with their upper-right corner hitting seminiferous epithelium were registered (dis^+) and the volume fraction of tubules represented by the ratio between the amount of dis^+ and the total number of disectors scanned (distot) was calculated. Following this step, the tubular volume (V tubular) was obtained by multiplying this ratio by the testicular fresh volume (V testis). The absolute number of seminiferous cells (N G, N CYT, N IM-SP, N M-SP, and N SC) was then calculated multiplying N_V by V tubular. Labeling indices for PCNA and apoptotic cells were calculated: LI PCNA = N G PCNA / N G; LI apoptosis = N apoptotic cells / N G. Only the V tubular, N total for germ and Sertoli cells, and the labeling indices (LI PCNA and LI apoptosis) were employed for the statistical analyses.

Volume density (V_V), i.e., the ratio between the area occupied by a phase or tissue component and a reference area, was employed to estimate the volume fractions of epithelial cytoplasm immunostained by caspase-3 (V_V caspase -3), UB (V_V UB), UCHL1 (V_V UCHL1), and metallothionein (V_V MT). The measurements of both immunoreactive and reference areas were estimated as follows: five sections per each immunostaining were chosen by systematic random sampling from each 20 sections sampled from the pool of sections obtained by complete sectioning of each specimen. Measurements were carried out using the same stereologic system employed to estimate numerical densities. This system allowed us to capture the fields to be measured, previously selected by random systematic sampling. An average of 20 fields / section was captured using a color videocamera TK-C1480B (JVC, Japan). Grey-level image transformation and binary image processing were carried out using the public domain NIH Image program, developed at the US National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/ [11].The volume fractions of immunoreactive cytoplasm were given by the formula:

V_V caspase-3, UB, UCHL1, or MT = Σ area of immunostained cytoplasm / Σ area of immunostained plus non-immunostained cytoplasm [11]. The absolute volumes in cm^3 (V) of immunostained cytoplasm for caspase-3, UB, UCHL1, and MT were then calculated, multiplying their respective V_V by V tubular.

Statistical Analysis

For each parameter studied, the mean ± SD was calculated. The differences among controls, cadmium-exposed rats, and cadmium+zinc- treated rats were evaluated by ANOVA. Comparison between each pair of means was performed using the Fisher & Behrens test ($p < 0.05$).

RESULTS

Qualitative results

No histologic changes were detected in either the Cd or Cd+Zn groups, in comparison with the CTRL group (Fig. **1a-c**). The seminiferous epithelium shows abundant spermatogonia and scattered primary spermatocytes with nuclei immunoreactive to PCNA in all the groups studied (controls and treated) (Fig. **1a-c**). Nuclei from germ cells positive to TUNEL were observed in all the groups. However, from a qualitative point of view, the positive nuclei were more abundant in treated animals in comparison with control rats (Fig. **1d-f**). Immunoreaction to UB was observed in the cytoplasm of spermatocytes and spermatids from all the groups studied. This immunostaining was more evident in the Cd group in comparison with both CTRL and Cd+Zn groups (Fig. **1g-i**). In both CTRL and Cd+Zn groups, the cytoplasm of spermatogonia and Sertoli cells was immunoreactive to UCHL1, whereas in the Cd group, the immunostaining for UCHL1 was more intense and also detected in the cytoplasm of spermatids (Fig. **2a-c**). Diffuse caspase-3 immunostaining was detected in the cytoplasm of the Sertoli cells and spermatids. Moreover, granular immunostaining for caspase-3 was observed in the cytoplasm of spermatogonia. No qualitative differences were noted among controls and treated rats (Fig. **2d-f**). MT-immunostaining was observed in the cytoplasm of Sertoli cells, and in some spermatogonia. The MT-immunoreactivity was more evident in the Cd+Zn group than in either the CTRL or Cd groups (Fig. **2g-i**).

Quantitative Results

The tubular volume did not change significantly when CTRL, Cd, and Cd+Zn groups were compared (Fig. **3**). When the number of germ cells (G, CYT, IM-SP, and M-SP) (Fig. **4a**) and Sertoli cells (Fig. **4b**) was compared among the three groups, no significant differences were observed per each type of cells.

