Quantitative Study of Leydig Cell Populations in Mice Exposed to Low Doses of Cadmium

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Abstract Leydig cell morphological changes were evaluated using morphometric and stereological methods in male mice exposed to low doses of cadmium. A possible reversibility of the changes after cadmium withdrawal was also considered. Nuclear morphological parameters and stereological densities of the Leydig cell population were lower in the cadmium-exposed groups than in the control. The withdrawal of cadmium did not lead to any significant recovery of the morphological parameters. Nevertheless, numerical density increased significantly in the withdrawn groups, suggesting that the hyperplasia of interstitial cells could try to relieve morphological damage after cadmium withdrawal.

Keywords Cadmium · Testes · Stereology · Leydig

Cadmium is a naturally occurring metal widely used in various chemical forms in metallurgical and other industrial processes. In recent years, there has been increasing concern about this metal being an environment pollutant, mainly derived from its extremely long biological half life, so that long-term past exposure could still result in direct toxic effects from the residual metal (Benoff et al. 2000).

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Several authors have reviewed the toxicological effect of cadmium on vital organs after acute and chronic exposure, highlighting the incidence of nephropathies, hepatic necrosis, pulmonary emphysema, osteoporosis and increased prevalence of several cancers (Swiergosz-Kowalewska 2001).

The adverse consequences on testes of exposure to this heavy metal have also been widely considered in many species, using a wide range of doses and administration routes (Benoff et al. 2000). The most widely-known and important lesion of Leydig cells following cadmium exposure is the appearance of interstitial tumours (Waalkes 2003). Certain studies have pointed out that cadmium can be taken up into Leydig cells by a transport system, which supports the hypothesis that Leydig cells may be a direct target of cadmium in male reproduction (Koizumi et al. 1992).

Nevertheless, apart from those on carcinogenic lesions, few data are available on the morphological consequences of subclinic and chronic cadmium exposure on the Leydig cell population.

The purpose of this work was to evaluate, by using morphometric and stereological methods, if long-term exposure to low doses of cadmium caused any morphological alteration in the Leydig cell population. The possible reversibility of the changes after cadmium withdrawal was also considered.

Materials and Methods

Forty-eight 12-week-old sexually mature male mice (*Mus musculus*, Swiss OF-1) were acquired from the University of Granada. Mice were divided randomly into four groups, which were controlled for 1 month (n = 10), 3 months (n = 10), 6 months (n = 14) and 12 months (n = 14), respectively. The animals were housed in plastic cages at an ambient temperature of 20–24°C at humidity of 45%–

65%, with a 12 h light/dark cycle and access to a standard food pellet diet and drinking ad libitum. All experiment protocols were approved by the Ethical Committee of the University of Córdoba, and experiments were performed in accordance with the rules of laboratory animal care and international laws on animal experimentation.

In Cd-Exposed animals Cadmium (Cadmium chloride, purity 99%, Acros Organics, New Jersey, USA) was administered in drinking water (0.015 g/L CdCl2). This dissolution was calculated so that the sum of individual doses ingested per animal over 12 months was 7.8 mg/kg (one-tenth times the oral LD50 dose of 63–88 mg/kg). Cadmium withdrawal was also considered in the 6 and 12 month groups, as summarised in Table 1.

Animals were sacrificed by cervical dislocation and both testes were removed. For morphological estimations, one testis was fixed in 10% neutral phosphate-buffered formalin; the other testis was frozen for analytical cadmium determinations.

The cadmium concentration in testes was estimated in samples of approximately 0.5 g of tissue, digested twice with 6 mL of concentrated (65%, w/v) nitric acid (Merck) and 1 mL 30% (w/v) hydrogen peroxide (*Fluka*) by an analytical microwave digestion system, and applied to a graphite tube atomiser for detection of cadmium by atomic absorption spectrophotometer (Varian Spectra A-600, Varian, Inc., USA).

For the morphological study, the fixed testis was cut into top, middle and bottom sections. Each portion was then processed and embedded in paraffin as for routine histology. The first section (4 μm thick) of each block was stained with hematoxylin and eosin and used for the stereological study.

The quantitative study was performed using an image analysis system consisting of a Leitz Ortholux triocular microscope connected by means of a SONY SSC-C370P® colour video camera to an IBM-compatible personal computer equipped with a frame grabber board. Each specimen was sampled systematically for the selection of microscopic images that were then digitized; a 100× lens (N.A. 1.25) was used for this procedure. An average of 50 microscopic fields per slab was chosen in each specimen. Each microscopic image was processed using Visilog 5® software. Quantification was performed by an observer experienced in the use of the analysis system (J.G.M) but with no previous knowledge of which group was being analysed. The system was initially, and regularly, calibrated using a millimetre slide, and then the images were measured to obtain morphometric and stereological parameters.

