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Melatonin increases tissue accumulation and toxicity of cadmium in the bank vole (*Clethrionomys glareolus*)

Ewa Chwełatiuk, Tadeusz Włostowski*, Alicja Krasowska & Elżbieta Bonda
*Institute of Biology, University of Białystok, Świerkowa 20B, 15-950, Białystok, Poland; *Author for correspondence (Tel: +48-85-745-7346; Fax +48-85-745-7302; E-mail: twlostow@uwb.edu.pl)*

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Abstract

Recent study has shown that a short photoperiod increases the accumulation and toxicity of cadmium (Cd) in the bank vole as compared to a long photoperiod. Since many of the effects of photoperiod on physiological processes in small mammals are transduced by the pineal gland and its hormone melatonin, in this study the effect of subchronic melatonin injection (7 μmol/kg/day for 6 weeks) on the hepatic, renal and intestinal Cd accumulation in the bank voles raised under a long photoperiod and exposed to dietary Cd (0.9 μmol/g) was examined. Simultaneously, histological examinations of the liver and kidneys, and analyses of metallothionein (MT) and lipid peroxidation were carried out. Melatonin co-treatment brought about a significant increase in the hepatic (61%), renal (79%) and intestinal (77%) Cd concentrations as compared to those in the Cd alone group. However, the concentrations of MT in the liver and kidneys of the Cd + melatonin co-treated bank voles did not differ from those in the Cd alone group. Also, histopathological changes in the liver (infiltration of leukocytes) and kidneys (glomerular swelling and a focal tubular cell degeneration) as well as an increase (2-fold) in the renal lipid peroxidation occurred only in animals from the Cd + melatonin group. These data indicate that (1) subchronic melatonin injection has similar effect on the tissue accumulation and toxicity of Cd to that produced by a short photoperiod and (2) the Cd-induced toxicity in the liver and kidneys of melatonin co-treated bank voles is probably due to increased Cd accumulation and decreased synthesis of MT.

Introduction

Cadmium (Cd) is one of the most important toxic chemicals due to its increasing level in the environment as a result of industrial and agricultural practices (Goering *et al*. 1995; Satarug *et al*. 2003; Waisberg *et al*. 2003). The source of Cd intake is mostly food and drinking water, and most of the Cd that is absorbed after oral exposure preferentially accumulates in the liver and kidneys, where it induces production of metallothionein (MT), a low-molecular-weight protein that has a high affinity for the metal (Klaassen *et al*. 1999; Nordberg & Nordberg 2000). It is commonly thought that intracellular sequestration of Cd into an inert Cd–MT complex produces tolerance to the toxic effects of this metal (Masters *et al*. 1994; Klaassen *et al*. 1999). When the amount of Cd in the liver and kidneys exceeds the binding capability of MT, the non-MT-bound Cd ions are believed to cause hepato- and nephrotoxicity (Cherian *et al*. 1976; Goyer *et al*. 1989; Nomiyama & Nomiyama 1998; Nordberg & Nordberg 2000). One mechanism by which these ions produce injury is assumed to be through generation of free radicals and lipid peroxidation because co-treatments with antioxidants such as vitamin E and *N*-acetyl cysteine greatly reduce the Cd-induced hepatotoxicity as well as nephrotoxicity (Nomiyama & Nomiyama 1998; Shaikh *et al*. 1999). Since elevated levels of renal and hepatic Cd can cause renal and hepatic dysfunction (Cherian *et al*. 1976; Min *et al*. 1986),

factors influencing Cd absorption and tissue accumulation are important aspects of Cd toxicity.