The LI PCNA did not show significant changes when CTRL, Cd, and Cd+Zn groups were compared (Fig. **5a**). The LI apoptosis in both Cd and Cd+Zn groups was significantly

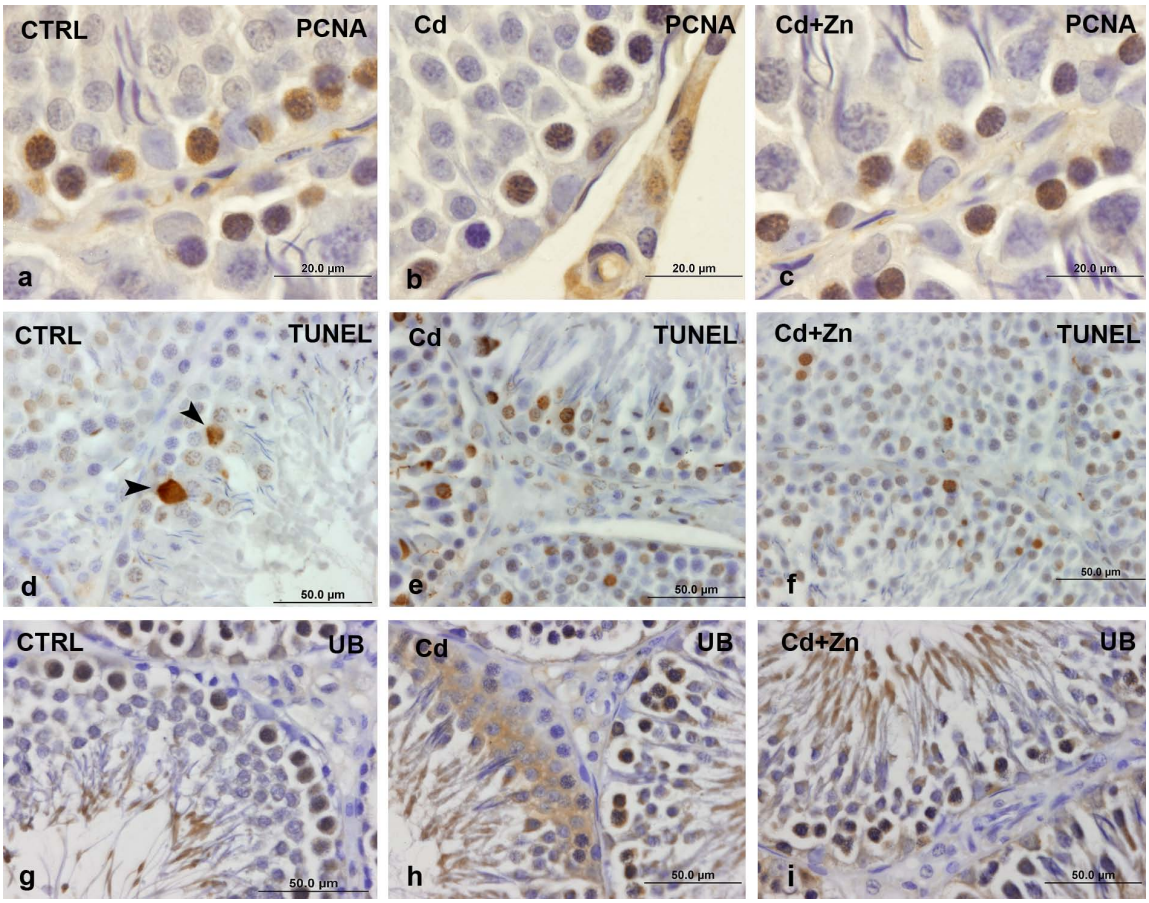


Fig. (1). (**a**) Seminiferous epithelium from a control rat; nuclear immunoreactivity to PCNA was observed in spermatogonia. (**b**) Seminiferous epithelium from a rat exposed to cadmium for 12 months; no changes in the amount of the nuclei immunoreactive to PCNA in comparison with controls were observed. (**c**) Seminiferous epithelium from a rat exposed to cadmium plus zinc for 12 months; no changes in the amount of the nuclei immunoreactive to PCNA in comparison with controls and Cd-treated animals were observed. No histological changes were detected in (**b**) and (**c**) compared with (**a**). In (**a-c**) images, bar represents 20 µm.

