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Testicular toxicity induced by dietary cadmium is associated with decreased testicular zinc and increased hepatic and renal metallothionein and zinc in the bank vole *(Clethrionomys glareolus)*

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Abstract

Mechanism of testicular toxicity induced by dietary cadmium (Cd) has been less investigated than that following acute Cd injection. In the present study we characterized testicular injury in a small rodent, the bank vole, exposed subchronically to dietary Cd in a quantity of 0.9 μmol/g, and determined the importance of some factors (Cd accumulation, metallothionein (MT), oxidative stress, and zinc (Zn)) in the injury. Dietary Cd induced moderate histopathological changes (hemorrhage in interstitium, necrosis and apoptosis in seminiferous tubule epithelium) in young (1 month old) bank voles fed, for 6 weeks, Fe-adequate (1.1–1.4 μmol/g) and Fe-enriched (4.5–4.8 μmol/g) diets. In contrast, adult (5 months old) bank voles appeared to be resistant to the toxic effects of dietary Cd, despite the fact that testicular Cd contents were higher and MT levels lower than those in the young animals. The Cd-induced histopathological changes and apoptosis were accompanied by increased testicular lipid peroxidation, decreased testicular Zn concentration and elevated levels of hepatic and renal MT and Zn. Supplemental dietary Zn (1.7–1.8 μmol/g) prevented the Cd-induced testicular Zn depletion and injury. The data indicate that dietary Cd produces testicular lesions indirectly, through decreasing testicular Zn, which seems to be due to the sequestration of this element by the Cd-induced hepatic and renal MT.

Introduction

Cadmium (Cd) is an industrial and environmental pollutant that is toxic to several organs and cells (Goering *et al.* 1995; Beyersmann & Hechtenberg 1997). Depending on acute exposure route, Cd produces apoptosis and necrosis in the liver, edema in the lungs, and apoptosis and hemorrhagic necrosis in the testes (Parizek & Zahor 1956; Bus *et al.* 1978; Xu *et al.* 1996; Habeebu *et al.* 1998). Following chronic exposure, Cd damages primarily the kidneys but is also toxic to the liver, bone and reproductive system (Kotsonis & Klaassen 1978; Waalkes *et al.* 1999; Habeebu *et al.* 2000a, b). It is commonly thought that an intracellular metallothionein (MT), a low-molecular-weight thiol-rich, Cd-induced protein, because of its high affinity for Cd, provides a mechanism by which the metal can be sequestered in a relatively inert and thus nontoxic form (Masters *et al.* 1994; Liu *et al.* 1995). When the amount of Cd in tissues exceeds the binding capacity of MT, the non-MT-bound Cd ions are believed to cause toxicity (Cherian *et al.* 1976; Goyer *et al.* 1989; Nomiyama & Nomiyama 1998; Nordberg & Nordberg 2000). The protein has been associated with Cd detoxification especially in the liver and kidneys where accumulation of Cd is relatively high and synthesis of MT is efficiently induced after Cd exposure (Klaassen *et al.* 1999). In contrast, the mammalian testes, one of the more remarkable sites of Cd toxicity, have shown either no induction or a reduced expression of the MT gene, when animals were exposed to Cd (Shiraishi *et al.* 1993; McKenna *et al.* 1996; Xu *et al.* 1999). This fact is thought to explain a higher sensitivity of testes to Cd-induced toxicity than many

other tissues. However, this hypothesis appears not to agree with the findings of other studies that MT is expressed in the testes after Cd exposure (Nordberg 1971; Chellman *et al.* 1985; Nolan & Shaikh 1986). Although non-MT-bound Cd ions can interact directly with various molecules inside the cell thereby causing an injury, one cannot exclude that the toxic effects of Cd on testes may also result from its indirect action. For example, acute Cd administration has been shown to reduce testicular zinc (Maitani & Suzuki 1986) and elevate the iron (Koizumi & Li 1992; Yiin *et al.* 1999), which in turn can lead to an increased production of reactive oxygen species (ROS), being probably involved in the tissue damage (Oteiza *et al.* 1999).