It has been shown that many factors such as age, gender, metal ions (Fe, Zn, Ca) and chelators affect the absorption of Cd from gastrointestinal tract and its accumulation in the liver and kidneys (Taguchi & Suzuki 1981; Klaassen *et al.* 1984; Rummler *et al.* 1989; Groten *et al.* 1991; Elsenhans *et al.* 1997). A recent study from our laboratory revealed further that hepatic and renal accumulation of Cd in a small rodent, the bank vole, is photoperiod-dependent; the animals exposed subchronically to dietary Cd under a short photoperiod accumulate more Cd (up to 100% in the liver and 65% in the kidneys) than the bank voles raised under a long photoperiod, despite the fact that the relative amount of ingested dietary Cd is similar in the short- and long-photoperiod bank voles (Włostowski *et al.* 2000, 2004). Thus, the mechanism by which photoperiod affects hepatic and renal accumulation of Cd in the bank voles is unknown and remains to be elucidated.

It is well known that many of the effects of photoperiod on physiological processes (e.g. body growth and sexual maturation) in small mammals, including voles are transduced by the pineal gland and its hormone melatonin (Bartness *et al.* 2002). A question arises as to whether melatonin, synthesis of which increases during a dark phase, is also responsible for differential accumulation of Cd in bank voles held under different photoperiods. Therefore, in the present study the effect of subcutaneously administered melatonin on hepatic, renal and intestinal Cd accumulation in the bank vole raised under a long photoperiod and exposed to dietary Cd was examined. In addition, histological examinations of the liver and kidneys as well as analyses of the tissue MT and lipid peroxidation were carried out.

Materials and methods

Animals and experimental design

The procedures used in this study were approved by the Local Ethical Committee for conducting an experimental study on laboratory animals (Medical Academy in Białystok). Male bank voles (1 month old, weighing 10–13 g), being the F_1 offspring of the wild-caught population (Knyszyn Old Forest near Białystok, northeastern Poland), were used throughout the study. They were housed in stainless-steel cages (three animals per cage) in a windowless room with automatically regulated temperature (18–20 °C) and lighting (16 h light/8 h dark), with lights on from 06:00 to 22:00 h. The bank voles were randomly assigned into four groups of six animals each: (1) control, (2) melatonin alone, (3) Cd alone, and (4) Cd + melatonin. The animals of groups (2) and (4) were injected subcutaneously (sc) with melatonin (7 μmol/kg) in a freshly prepared solution of 0.9% NaCl–ethanol (13:1 v/v) in a volume of 5 ml/kg body wt. The voles of groups (1) and (3) were injected sc with 5 ml/kg of the solution of 0.9% NaCl–ethanol (13:1 v/v). The injections were performed at 14:00–15:00 h, 7 times/week for 6 weeks. The pilot experiment showed that the selected dose of melatonin (7 μmol/kg/day) was the lowest one that had comparable suppressive effects on the gonads and body growth of bank voles to those seen at short photoperiod (8 h light/16 h dark).

The bank voles received distilled water *ad libitum* and control (groups (1) and (2)) or Cd-containing wheat grains (0.9 μmol/g dry wt.) (groups (3) and (4)), 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 weekly throughout the experiment. Prior to the experiment the grains were contaminated with Cd (soaked in $CdCl_2$ solution) (Włostowski *et al.* 2004). Atomic absorption spectrophotometry (AAS) analysis of the grains revealed that actual levels of Cd were between 0.87–0.93 μmol/g dry wt. This dose of Cd was chosen to compare the data with those obtained in a study performed on bank voles kept under short and long photoperiods (Włostowski *et al.* 2004).

Assays

At the end of the 6-week exposure period, the animals were weighed, euthanized by cervical dislocation and the liver, kidneys, testes and duodenum were removed, rinsed in ice-cold saline and blotted dry. A portion of the liver (0.25 g) and one kidney 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 Cd concentrations, respectively. The remaining homogenate was centrifuged at 20 000 × *g* for 20 min at 4 °C, and the resulting supernatant was removed for MT assay.

Cd determination was performed as described previously (Włostowski *et al*. 2004). Briefly, the homogenates of the liver and kidneys (1.0 ml) and duodenum were 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). Cadmium analyses of these solutions were carried out by electrothermal atomic absorption spectrometry (AAS) using an AAS3 Zeiss Jena instrument with an EA3 furnace attachment. Samples of bovine liver 1577b (NIST) were also analyzed in an identical manner to check accuracy of the method. The recovery of Cd was 91–95%.