The morphometric data were obtained by tracing the outline of the nuclear profiles of Leydig cells with the cursor of the analysis system. The quantitative information on the nuclear profiles included their area, average diameter and perimeter. The shape factor (*minimal diameter/maximal diameter*) was also estimated with values of 1.00 for a circle and <1.00 for elliptical structures. For the stereological estimators, several adapted test systems were superimposed onto each microscopic image. The nuclear volume fraction (V_V) of the Leydig cell population was obtained following the equations: V_V (Leydig/tissue) = ΣP(Leydig)/ΣP(tissue), where ΣP(Leydig) and ΣP(tissue) are the total number of points which hit the nucleus of the Leydig cells and the tissue, respectively (Fig. 1a). For the nuclear surface density (S_V) of the Leydig cells, the calculation was: S_V(Leydig/tissue) = $2 \times$ (*p/l*) $\times \Sigma I$(Leydig)/ΣP(tissue), where *p/l* is the test line length per point in the test system (in our study, *p/l* = 0.4 μm^{-1}), ΣI(Leydig) the total number of intersects between test lines and the nuclear profile boundary of Leydig cells, and ΣP(tissue) is the total number of points which hit the tissue (Fig. 1b). The numerical density (Q_A) of Leydig cells in the plane was also estimated using a test system of rectangular counting frames superimposed onto each microscopic image (Fig. 1c). Thus, the number of profiles per area Q_A(Leydig/tissue) was estimated according to Q_A(Leydig/tissue) = ΣQ(Leydig)/ΣP(tissue) $\times$ *a/p*, where ΣQ(Leydig) is the total number of nuclear profiles of the Leydig cells counted within the counting frames, ΣP(tissue) is the total number of points which hit the tissue, and *a/p* is the area associated with one point on the test system (in our study, *a/p* = 125 μm^2). The Leydig cell nuclear volume was obtained using the point-sampled intercepts method (Gundersen and Jensen 1985); the

Table 1 Animal distribution in experimental the groups regarding the time that they were raised with (Y) or without (N) cadmium administration

	Time (months)											
	1	2	3	4	5	6	7	8	9	10	11	12
1 month group												
Control (n = 4)	N											
Cd-Exposed (n = 6)	Y											
3 month group												
Control (n = 4)	N	N	N									
Cd-Exposed (n = 6)	Y	Y	Y									
6 month group												
Control (n = 4)	N	N	N	N	N	N						
Cd-Exposed (n = 6)	Y	Y	Y	Y	Y	Y						
Cd-Withdrawn (n = 4)	Y	Y	N	N	N	N						
12 month group												
Control (n = 4)	N	N	N	N	N	N	N	N	N	N	N	N
Cd-Exposed (n = 6)	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Cd-Withdrawn (n = 4)	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N

Fig. 1 Photomicrographs of Leydig cells of Cd-Exposed animals. The test systems superimposed on **a–d** were used in the determination of the nuclear volume fraction V_V(nucl/tis), nuclear surface density S_V(nucl/tis), nuclear numerical density Q_A(nucl/tis) and nuclear volume ($\bar{v}_v$), respectively
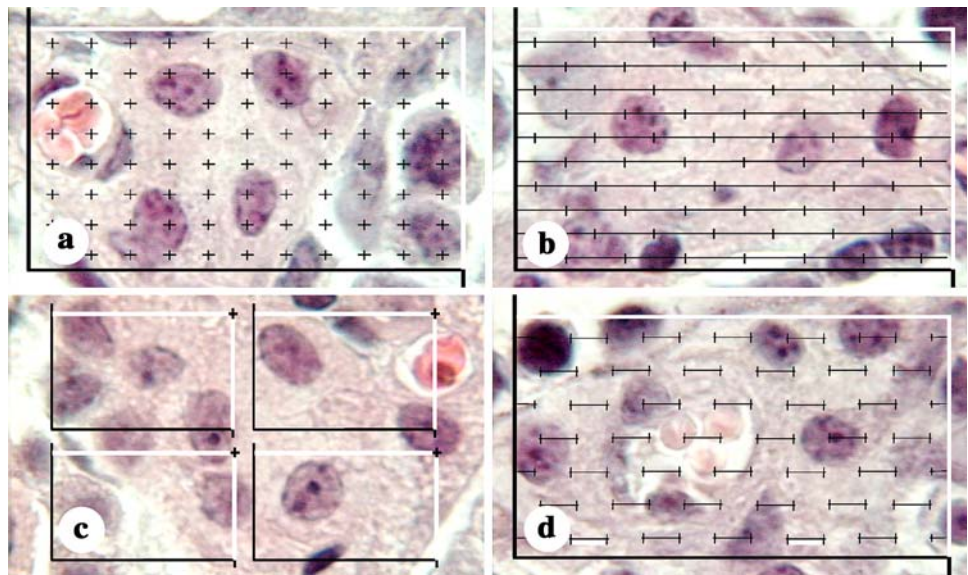
volume-weighted mean nuclear volume ($\bar{v}_v$) was obtained by the formula: $\bar{v}_v = l_o^3 \times \pi/3$, where l_o is the intercept length of the test lines through the sampled nucleus of Leydig cells (Fig. 1d).