As in acute Cd toxicity, testis has been shown to be also a target organ following chronic Cd exposure (Saygi *et al.* 1991; Świergosz *et al.* 1998). However, compared to acute Cd-induced testicular injury, the pathology of testicular injury following repeated Cd exposure has not been fully investigated. Data from our laboratory indicate that hemorrhagic necrosis is a common characteristic of testicular injury induced by dietary Cd (0.4–1.1 μmol/g) in a small rodent, the bank vole, although the liver and kidneys appear to be resistant to the toxic effects of these doses of Cd (Włostowski *et al.* 2004; unpublished observation); still the mechanism by which dietary Cd produces exclusively testicular injury remains to be elucidated. Therefore, the main purpose of this work was to study histopathological changes and apoptosis in the testes of bank voles exposed subchronically to dietary Cd. Analyses of Cd, MT, Zn, Fe and lipid peroxidation were also carried out to determine their possible role in the injury. Because supplemental dietary Fe has been shown to protect against Cd-induced hepato- and nephrotoxicity (Groten *et al.* 1991; Włostowski *et al.* 2003), in this study the animals were fed Fe-adequate and Fe-enriched diets. Furthermore, as Zn status determines sensitivity of the animal to Cd-induced testicular damage, and Zn pretreatment protects testes against injury (Wahba *et al.* 1994; Oteiza *et al.* 1999), the bank voles were also provided Zn-adequate and Zn-enriched diets. Since the animal age may affect Cd accumulation and MT synthesis (Wormser & Nir 1988; Shaikh *et al.* 1993) all experiments were performed on young and adult bank voles.

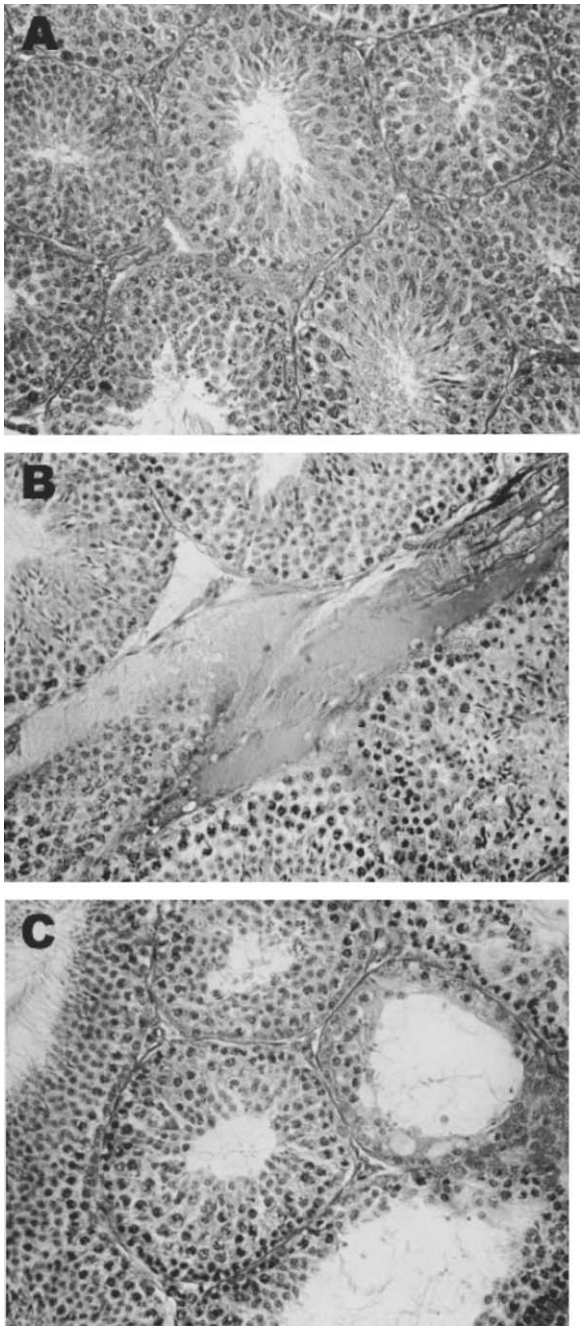

Figure 1. Representative photomicrographs of testis section from (A) control bank voles and (B, C) bank voles fed, for 6 weeks, a diet containing 0.9 μmol Cd/g (Cd group). Hemorrhage in interstitium (B), and vacuolization and necrosis of the seminiferous epithelium (C) in all Cd-treated voles were observed. Similar histopathological changes were seen in bank voles from the Fe+Cd group (not shown). No changes were noted in animals from the control, Fe, Zn, and Zn+Cd groups. H & E staining, $\times$ 200.

Table 1. Effect of dietary cadmium on body and testes weights, and testicular injury in bank voles fed diets supplemented and not supplemented with Fe and Zn[#].