Metallothionein content was determined by a Cd-saturation method (Onosaka & Cherian 1982) with minor modification (Włostowski & Krasowska 1999). Briefly, in a 1.5 ml vial, 0.1 sample was incubated for 10 min at room temperature with 1.0 ml Tris–HCl buffer (0.03 M, pH 7.8) containing 1.0 μg Cd/ml as $CdCl_2$. 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 and centrifuged for 5 min at 10 000 × *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).

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 h. 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).

Histological examinations

A portion of the liver and one kidney from each animal were fixed in 10% phosphate-buffered formalin, dehydrated in ethanol and xylene, embedded in paraffin, cut into 8 μm sections, and stained with hematoxylin and eosin (H & E) for microscopic examination.

Statistical analysis

Data were expressed as means ± SD. The values were analyzed by two-way analysis of variance (ANOVA) followed by the Duncans multiple-range test (MS Statistica 5.1). Differences at $P < 0.05$ were considered statistically significant.

Results

The 6-week exposure to dietary Cd did not affect the body and organs weights of the bank voles, but melatonin treatment reduced significantly the growth of the body as well as the liver and kidneys; in particular, this molecule suppressed completely development of the testes (Table 1). Cd and melatonin treatments had no effect on relative food consumption; the food intake in the control and Cd alone groups was 0.154 ± 0.01 g/g body wt./day, whereas that in the melatonin and Cd + melatonin bank voles appeared to be 0.150 ± 0.005 g/g body wt./day.

Tissue Cd and MT concentrations following subchronic Cd and melatonin administration are shown in Table 2. The hepatic, renal and intestinal Cd levels were significantly affected by dietary Cd, melatonin and their interaction. Melatonin

Table 1. Effect of cadmium and melatonin on body and organs weights in the bank vole[#].

Group	Body weight (g)	Liver wet weight (mg)	Kidneys wet weight (mg)	Testes wet weight (mg)
Control	19.8 ± 2.0^{a}	1068 ± 100^{a}	223 ± 18^{a}	475 ± 110^{a}
Melatonin	15.1 ± 1.1^{b}	920 ± 70^{b}	190 ± 15^{b}	25 ± 9^{b}
Cd	21.3 ± 2.1^{a}	1140 ± 120^{a}	245 ± 20^{a}	455 ± 100^{a}
Cd + Melatonin	14.4 ± 1.0^{b}	900 ± 95^{b}	188 ± 12^{b}	30 ± 11^{b}
Source of variation: ANOVA – *P* values				
Cd	NS	NS	NS	NS
Melatonin	0.0000	0.0000	0.0000	0.0000
Cd × Melatonin	NS	NS	NS	NS

[#]Values represent the mean ± SD for n = 6. The 1-month old male bank voles received, for 6 weeks, control diet or diet contaminated with Cd (0.9 μmol/g). Melatonin was administered subcutaneously (7 μmol/kg/day for 6 weeks). Means in the same column marked with different superscript letters a significantly different ($P < 0.05$). NS: nonsignificant.

Table 2. Effect of cadmium and melatonin on tissue Cd accumulation and metallothionein levels in the bank vole[#].

Group	Hepatic Cd (nmol/g)	Renal Cd (nmol/g)	Intestinal Cd (nmol/g)	Hepatic MT (nmol/g)	Renal MT (nmol/g)
Control	0.89 ± 0.27^{a}	1.78 ± 0.27^{a}	1.16 ± 0.18^{a}	1.52 ± 0.83^{a}	1.98 ± 0.27^{a}
Melatonin	0.80 ± 0.36^{a}	1.87 ± 0.27^{a}	0.98 ± 0.19^{a}	1.46 ± 0.70^{a}	1.94 ± 0.19^{a}
Cd	146 ± 29^{b}	212 ± 26^{b}	93 ± 14^{b}	31.1 ± 3.1^{b} (0.21)	36.3 ± 2.1^{b} (0.17)
Cd + Melatonin	236 ± 38^{c}	380 ± 33^{c}	164 ± 23^{c}	29.6 ± 4.4^{b} (0.12)	34.8 ± 4.8^{b} (0.09)
Source of variation: ANOVA – *P* values					
Cd	0.0000	0.0000	0.0000	0.0000	0.0000
Melatonin	0.0000	0.0000	0.0000	NS	NS
Cd × Melatonin	0.0000	0.0000	0.0000	NS	NS