Data were analysed using the GLM (General Linear Model) included in the Statistica software. (Statistica, version 6. Statsoft inc) to determine the effects of cadmium on each time exposure group and the overall effects of cadmium, time, and the cadmium over time interaction. The Fisher LSD post-hoc test was used to perform multiple comparisons between groups. Results are expressed as the mean ± standard deviation and $p < 0.05$ was considered to be significant.

Results and Discussion

Figure 2 shows the levels of cadmium concentration in the testes of mice at the different times of cadmium exposure. Whereas no cadmium was detected in the controls, a time-dependent increase in the cadmium concentration was observed in the testes of exposed animals so that differences were highly significant compared to the control as from 3 months of exposure. The figures recorded for cadmium withdrawn at 6 and 12 months reflected a significant drop in cadmium levels. These results are consistent with data from previous studies that have reported the strong tendency of cadmium to concentrate in the testes in cases of chronic exposure (Bench et al. 1999; Teiichiro et al. 2002).

Morphological variables of Leydig cells are summarised in Table 2. Cadmium exposure caused a decrease in the linear parameters of the Leydig cells so that, after 12 months of cadmium exposure, all the variables were
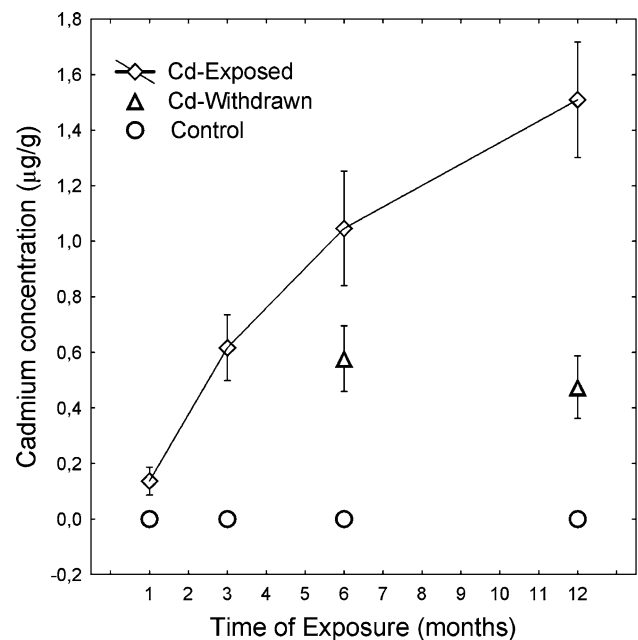

Fig. 2 Concentrations of cadmium in the testes at the different group times of cadmium exposure

significantly lower in the cadmium-exposed groups than in the control. The shape and form data also showed a decrease in the Cd-Exposed groups, indicating that the Leydig nuclear shape was less circular in the exposed groups compared to the control in accordance with the Leydig cell nuclear shrinkage and hyperchromatic images often observed in the cadmium-exposed groups. Although no other severe lesions were seen in our study, these morphological changes indicated that the cadmium exposure caused a degeneration process in the Leydig cells, and this agrees with other studies, which have pointed out that

the degenerative effect of cadmium on this cell population might be the previous step to the appearance of tumour lesions (Selypes et al. 1992; Yang et al. 2003). In our study, the cadmium withdrawal was hardly reflected in the recovery of Leydig cell morphological data, suggesting that the morphological damage induced by cadmium remained fairly stable even after cadmium was withdrawn.