Group	Body weight (g)		Testes wet weight (g)		Apoptosis		Histopathology	
	1 mo	5 mo	1 mo	5 mo	1 mo	5 mo	1 mo	5 mo
Control	20.7 ± 3.7^{a}	22.2 ± 2.0^{a}	0.47 ± 0.22^{a}	0.62 ± 0.16^{a}	0.13 ± 0.03^{a}	0.10 ± 0.02^{a}	(−)	(−)
Cd	20.1 ± 1.6^{a}	21.4 ± 3.4^{a}	0.40 ± 0.19^{a}	0.51 ± 0.24^{a}	0.23 ± 0.06^{b}	$0.10 \pm 0.03^{a*}$	(+)	(−)
Fe	21.0 ± 2.5^{a}	21.9 ± 3.2^{a}	0.48 ± 0.21^{a}	0.50 ± 0.24^{a}	0.12 ± 0.04^{a}	0.09 ± 0.05^{a}	(−)	(−)
Fe+Cd	20.8 ± 3.7^{a}	20.7 ± 2.5^{a}	0.48 ± 0.17^{a}	0.55 ± 0.23^{a}	0.25 ± 0.07^{b}	$0.09 \pm 0.04^{a*}$	(+)	(−)
Zn	21.1 ± 2.5^{a}	21.5 ± 2.0^{a}	0.49 ± 0.21^{a}	0.60 ± 0.20^{a}	0.12 ± 0.04^{a}	0.10 ± 0.04^{a}	(−)	(−)
Zn+Cd	20.5 ± 2.0^{a}	22.0 ± 3.0^{a}	0.50 ± 0.20^{a}	0.57 ± 0.15^{a}	0.10 ± 0.04^{a}	0.11 ± 0.05^{a}	(−)	(−)

[#]Values represent the mean ± SD for $n = 7$. The 1- and 5-months (mo) old bank voles received, for 6 weeks, control diet or diets enriched with Fe (4.5–4.8 μmol/g) or Zn (1.7–1.8 μmol/g) and contaminated with Cd (0.9 μmol/g). Apoptosis is expressed as TUNEL-positive cells per seminiferous tubule (see Figure 2). Histopathology: normal morphology (−), moderate histopathological changes (+) (see Figure 1). Means in the same column marked with different superscript letters are significantly different ($P < 0.05$). * Significantly different from the 1-month (mo) old vole ($P < 0.05$).

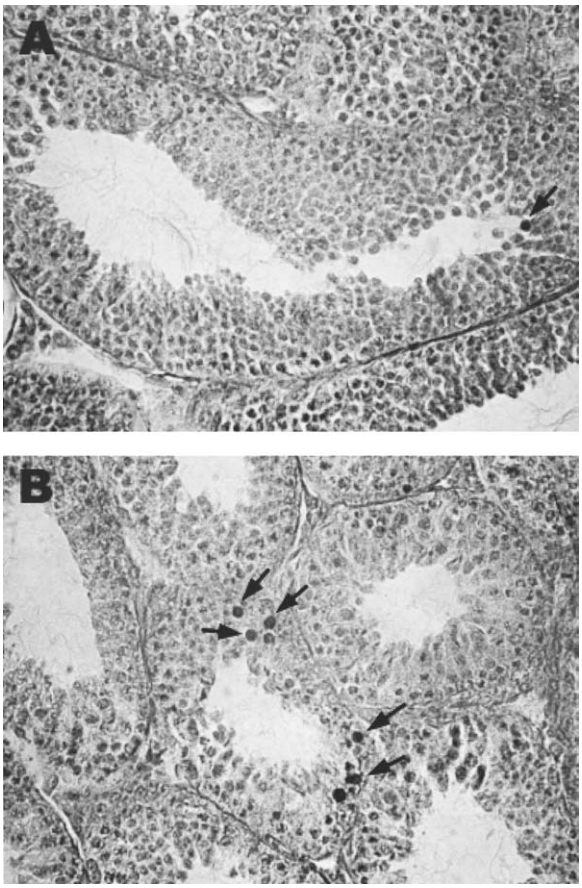

Figure 2. Immunohistochemical demonstration of apoptotic cells in testes by the TUNEL technique. (A) Control bank voles showing the normal level of apoptosis. (B) Bank voles fed, for 6 weeks, a diet containing 0.9 μmol Cd/g (Cd group) showing the increased number of apoptotic cells (arrows). × 200.

Materials and methods

Animals and experimental design

Male bank voles (1 month old, weighing 10–13 g and 5 month old, weighing 19–24 g), being F1 offspring of the wild-caught population (Knyszyn Old Forest, north-eastern Poland) were used throughout the study. The bank voles from the two age groups were randomly divided into six groups (7 animals each) according to dietary Cd, Fe and Zn: (1) control; (2) Cd – receiving diet containing 0.9 μmol Cd/g; (3) Fe – receiving diet enriched with Fe (4.5–4.8 μmol/g; (4) Fe+Cd– receiving Fe-enriched diet (4.5–4.8 μmol/g) containing 0.9 μmol Cd/g; (5) Zn – receiving diet enriched with Zn (1.7–1.8 μmol/g), and (6) Zn+Cd – receiving Zn-enriched diet (1.7–1.8 μmol/g) containing 0.9 μmol Cd/g.