[#]Values represent the mean ± SD for n = 6. The 1-month old male bank voles received, for 6 weeks, control diet or diet contaminated with Cd (0.9 μmol/g). Melatonin was administered subcutaneously (7 μmol/kg/day for 6 weeks). In parentheses the ratio of MT levels to Cd content (nmol MT/nmol Cd) is presented. Means in the same column marked with different superscript letters are significantly different ($P < 0.05$). NS: nonsignificant.

co-treatment brought about a significant rise in the hepatic (61%), renal (79%) and intestinal (77%) Cd concentrations as compared to those in the Cd alone group. Importantly, the concentrations of MT in the liver and kidneys of the Cd + melatonin co-treated bank voles did not differ from those in the Cd alone group. However, the ratio of MT levels to hepatic and renal Cd content (nmol MT/nmol Cd) was nearly doubled in the Cd alone bank voles compared to the Cd + melatonin animals, indicating that proportionally more MT was produced relative to the available amount of Cd in the liver and kidneys of bank voles not treated with melatonin. As a result, the hepatic and renal MT capacity exceeded by 72 and 42 nmol Cd/g, respectively, the concentrations of Cd in the bank voles from the Cd alone group, while in the animals from the Cd + melatonin group the levels of Cd in the liver and kidneys exceeded by 29 and 136 nmol Cd/g, respectively, the MT capacity.

Hepatic and renal lipid peroxidation (measured as TBARS) as well as histopathological results are presented in Table 3. ANOVA revealed that lipid peroxidation in the liver was not affected, while in the kidneys this process was significantly influenced by dietary Cd, melatonin and their interaction. Melatonin co-treatment increased significantly (2-fold) the renal TBARS level above that in bank voles from the Cd alone as well as the other groups. Likewise, the histopathological changes occurred only in the animals from the Cd + melatonin group. The lesions observed in the liver included primary foci of inflammation (infiltration of leukocytes) (Figure 1). In the kidneys the damage included glomerular swelling and a focal tubular cell degeneration (Figure 2).