In relation to the stereological variables (Table 3), the V_V (Leydig/tissue) reflected that the percentage of the

testicular tissue occupied by the Leydig cells was 28% lower in the Cd-Exposed groups than in the control. However, the Cd-Withdrawn groups showed a recovery of the Leydig cell percentage that reached similar values to the control. Similarly, the nuclear surface density S_V (Leydig/tissue) of the Leydig cells also decreased in the cadmium-exposed groups. Apart from the diversity of routes and doses of cadmium exposure, along with other factors that could obviously affect the results, previous

Table 2 Data (mean ± SD) of nuclear morphometric parameters of the Leydig cells

	Groups			
	1 month	3 months	6 months	12 months
Volume $\bar{v}_v$ (μm^3)				
Control	124.49 ± 13.90	125.39 ± 13.06	139.61 ± 32.34	145.94 ± 26.03
Cd-Exposed	123.80 ± 17.16	123.01 ± 16.46	127.69 ± 25.15	123.85 ± 7.74
Cd-Withdrawn			120.75 ± 10.13	118.14 ± 8.88*
Area (μm^2)				
Control	22.63 ± 1.82	22.69 ± 0.57	24.69 ± 2.55	25.19 ± 1.64
Cd-Exposed	22.53 ± 1.60	21.75 ± 2.22	22.36 ± 1.40*	22.00 ± 1.04**
Cd-Withdrawn			21.91 ± 2.05*	21.97 ± 2.30*
Average diameter (μm)				
Control	5.27 ± 0.22	5.28 ± 0.03	5.51 ± 0.27	5.57 ± 0.17
Cd-Exposed	5.28 ± 0.19	5.19 ± 0.25	5.26 ± 0.14	5.20 ± 0.12**
Cd-Withdrawn			5.20 ± 0.24*	5.20 ± 0.26*
Perimeter (μm)				
Control	17.27 ± 0.75	17.40 ± 0.13	18.08 ± 0.89	18.32 ± 0.55
Cd-Exposed	17.35 ± 0.58	17.14 ± 0.82	17.30 ± 0.47	17.12 ± 0.38**
Cd-Withdrawn			17.18 ± 0.74	17.16 ± 0.77*
Shape factor FFELL				
Control	0.75 ± 0.04	0.76 ± 0.03	0.76 ± 0.03	0.76 ± 0.01
Cd-Exposed	0.72 ± 0.02	0.71 ± 0.02**	0.72 ± 0.02*	0.71 ± 0.02*
Cd-Withdrawn			0.73 ± 0.04	0.73 ± 0.06

*,** Significantly different from control with $p < 0.05$ and $p < 0.01$, respectively

Table 3 Data (mean ± SD) of nuclear stereological variables of the Leydig cells

	Groups			
	1 month	3 months	6 months	12 months
V_V (Leydig/tissue) (%)				
Control	6.34 ± 0.58	6.32 ± 1.11	6.34 ± 0.50	6.69 ± 0.66
Cd-Exposed	5.97 ± 0.45	4.55 ± 0.70***	4.68 ± 0.53***	4.41 ± 0.89***
Cd-Withdrawn			6.38 ± 0.69	5.69 ± 0.34*
S_V (Leydig/tissue) (cm^3/cm^2)				
Control	65.95 ± 9.33	65.46 ± 13.12	65.48 ± 7.25	71.02 ± 5.93
Cd-Exposed	63.04 ± 6.44	48.02 ± 6.83**	51.30 ± 6.87**	48.07 ± 8.14***
Cd-Withdrawn			65.56 ± 7.50	58.79 ± 4.25*
Q_A (Leydig/tissue) (in mm^2)				
Control	346.69 ± 28.83	302.57 ± 37.32	298.59 ± 25.23	298.12 ± 25.53
Cd-Exposed	310.16 ± 43.05	262.50 ± 21.92*	234.16 ± 11.16**	242.28 ± 27.43**
Cd-Withdrawn			322.32 ± 29.98	320.91 ± 36.13

*,**,*** Significantly different from control with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

studies have reported that cadmium exposure induces significant reductions in the testicular interstitium (Gouveia 1988; Biswas et al. 2001; Blanco et al. 2007). The Q_A (Leydig/tissue) values showed that the numerical density of the Leydig cells decreased significantly as from 3 months of cadmium exposure with respect to the controls, but in both Cd-Withdrawn groups, the numerical density of the Leydig cells rose to figures above those recorded for the control group. This recovery of the numerical density of Leydig cells recorded in Cd-Withdrawn groups suggests that the cadmium withdrawal led to a hyperplasia of this cell population, which could try to offset the morphological damage.

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