The animals were housed in groups of 2 or 3 in stainless-steel cages and kept for 6 weeks on a 16 h light/8h dark cycle in a room maintained at 18–20 °C and at 50–70% relative humidity. For 6 weeks, the bank voles received ad libitum distilled water and control or Cd-containing wheat grains which are considered to be an adequate quality food for these rodents (Włostowski *et al.* 1996). In addition, an identical amount of apple was offered to all animals (3 g/vole/week) and was eaten completely. The food intake was measured in the last 2 weeks of the experiment. Prior to the experiment the grains were contaminated with Cd (soaked in $CdCl_2$ solution) and supplemented with Fe or Zn (soaked in $FeSO_4$ or $ZnCl_2$ solution) (Świergosz *et al.* 1998). Atomic absorption spectrophotometry (AAS) analysis of the grain revealed that actual levels of Cd were

Table 2. Effect of dietary cadmium on testicular Cd, metallothionein (MT), Zn, lipid peroxidation (TBARS) and Fe in bank voles fed diets supplemented and not supplemented with Fe and Zn[#].

Group	Cd (nmol/g)		MT (nmol/g)		Zn (nmol/g)		TBARS (nmol/g)		Fe (nmol/g)	
	1 mo	5 mo	1 mo	5 mo	1 mo	5 mo	1 mo	5 mo	1 mo	5 mo
Control	0.27 ± 0.18^{a}	$0.54 \pm 0.27^{a*}$	3.52 ± 0.90^{a}	$1.12 \pm 0.14^{a**}$	555 ± 84^{a}	462 ± 67^{a}	19.3 ± 15.0^{a}	$86.9 \pm 30.8^{a*}$	1934 ± 895^{a}	$720 \pm 308^{a*}$
Cd	2.14 ± 0.89^{b}	$4.00 \pm 1.42^{b*}$	3.06 ± 0.74^{a}	$1.30 \pm 0.13^{a**}$	245 ± 124^{b}	$456 \pm 76^{a*}$	67.0 ± 18.0^{b}	71.6 ± 43.2^{a}	1432 ± 1074^{a}	797 ± 449^{a}
Fe	0.27 ± 0.18^{a}	$0.63 \pm 0.31^{a*}$	3.32 ± 0.61^{a}	$1.11 \pm 0.19^{a**}$	538 ± 76^{a}	448 ± 69^{a}	25.0 ± 10.0^{a}	$79.3 \pm 20.0^{a*}$	1773 ± 806^{a}	$743 \pm 269^{a*}$
Fe+Cd	2.22 ± 0.36^{b}	$4.45 \pm 0.98^{b*}$	4.05 ± 0.60^{a}	$1.32 \pm 0.17^{a**}$	310 ± 76^{b}	$460 \pm 69^{a*}$	97.0 ± 36.1^{b}	94.0 ± 26.2^{a}	2256 ± 1146^{a}	$824 \pm 367^{a*}$
Zn	0.18 ± 0.18^{a}	$0.58 \pm 0.27^{a*}$	3.57 ± 0.66^{a}	$1.21 \pm 0.19^{a**}$	573 ± 101^{a}	495 ± 87^{a}	20.3 ± 12.5^{a}	$80.1 \pm 32.0^{a*}$	1826 ± 859^{a}	$809 \pm 278^{a*}$
Zn+Cd	2.05 ± 0.53^{b}	$4.54 \pm 1.07^{b*}$	3.94 ± 0.71^{a}	$1.32 \pm 0.19^{a**}$	466 ± 89^{a}	451 ± 73^{a}	30.2 ± 15.3^{a}	$89.3 \pm 26.5^{a*}$	1880 ± 931^{a}	$761 \pm 358^{a*}$

[#]Values represent the mean $\pm$ SD for $n = 7$. The 1- and 5-months (mo) old bank voles received, for 6 weeks, control diet or diets enriched with Fe (4.5–4.8 μmol/g) or Zn (1.7–1.8 μmol/g) and contaminated with Cd (0.9 μmol/g). Means in the same column marked with different superscript letters are significantly different ($P < 0.05$).

*, **Significantly different from the 1-month (mo) old vole ($P < 0.05$; $P < 0.01$, respectively).