(**d**) Seminiferous tubules from a control rat. TUNEL method detects two positive nuclei from spermatocytes (arrowheads). (**e**) Seminiferous tubules from a rat treated with Cd for 12 months; an increasing number of TUNEL-positive spermatogonia and spermatocytes were detected in comparison with (d). (**f**) Seminiferous tubules from a rat treated with Cd plus Zn for 12 months. There is a similar amount of TUNEL-positive nuclei in comparison with (e). (**g**) Seminiferous tubule from a control rat; cytoplasmic immunoreactivity to UB was observed in spermatocytes and spermatids. (**h**) Seminiferous tubules from a rat treated with Cd for 12 months; the cytoplasmic immunostaining for UB detected in both spermatocytes and spermatids was more intense than in (**g**).

(**i**). Seminiferous tubules from a rat treated with Cd plus Zn for 12 months; the cytoplasmic immunostaining for UB detected in both spermatocytes and spermatids was decreased in comparison to that observed in (h). In (a-c) images, bars represent 20 µm and in (d-i) images, bars represent 50 µm.

greater than in the CTRL group. However, between the Cd and Cd+Zn groups, no significant differences for LI apoptosis were observed (Fig. **5b**).

The cytoplasmic volume of germ cells immunostained for UB was significantly increased in the Cd group in comparison with the CTRL and Cd+Zn groups, whereas no significant differences for V UB were observed between the CTRL and Cd+Zn groups (Fig. **6a**). The cytoplasmic volume of seminiferous epithelium immunostained for UCHL1 was significantly increased in the Cd group in comparison with both CTRL and Cd+Zn groups. Moreover, V UCHL1 was also significantly greater in the Cd+Zn group than in the CTRL group (Fig. **6b**). In relation to V caspase-3, no significant differences among the three groups were observed (Fig. **6c**). The cytoplasmic volume of seminiferous epithelium immunostained for MT was significantly increased in the Cd+Zn group in comparison with both CTRL and Cd groups. No significant differences in V MT were observed between the CTRL and Cd groups (Fig. **7**).

DISCUSSION

The protocol of Cd and Zn exposure employed in this study (long time, low oral doses) did not produce either histological or quantitative changes in rat seminiferous epithelium with respect to an eventual seminiferous tubule atrophy (there were no changes in the tubular volume) or in relation to the amount of germ cells (similar to the amount observed in control testes). These findings disagree with those described in other studies, where higher dietary Cd