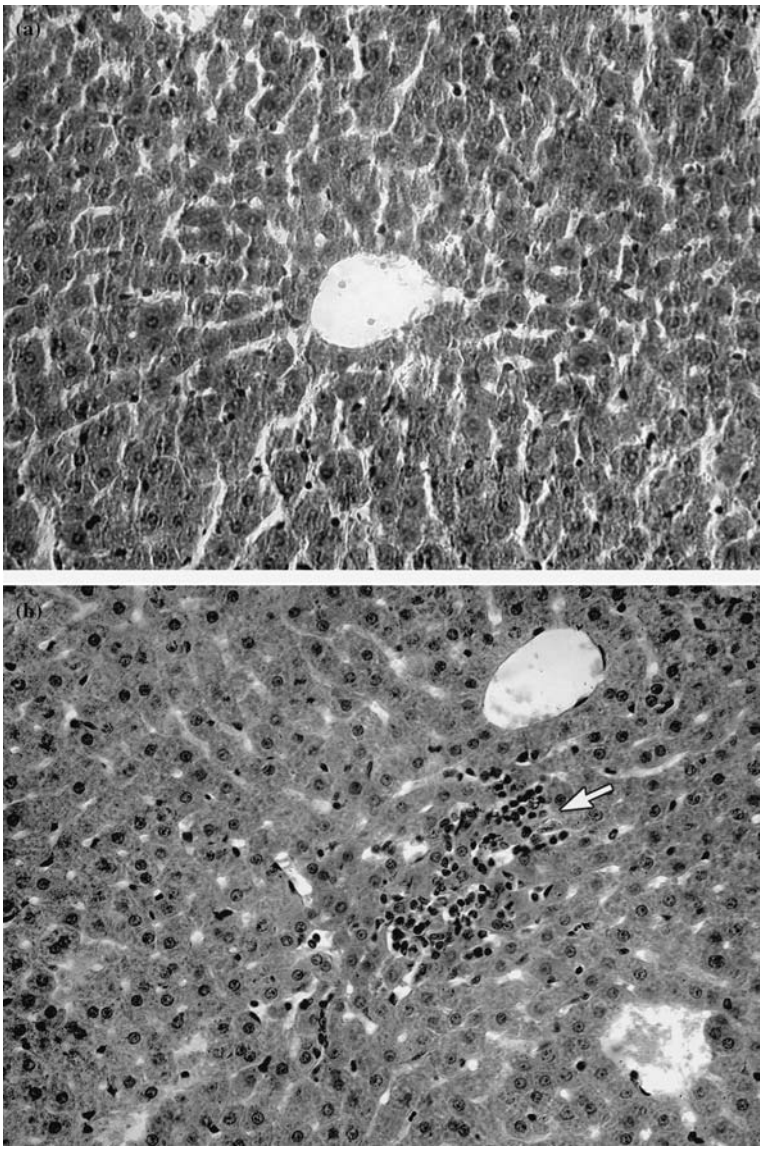

Figure 1. Representative photomicrographs of liver section from (a) control bank voles and (b) bank voles fed, for 6 weeks, a diet containing 0.9 μmol Cd/g and co-treated with melatonin (7 μmol/kg/day for 6 weeks). Foci of inflammation in the Cd + melatonin co-treated animals were observed (arrow). H & E staining, ×200.

Discussion

The present study demonstrates that exogenous melatonin injected subcutaneously to bank voles raised under a long photoperiod produces physiological effects (reduction of body mass and suppression of testicular development) similar to those observed under a short photoperiod (Włostowski *et al.* 2000, 2004). In addition, the subchronic melatonin administration has many similarities to short photoperiod (SP) regarding the accumulation and toxicity of Cd (Włostowski *et al.* 2004). The four most important similarities are listed below: (1) both the melatonin co-treated and SP bank voles accumulate significantly more Cd in the liver and kidneys than the animals kept under a long photoperiod (LP), (2) the capability to produce MT under the Cd exposure is lower in the melatonin co-treated and SP voles as compared with the LP animals, (3) Cd exposure induces lipid