Table 3. Effect of dietary cadmium on hepatic Cd, metallothionein (MT), and Zn levels in bank voles fed diets supplemented and not supplemented with Fe and Zn[#].

Group	Cd (nmol/g)		MT (nmol /g)		Zn (nmol/g)	
	1 mo	5 mo	1 mo	5 mo	1 mo	5 mo
Control	0.89 ± 0.18^{a}	$2.23 \pm 0.49^{a*}$	2.12 ± 1.13^{a}	$0.52 \pm 0.11^{a**}$	413 ± 67^{a}	517 ± 50^{a}
Cd	233.08 ± 44.48^{b}	$85.40 \pm 29.36^{b*}$	51.60 ± 10.68^{b}	$6.23 \pm 3.94^{b**}$	853 ± 104^{b}	$561 \pm 47^{a*}$
Fe	1.07 ± 0.44^{a}	$2.67 \pm 0.54^{a*}$	2.22 ± 1.14^{a}	$0.66 \pm 0.10^{a**}$	459 ± 76^{a}	535 ± 109^{a}
Fe+Cd	218.84 ± 35.58^{b}	$93.41 \pm 4.45^{b*}$	48.42 ± 10.29^{b}	$9.91 \pm 2.16^{b**}$	959 ± 115^{b}	$529 \pm 66^{a*}$
Zn	0.98 ± 0.18^{a}	$2.45 \pm 0.45^{a*}$	2.16 ± 1.14^{a}	$0.71 \pm 0.15^{a**}$	466 ± 61^{a}	521 ± 64^{a}
Zn+Cd	225.07 ± 35.58^{b}	$89.85 \pm 10.68^{b*}$	52.74 ± 11.56^{b}	$10.42 \pm 3.81^{b**}$	936 ± 125^{b}	$555 \pm 110^{a*}$

[#]Values represent the mean $\pm$ SD for $n = 7$. The 1- and 5-months (mo) old bank voles received, for 6 weeks control diet or diets enriched with Fe (4.5–4.8 μmol/g) or Zn (1.7–1.8 μmol/g) and contaminated with Cd (0.9 μmol/g). Means in the same column marked with different superscript letters are significantly different ($P < 0.05$). *, **Significantly different from the 1-month (mo) old vole ($P < 0.05$; $P < 0.01$, respectively).

Table 4. Effect of dietary cadmium on renal Cd, metallothionein (MT), and Zn levels in bank voles fed diets supplemented and not supplemented with Fe and Zn[#].

Group	Cd (nmol/g)		MT (nmol/g)		Zn (nmol/g)	
	1 mo	5 mo	1 mo	5 mo	1 mo	5 mo
Control	2.22 ± 1.07^{a}	$4.67 \pm 1.34^{a*}$	2.38 ± 0.56^{a}	$1.00 \pm 0.31^{a*}$	512 ± 112^{a}	566 ± 92^{a}
Cd	409.22 ± 80.06^{b}	$177.92 \pm 26.69^{b*}$	72.57 ± 23.0^{b}	$10.93 \pm 2.29^{b**}$	988 ± 184^{b}	$592 \pm 99^{a*}$
Fe	1.78 ± 0.71^{a}	$5.12 \pm 1.60^{a*}$	2.10 ± 0.42^{a}	$1.08 \pm 0.27^{a*}$	529 ± 99^{a}	590 ± 107^{a}
Fe+Cd	343.39 ± 62.27^{b}	$200.16 \pm 48.04^{b*}$	66.47 ± 15.89^{b}	$13.47 \pm 4.07^{b**}$	856 ± 142^{b}	$621 \pm 89^{a*}$
Zn	1.78 ± 0.89^{a}	$4.90 \pm 1.65^{a*}$	2.16 ± 0.32^{a}	$1.02 \pm 0.32^{a*}$	535 ± 87^{a}	599 ± 115^{a}
Zn+Cd	358.51 ± 71.17^{b}	$171.69 \pm 36.47^{b*}$	70.53 ± 19.32^{b}	$15.63 \pm 4.45^{b**}$	921 ± 153^{b}	$630 \pm 93^{a*}$

[#]Values represent the mean y $\pm$ SD for $n = 7$. The 1- and 5-months (mo) old bank voles received, for 6 weeks, control diet or diets enriched with Fe (4.5–4.8 μmol/g) or Zn (1.7-1.8 μmol/g) and contaminated with Cd (0.9 μmol/g). Means in the same column marked with different superscript letters are significantly different ($P < 0.05$). *, **Significantly different from the 1-month (mo) old vole ($P < 0.05$; $P < 0.01$, respectively).