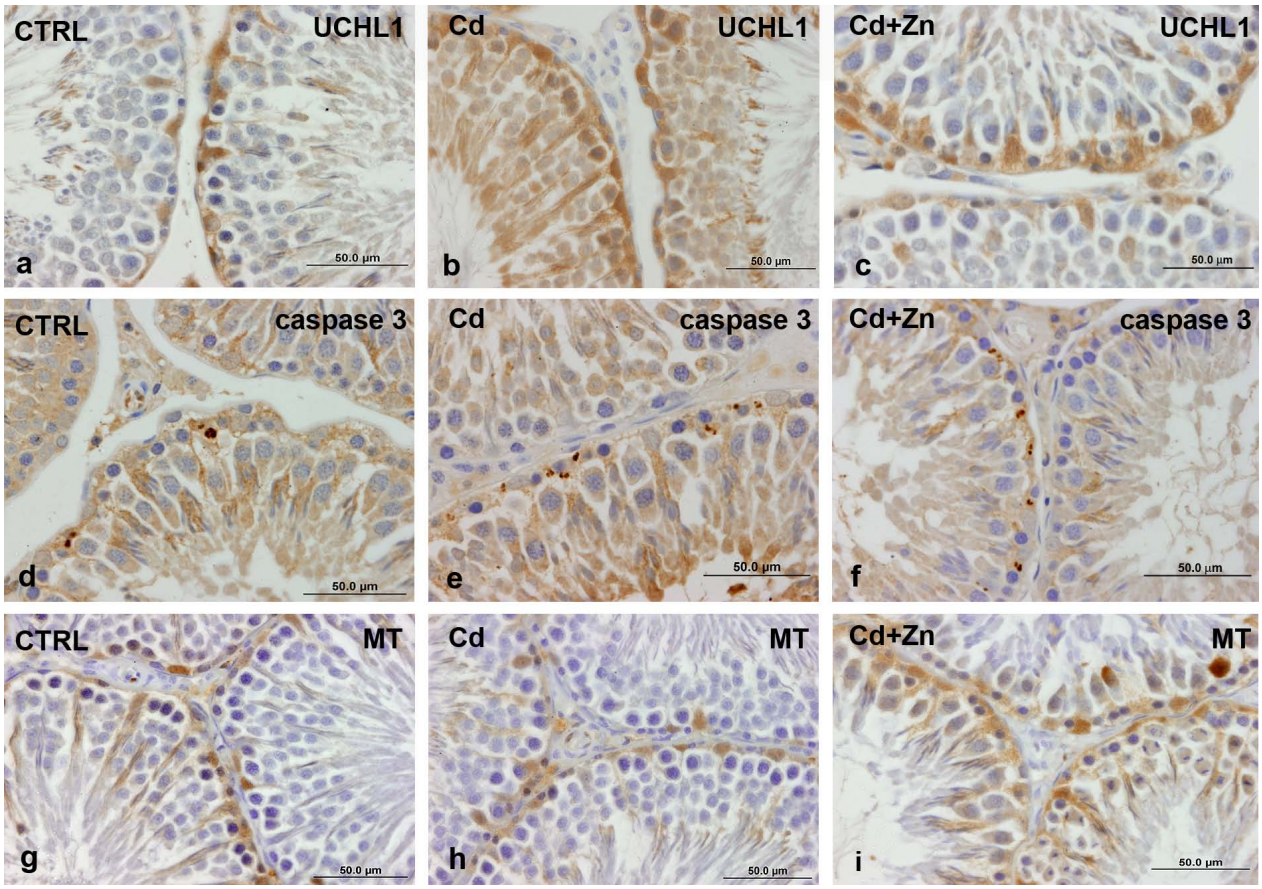


Fig. (2). (**a**) Seminiferous epithelium from a control rat; cytoplasmic immunoreactivity to UCHL1 was observed in spermatogonia and in Sertoli cells. (**b**) Seminiferous epithelium from a rat exposed to cadmium for 12 months; more intense immunostaining for UCHL1 in comparison with image (a) was observed in Sertoli cells, spermatogonia, and spermatids. (**c**) Seminiferous epithelium from a rat exposed to cadmium plus zinc for 12 months; the immunostaining for UCHL1 detected was similar to that observed in (a). (**d**) Seminiferous tubules from a control rat; granular immunostaining for caspase-3 is observed in the spermatogonia. Moreover, a diffuse immunoreactivity to caspase-3 is observed in the cytoplasm of Sertoli cells and spermatids. (**e**) Seminiferous tubules from a rat treated with Cd for 12 months; the amount and distribution of caspase-3 immunoreactivity was similar to that observed in (d). (**f**) Seminiferous tubules from a rat treated with Cd plus Zn for 12 months; the amount and distribution of caspase-3 immunoreactivity was similar to that observed in (d) and (e). (**g**) Seminiferous tubules from a control rat; cytoplasmic immunoreactivity to MT was observed in the cytoplasm of the Sertoli cells, and in some spermatogonia. (**h**) Seminiferous tubules from a rat treated with Cd for 12 months; the cytoplasmic immunostaining for MT is similar in amount and distribution to that detected in (g). (**i**) Seminiferous tubules from a rat treated with Cd plus Zn for 12 months; the cytoplasmic immunostaining for MT detected in both Sertoli cells and spermatogonia was increased in comparison to that observed in (g and h). For all images, bars represent 50 μm.

doses [29] or parenteral/intraperitoneal exposure were employed [30]. The preservation of the size of seminiferous epithelium might be related to the absence of impairment of the cell proliferation index of the germ cells in rats treated with Cd or Cd + Zn. However, other authors [11] have stated the influence of cadmium in the increase of cell proliferation of prostate epithelium, which could be related to its carcinogenic potential [7, 31].