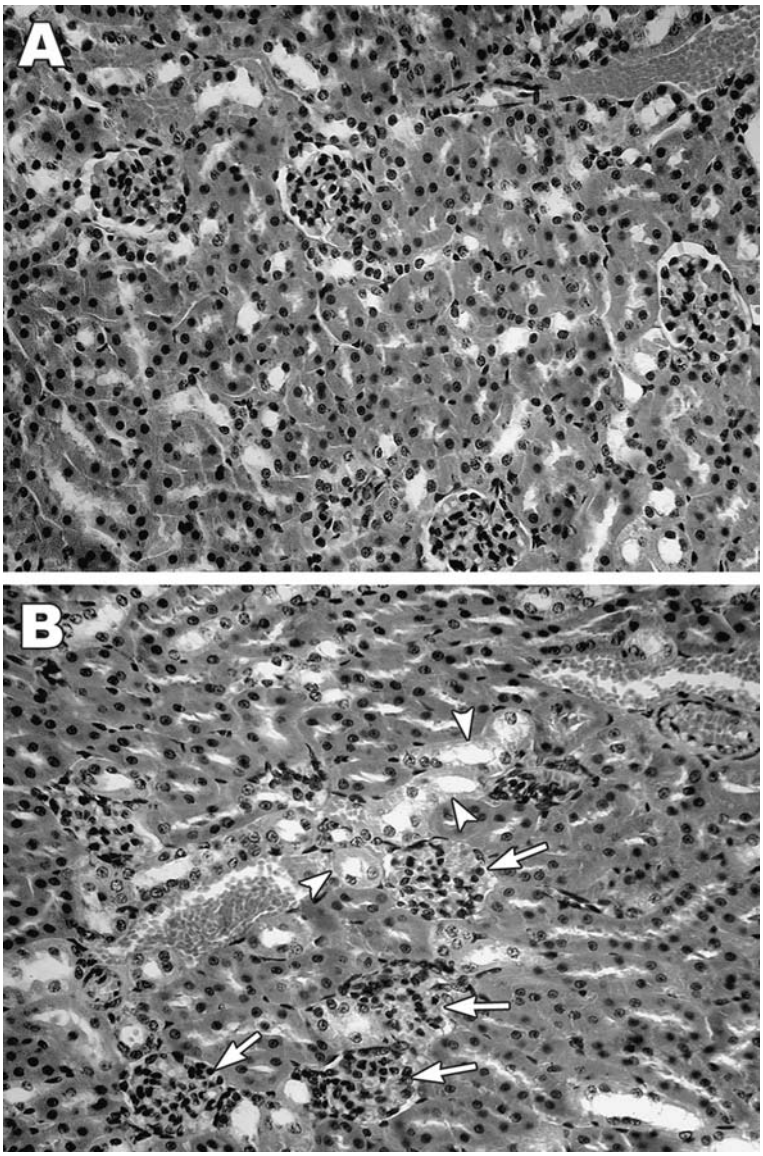

Figure 2. Representative photomicrographs of kidney section from (a) control bank voles and (b) bank voles fed, for 6 weeks, a diet containing 0.9 μmol Cd/g and co-treated with melatonin (7 μmol/kg/day for 6 weeks). Glomerular swelling (arrows) and a focal degeneration of tubular cells (arrowheads) in the Cd + melatonin co-treated animals were observed. H & E staining, ×200.

peroxidation in the kidneys of bank voles co-treated with melatonin or raised under a short photoperiod but does not in the LP animals, and (4) similar histopathological changes occur in the liver (infiltration of leukocytes) and kidneys (glomerular swelling and a focal tubular cell degeneration) of the melatonin co-treated and SP bank voles exposed to dietary Cd, but do not occur in the LP animals. This comparison indicates that melatonin of pineal origin is most likely responsible for the effects of short photoperiod.

The exact mechanism by which melatonin or short photoperiod increases the tissue accumulation of Cd is not known at present. It is possible, however, that melatonin increases Cd uptake from the intestinal lumen into the mucosa, thereby increasing absorption of the metal. A significant rise in the intestinal Cd concentration in the bank voles co-treated with melatonin (Table 2) supports the possibility. It has been recently shown that melatonin increases also plasma zinc in rats, and the presence of specific binding sites for melatonin

in the intestine is thought to be a fundamental mechanism by which this hormone increases the absorption of zinc (Baltaci *et al*. 2004). Since Cd utilizes transporters for essential metals such as zinc and iron (Takiguchi *et al*. 2001; Ryu *et al*. 2004), it is conceivable that they could be responsible for an increased Cd absorption in the bank voles co-treated with melatonin. However, it is not known whether and how melatonin or short photoperiod affects metal transporters in the intestine or other tissues of the bank vole. Nevertheless, recent study from our laboratory revealed that energy assimilation efficiency (an indicative of general digestive and absorptive processes) in the bank voles raised under a long photoperiod, amounting to 75%, increases to 90% in the animals kept under a short photoperiod or in those treated with exogenous melatonin (in preparation). These data suggest that melatonin or short photoperiod increases the tissue accumulation of Cd in the bank voles by enhancing its absorption. However, more direct research is needed to explore the mechanism of melatonin action on Cd accumulation in these animals.