between 0.87–0.93 μmol Cd/g dry wt. The same analysis showed that the grains contained 1.1–1.4 μmol Fe/g dry wt and 0.3–0.4 μmol Zn/g dry wt (Fe- and Zn-adequate diets) and 4.5–4.8 μmol Fe/g dry wt and 1.7–1.8 μmol Zn/g dry wt (diets supplemented with Fe or Zn in excess of nutritional requirements (Groten *et al.* 1991)). Based on our recent experiments, the selected dose of Cd appeared to be nontoxic to the liver and kidneys but toxic to the testes of bank voles raised under a long photoperiod (Włostowski *et al.* 2004; unpublished observation). The experimental protocols were approved by the local ethical committee for performing an experimental study on laboratory animals (Medical Academy in Białystok).

Assays

After 6 weeks of Cd exposure, the animals were euthanized by cervical dislocation and both testes as well as the liver and kidneys were removed and weighed. One testis, a portion of the fresh liver (0.25 g) and kidneys were transferred to 2.0 ml chilled 0.25 M sucrose and homogenized with a Teflon pestle in a glass homogenizer. Aliquots (0.2 and 1.0 ml) of the homogenate were taken for determination of lipid peroxidation and metal concentrations, respectively. The remaining homogenate was centrifuged at 20 000 $\times$ g for 20 min at 4 °C, and the resulting supernatant was removed for MT assay.

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) formation, using the thiobarbituric acid (TBA) assay (Ohkawa *et al.* 1979). To 0.2 ml of the tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% thiobarbituric acid, and 0.6 ml of distilled water were added and vortexed. The reaction mixture was placed in a water bath at 95 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of butanol/pyridine mixture (15:1, v/v) were added and vortexed. After centrifugation, absorbance

of the organic phase was determined at 532 nm. Tetraethoxypropane was used to prepare a calibration curve. The results were expressed as TBARS (nmol/g wet wt).

MT in the testes, liver and kidneys was determined by using a Cd-hem method (Onosaka & Cherian 1982) with minor modification (Włostowski & Krasowska 1999). Briefly, in a 1.5 ml vial 0.1 ml sample was incubated for 10 min at room temperature with 1.0 ml Tris-HCl buffer (0.03 M, pH 7.8) containing 1 μg Cd/ml. To remove non-MT-bound Cd, bovine hemoglobin (Sigma) (0.1 ml of a 5% solution in H_2O) was added and the sample was heated for 1.5 min at 95 °C, cooled in ice and centrifuged for 5 min at 10 000 $\times$ g. Addition of hemoglobin, heating and centrifugation of the sample was repeated three times. Cd bound to MT in the resulting clear supernatant was determined by electrothermal AAS. MT content was expressed in nmol of the protein per gram of wet tissue, assuming that 1 mol of MT (6600) binds 7 moles of Cd (Winge & Miklossy 1982).

Metal determinations were performed as described previously (Włostowski *et al.* 1996). The homogenate (1.0 ml) was placed in a glass tube with 2.0 ml of concentrated nitric acid. After 20 h of sample digestion at room temperature, 72% perchloric acid (0.5 ml) was added and the mixture was heated at 100 °C for 3 h. Finally, the temperature was raised to 150 °C and digestion continued for another 4 h. Deionized water was added to the residue after digestion to a volume of 3.0 ml (first solution). A portion of the first solution (200 μl) was evaporated to dryness in a quartz crucible at 130 °C and the residue was redissolved in an appropriate amount of deionized water (second solution). Cd analyses of these solutions were carried out by electrothermal AAS using AAS3 Carl Zeiss Jena instrument with an EA3 furnace attachment. The concentrations of Fe and Zn of the first solution were determined by AAS in an air-acetylene flame. Quality assurance procedures included the analysis of reagent blanks and appropriate standard reference material (NIST bovine liver 1577b). The recovery of Cd, Fe and Zn was 91–95, 97–104 and 90–94%, respectively.

Histological examinations

One testis from each animal was fixed in Bouin's fluid, dehydrated in ethanol and xylene, embedded in paraffin, cut into 8 μm sections, and stained with hematoxylin and eosin for microscopic examination.