Although no changes in number and proliferation were stated, a significant increase in the rate of apoptosis in the spermatogonia of Cd-treated rats was evidenced by the TUNEL technique. This increase of programmed death rate of spermatogonia was not reversed by Zn exposure. The increment of apoptosis mediated by Cd was also described by other authors either in testes [32-35] or in prostate [10, 11].

Ubiquitins are proteins that can bind other protein components and form protein-ubiquitin conjugates. These conjugates display proteolytic activity and may be involved in cellular processes such as apoptosis [16]. Ubiquitins and their carboxyl-terminal hydrolases have been detected in the testis, where they may play a role in modulating changes in the chromatin structure of the spermatids [36]. In our study, the increase of the apoptotic rate was accompanied by a significant increase in the expression of UB in the cytoplasm of germ cells exposed to Cd, and in this case, the Zn reversed the increase of UB immunoexpression. This increase of ubiquitin, modulated by zinc intake, might be correlated to the findings of some authors [37] that indicate an increase of the transcription factors for the proteolysis-dependent poly-ubiquitination in yeast cells exposed to cadmium. This can be interpreted as a means of impeding genetic mutations

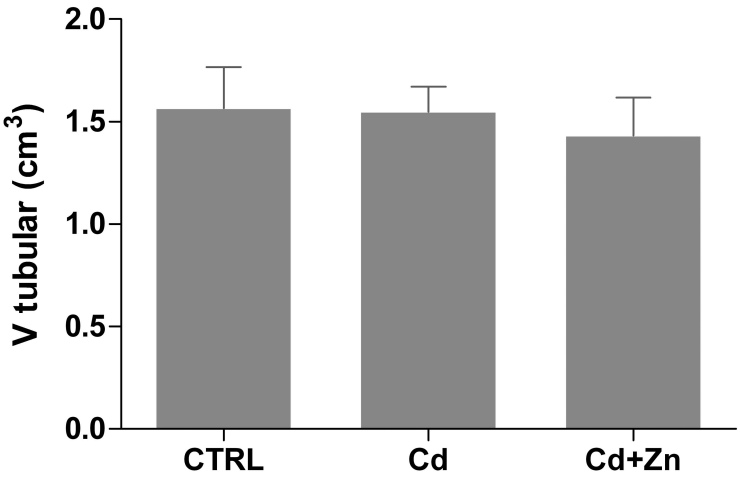


Fig. (3). Bar graph of the values of the volume (V tubular) of seminiferous tubules in control (CTRL), cadmium-exposed (Cd), and cadmium plus zinc (Cd+Zn) -treated rats, after 12 months of the beginning of the experiment, expressed in cm^3. Results are expressed as mean ± SD. There were no significant differences among groups ($p < 0.05$).

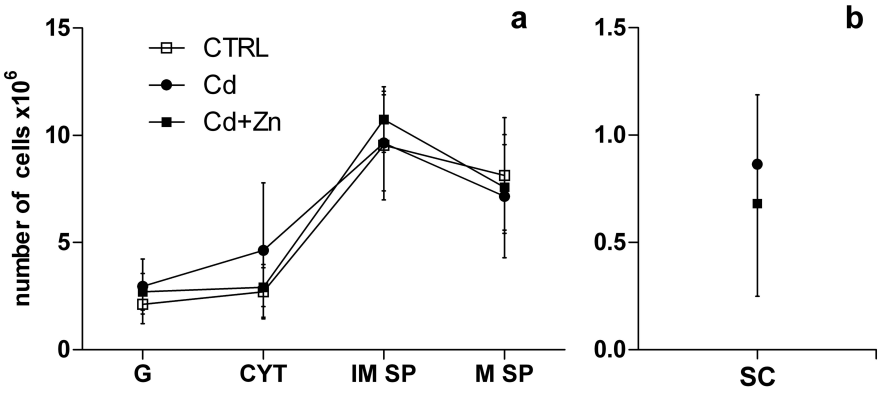
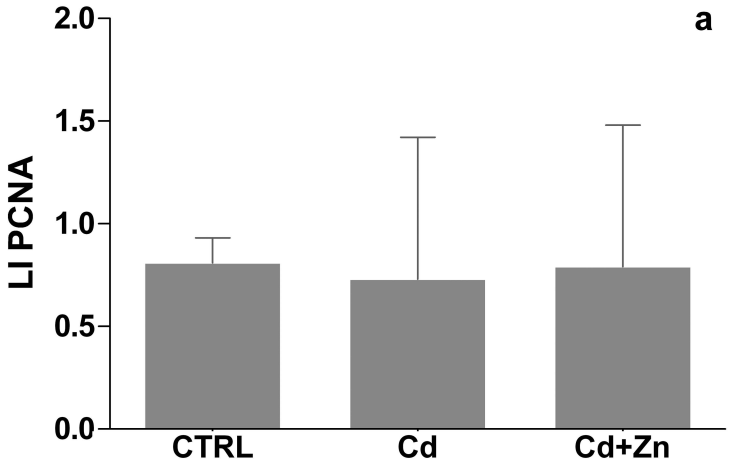


Fig. (4). (a) Graph indicating the mean number of germ cells per testis ($x10^6$): spermatogonia (G), spermatocytes (CYT), immature spermatids (IM SP), and mature spermatids (M SP), in rat controls (CTRL), rats exposed to cadmium (Cd), and rats treated with cadmium plus zinc (Cd+Zn). The error bars indicate the confidence limits (95%) from the mean values of the CTRL group. **(b)** Graph showing the mean number of Sertoli cells per testis ($x10^6$), in CTRL, Cd, and Cd+Zn groups. The error bar indicates the confidence limits (95%) from the mean values of the CTRL group.



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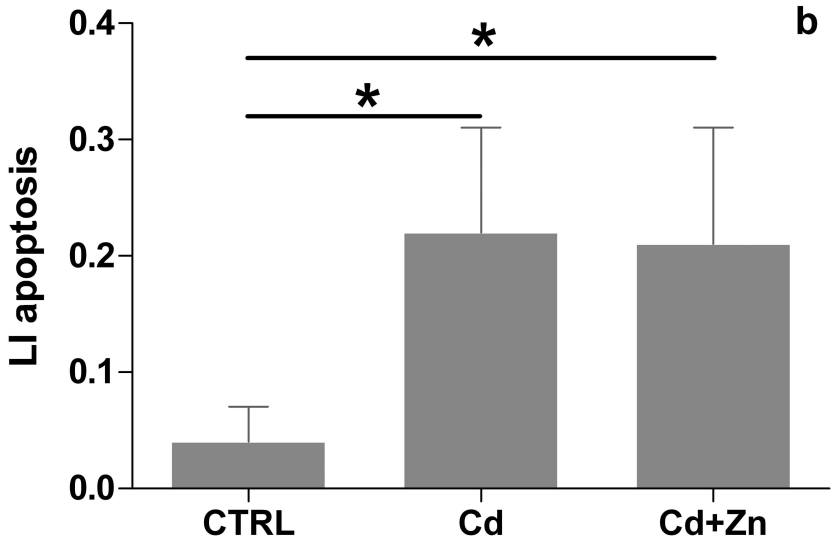
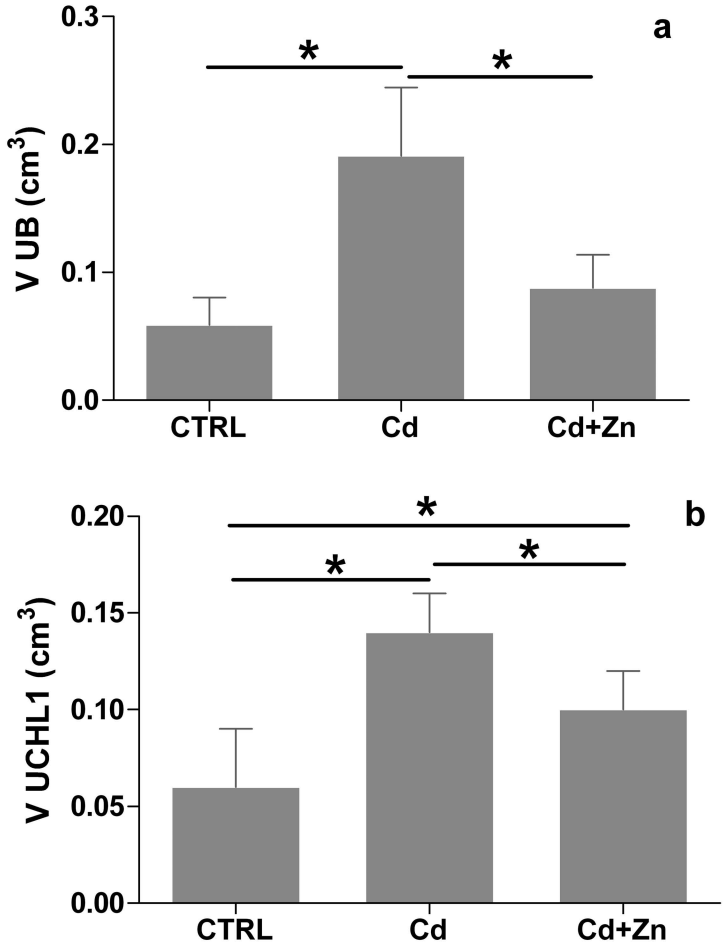