In situ *apoptosis detection*

Apoptosis in the testes was demonstrated in situ by the TUNEL (TdT-mediated dUTP-fluorescein Nick End Labeling) assay, using a kit from Roche Diagnostics (Mannheim, Germany) according to their instructions. Briefly, sections were dewaxed in xylene, hydrated in graded alcohol series and permeabilized in 0.1% Triton X-100/0.1% sodium citrate for 8 min. Terminal deoxynucleotidyl transferase (TdT) enzyme and fluorescein-labeled nucleotides were applied to the sections for 60 min at 37 °C. Sections were washed with PBS and treated with alkaline phosphatase-conjugated anti-fluorescein antibody for 30 min at 37 °C. They were next treated with substrate solution (NBT/BCIP) for 10 min in the dark. Sections were counterstained in hematoxylin prior to analysis by light microscope. Apoptosis was expressed as number of TUNEL-positive cells per total number of seminiferous tubules within each testis cross.

Statistical analysis

Data were expressed as means $\pm$ SD. The values were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range test (MS Statistica). Differences at $P < 0.05$ were considered statistically significant.

Results

The 6-week exposure to dietary Cd (0.9 μmol/g) alone or in combination with extra Fe and Zn had no effect on final body and testes weights, as well as on food intake (2.5–3.0 g/day) in the young (1 month old) and adult (5 month old) bank voles (Table 1). No significant differences in the final body and organ weights were found between the two age groups.

Moderate histopathological changes (hemorrhage in interstitium, vacuolization and necrosis of seminiferous epithelium) (Figure 1) and increased incidence of apoptosis (Figure 2) occurred in the testes of all young bank voles fed both a diet enriched only in Cd (Cd group) and the Cd diet supplemented with Fe (Fe+Cd group) (Table 1); however , in the young animals no lesions were produced by dietary Cd in the presence of extra dietary Zn (Zn+Cd group). In comparison, the testes of all adult bank voles fed the Cd diets showed normal morphology (Table 1).

Testicular Cd concentrations increased significantly to a similar level in bank voles fed all three

Cd diets, but the testes of adult voles exhibited significantly higher Cd contents than the testes of young animals (Table 2). In contrast, testicular Cd-binding protein (MT) concentrations were similar in all six groups of bank voles studied but the testes of adult voles showed lower levels of the protein than the testes of young animals (Table 2). In the young bank voles which displayed testicular lesions, dietary Cd alone or in combination with extra Fe brought about a remarkable decline in testicular Zn concentration (Table 2). This effect was prevented in animals fed the Cd diet supplemented with extra Zn. In contrast, no changes in testicular Zn concentrations were observed in all adult bank voles upon Cd exposure (Table 2). Likewise, testicular lipid peroxidation was affected significantly by dietary Cd only in the young bank voles (Table 2). In general, the process inversely correlated with the changes of testicular Zn. Noteworthy, TBARS levels in the testes of all control groups of the adult bank voles (control, Fe and Zn groups) were 3–4 times higher than those in the respective young animals, and were similar to those at which histopathological changes were seen (Cd and Fe+Cd groups). The lipid peroxidation was not correlated with testicular Fe which appeared to be similar in all six groups of bank voles and the testes of adult voles showed lower concentrations of this element than the testes of young animals (Table 2).

All Cd-treated groups showed Cd accumulation in the liver and kidneys (Tables 3 and 4). Neither supplemental dietary Fe nor Zn affected significantly the accumulation of Cd in the two age groups. However, in the young bank voles the Cd accumulation in the liver and kidneys was much greater (1.7–2.7-fold) compared with adult animals. The concentrations of hepatic and renal MT, which correlated closely with Cd accumulation, were even greater (4.5-8.3-fold) in the young than adult bank voles. These changes of MT content in the liver and kidneys of young bank voles were accompanied by a significant increase (1.6–2.1-fold) in the Zn concentrations; notably, no changes in hepatic and renal Zn levels were found in the adult animals upon Cd exposure (Tables 3 and 4).

Discussion

The purpose of the present study was (1) to characterize testicular injury in bank voles exposed subchronically to dietary Cd, and (2) to determine the importance of some factors (Cd accumulation, MT, oxidative stress and Zn) in the injury.

The present study demonstrates that dietary Cd induces similar histopathological changes in the testes (hemorrhage in interstitium, necrosis and apoptosis in seminiferous tubule epithelium) as observed following acute Cd injection (Liu *et al.* 2001); however, the injury produced by dietary Cd is only a moderate compared with a severe one after acute Cd exposure. Furthermore, Cd-induced testicular injury occurred only in the young bank voles, while the testes of adult animals appeared to be resistant to the toxic effects of dietary Cd (Table 1). This difference in sensitivity to Cd-induced testicular injury between the two age groups was accompanied by different levels of testicular Cd, MT, lipid peroxidation and Zn, which are considered to be critical factors in Cd-induced testicular toxicity (Liu *et al.* 2001).