Fig. (5). (a) Bar graph of the values of the labeling index of PCNA (LI PCNA) of spermatogonia, in controls (CTRL), cadmium-treated (Cd) and cadmium plus zinc-exposed (Cd+Zn) rats, 12 months after the beginning of the experiment. Results are expressed as mean ± SD. There was a non-significant difference among groups ($p < 0.05$).

(b) Bar graphs of the values of apoptotic index (LI apoptosis) of germ cells in controls (CTRL), cadmium-treated (Cd) and cadmium plus zinc-exposed (Cd+Zn) animals, 12 months after the beginning of the experiment. Results are expressed as mean ± SD. Asterisks indicate significant differences ($p < 0.05$) between CTRL and Cd groups, and between CTRL and Cd+Zn groups.



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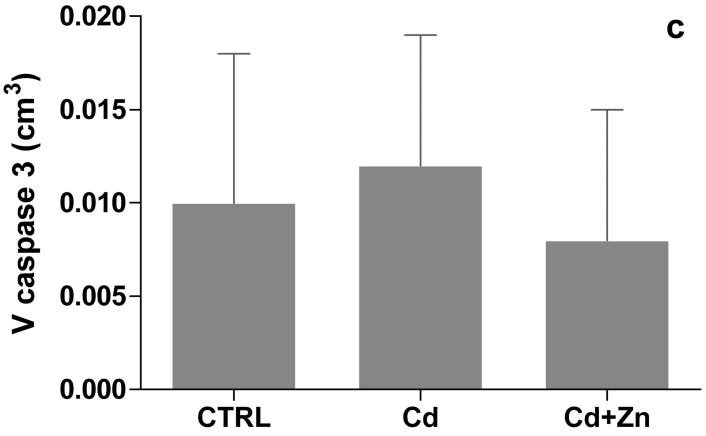

Fig. (6). (a) Bar graph of the values of volume of the seminiferous epithelium immunoreactive to ubiquitin (V UB) in cm^3 in controls (CTRL), cadmium- treated (Cd) and cadmium plus zinc-exposed (Cd+Zn) rats, 12 months after the beginning of the experiment. Results are expressed as mean ± SD. Asterisks indicate significant differences ($p < 0.05$) between CTRL and Cd groups, and between Cd and Cd+Zn groups.

(b) Bar graph of the values of volume of the seminiferous epithelium immunoreactive to UCHL1 (V UCHL1) in cm^3 in controls (CTRL), cadmium treated (Cd) and cadmium plus zinc exposed (Cd+Zn), 12 months after the beginning of the experiment. Results are expressed as mean ± SD. Asterisks indicate significant differences ($p < 0.05$) among CTRL and Cd groups, Cd and Cd+Zn groups, and CTRL and Cd+Zn groups, respectively.

(c) Bar graph of the values of volume of the seminiferous epithelium immunoreactive to caspase 3 (V caspase 3) in cm^3, in controls (CTRL), cadmium-treated (Cd) and cadmium plus zinc-exposed (Cd+Zn) rats, 12 months after the beginning of the experiment. Results are expressed as mean ± SD. There were no significant differences among groups ($p < 0.05$).

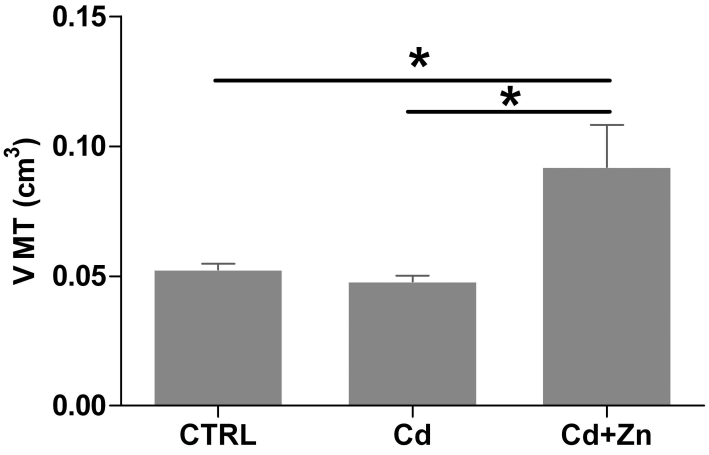

Fig. (7). Bar graph of the values of volume of the seminiferous epithelium immunoreactive to metallothionein (V MT) in cm^3, in controls (CTRL), cadmium-treated (Cd) and cadmium plus zinc-exposed (Cd+Zn) rats, 12 months after the beginning of the experiment. Results are expressed as mean ± SD. Asterisks indicate significant differences ($p < 0.05$) between CTRL and Cd+Zn groups, and between Cd and Cd+Zn groups.

mediated by cadmium, inhibiting the entry of the cells in the S-phase of the cell cycle [37].

Furthermore, the changes in the amount of cytoplasm immunoreactive to UCHL1, essential for maintaining ubiquitination activity by releasing UB from its substracts [16], paralleled the variation observed for UB immunostaining. These data suggest that Cd induces overexpression of UCHL1, and subsequently, the increase of mono-UB levels required to enhance the apoptosis [17]. Therefore, it seems that ubiquitin regulates the apoptosis mediated by Cd in seminiferous epithelium of the rat [38, 39]. In addition, although Zn exposure was able to decrease the UCHL1 and UB levels, it did not do so sufficiently to reverse the apoptotic rate measured by TUNEL at the control levels.

Caspases are important components of the mammalian apoptotic machinery; caspase-3 is a prototypical enzyme that becomes activated during apoptosis in a wide variety of tissues [40]. However, no significant changes were detected in the amount of caspase-3 immunostaining either in the Cd

or Cd+Zn groups in relation to the controls. This might be interpreted as an effect of the long-term duration of the experiment. When the testes were examined, 12 months after the beginning of the treatment, the wave of caspase-3 activation could have ceased, leaving only the fragmented DNA evidenced by TUNEL [41] remaining to be quantified.

On the other hand, the results of this study indicate that MT immunostaining increased significantly, in both spermatogonia and Sertoli cells, in the group of rats treated with Cd + Zn, but not in the animals exposed solely to Cd. Thus, it seems that MT might be induced either by Zn or by the combination of Cd plus Zn, but not by Cd alone, as was reported by other authors [19, 29].

In conclusion: a) The intake of low, oral doses of cadmium chloride over a long period of time induces quantitative changes in apoptosis of the seminiferous epithelium of the rats, without noticeable morphologic or proliferative alterations. b) The increase of mono-ubiquitin levels mediated by cadmium is caused by overexpression of UCHL1. c) Zinc exposure was able to decrease UCHL1 and ubiquitin, but not sufficiently to reverse the apoptotic rate of the spermatogonia at the control level. d) It seems that metallothionein might be induced either by zinc or by the combination of cadmium plus zinc, but not by cadmium alone.

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