The difference in susceptibility of testes to Cd toxicity has been attributed, among other things, to differential Cd uptake into the testes, as more Cd was found in the testes of some sensitive mouse strains than in resistant ones (Chellman *et al.* 1984; King *et al.* 1999). However, in the present study adult bank voles accumulated higher amounts of Cd in comparison to young animals, but in the absence of testicular injury (Table 1 and 2). Furthermore, pretreatment of animals with low doses of Cd, which resulted in higher Cd accumulation in the testes, protected against Cd-induced testicular injury (Waalkes *et al.* 1988). Moreover, a recent study has shown that testicular Cd accumulation is relatively low and Cd ions, by themselves, seem not to be directly responsible for the injury (Liu *et al.* 2001). Thus, it is conceivable that the toxic effects of dietary Cd on testes in the bank vole could result from its indirect action.

A Cd-binding protein, MT, has been proposed to play an important role in protecting against Cd-induced injury in a number of tissues, including the testes (Nordberg 1971; Chellman *et al.* 1985; Nolan & Shaikh 1986). In the present study, testicular MT levels appeared to be similar or much higher in the bank vole exhibiting testicular injury than in those with normal morphology (Table 2). These results and the fact that MT-1 transgenic mice producing relatively high amount of MT are not refractory to Cd-induced testicular injury (Dalton *et al.* 1996) suggest that MT does not play a protective role against Cd-induced testicular injury in the bank voles.

Several lines of evidence indicate that reactive oxygen species (ROS) are involved in Cd-induced tissue

damage, including the testes (Oteiza *et al.* 1999). Lipid peroxidation is considered as an indirect measure of generation of ROS (Suzuki *et al.* 2000). In this study, lipid peroxidation increased only in the testes of young bank voles, exhibiting at the same time the tissue injury and apoptosis (Tables 1 and 2). Thus, it is possible that Cd-induced oxidative stress, that was independent of the tissue Fe (Table 2), could be responsible for these processes. However, the fact that in all adult bank voles a relatively high TBARS level is formed and no histopathological changes are observed indicates that lipid peroxidation is not always involved in the tissue injury. Still, a possibility may exist that lipid peroxidation does not strictly depend on ROS (Casalino *et al.* 2002) or there are different targets of ROS attack in the testes of young and adult bank voles.

In this study, Cd-induced testicular lipid peroxidation, histopathological changes and increased incidence of apoptosis were associated with testicular Zn depletion and supplemental dietary Zn resulted in complete reversal of the Cd-mediated effects (compare Table 1 and 2). Our observations agree with previous studies (Parizek 1957; Wahba *et al.* 1994) and suggest that the Zn depletion in testes caused by dietary Cd may be a causal factor in inducing testicular injury in the bank vole. The assumption is supported by the fact that both lipid peroxidation and apoptosis in testes have been demonstrated to increase in animals fed Zn-deficient diets (Oteiza *et al.* 1995; Nodera *et al.* 2001) and dietary Zn deficiency produces similar histopathological changes in the testes as those observed in the present study (Mason *et al.* 1982). It is also worth noting that Zn supplementation prevents against Cd-induced disruption of vascular endothelium (Kaji *et al.* 1993), further supporting the notion that Zn plays an important role in the pathogenesis of Cd-induced testicular injury.

The deprivation of testicular Zn observed in the present study was accompanied by a dramatic increase in the liver and kidneys concentrations of MT and Zn (Tables 3 and 4). The increase of hepatic MT level is known to sequester Zn from the plasma, thereby increasing its concentration in the liver and probably restricting Zn supply of other tissues e.g. testes (Coyle *et al.* 2002). In the present study supplemental dietary Zn prevented the Cd-induced testicular Zn depletion and injury in the young bank voles. Furthermore, relatively low levels of Cd-induced MT in the liver and kidneys of adult bank voles reflected in no changes in the concentrations of Zn in the liver, kidneys as well as the testes (Tables 2, 3 and 4). Thus, these results indicate that elevated levels of hepatic and renal MT produced upon Cd exposure could be responsible for testicular Zn deprivation and the resultant injury. Noteworthy, similar mechanism of teratogenicity associated with the inappropriate induction of maternal hepatic MT has been proposed (Carey *et al.* 2000), further supporting the conclusion that high levels of hepatic MT may have detrimental effects on other tissues, most probably by restricting their Zn supply.

In summary, the data obtained indicate that dietary Cd induces histopathological changes and apoptosis in the testes of young bank voles, whereas the testes of adult animals are refractory to the toxic effects of dietary Cd. It seems that dietary Cd produces testicular lesions indirectly through decreasing testicular Zn, which may be due to the sequestration of this element by the Cd-induced hepatic and renal MT.

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