The results of the present study also indicate that melatonin-dependent increase of hepatic and renal Cd accumulation as well as a decrease in the capability to produce MT could be responsible for the tissue damage. Numerous reports have shown that elevations in the amounts of MT are directly related to reduced Cd toxicity both *in vivo* and *in vitro* (Rugstad & Norseth 1975; Goering & Klaassen 1984; Jin *et al*. 1987; Waalkes *et al*. 1988; Liu *et al*. 1995). Furthermore, animals that fail to synthesize MT are more sensitive to toxic effects of Cd (Michalska & Choo 1993; Masters *et al*. 1994). The MT protection is typically associated with its ability to sequester Cd in the cytoplasm, thereby reducing the amount of Cd available to interact with target organelles such as mitochondria and nuclei (Klaassen *et al*. 1999; Waisberg *et al*. 2003). When the amount of non-MT-bound Cd becomes sufficiently high, as was observed in the liver and kidneys of the bank voles co-treated with melatonin, the toxicity can occur. Similar to the bank voles raised under a short photoperiod (Włostowski *et al*. 2004), also in the melatonin co-treated voles the non-MT-bound Cd produces nephrotoxicity probably through induction of lipid peroxidation (Table 3); notably, this process has been shown to be associated with Cd-induced toxicity in many systems (Koizumi & Li 1992; Stohs & Bagchi 1995; Skaikh *et al*. 1999; Wätjen & Beyersmann 2004). Oxidative stress and lipid peroxidation have been also postulated to cause the liver injury in Cd-intoxicated animals (Sauer *et al*. 1997; Liu *et al*. 2002). However, in the liver of melatonin co-treated bank voles, Cd-induced inflammation was not accompanied by a rise in lipid peroxidation (Table 3).

The mechanism by which subchronic administration of melatonin decreases the capacity of the liver and kidneys of bank voles to synthesize MT is not known at present, but it may be related, at least to some extent, to the suppressive effects on gonads as well as on the hepatic and renal growth.

Table 3. Effect of cadmium and melatonin on hepatic and renal lipid peroxidation (TBARS) and histopathology in the bank vole[#].

Group	Hepatic TBARS (nmol/g)	Renal TBARS (nmol/g)	Histopathology	
			Liver	Kidneys
Control	189 ± 12^{a}	206 ± 11^{a}	(−)	(−)
Melatonin	196 ± 10^{a}	202 ± 15^{a}	(−)	(−)
Cd	200 ± 15^{a}	211 ± 11^{a}	(−)	(−)
Cd + Melatonin	210 ± 20^{a}	402 ± 16^{b}	(+)	(+)
Source of variation: ANOVA – *P* values				
Cd	NS	0.0000		
Melatonin	NS	0.0000		
Cd × Melatonin	NS	0.0000		

[#]Values represent the mean ± SD for n = 6. The 1-month old male bank voles received for, 6 weeks, control diet or diet contaminated with Cd (0.9 μmol/g). Melatonin was administered subcutaneously (7 μmol/kg/day for 6 weeks). Histopathology: normal morphology (−), histopathological changes (+) (see Figs. 1 and 2). Means in the same column marked with different superscript letters are significantly different ($P < 0.05$). NS: nonsignificant.

Notably, the capacity to synthesize MT in various tissues is associated with their growth and development (Panemangalore *et al*. 1983; Zalups & Cherian 1992; Studer *et al*. 1997). Thus, the reduction of hepatic and renal growth in the melatonin treated voles could be responsible for this effect. Furthermore, it has been found in mice that testosterone increases Cd-induced hepatic and renal MT above that seen with Cd alone (Shimada *et al*. 1997). It is thus reasonable to assume that testosterone secretion by testes of the sexually mature bank voles (Cd alone group) but not by the undeveloped testes of melatonin co-treated voles may also account for the difference in the capacity to synthesize MT in these animals.

In conclusion, the present study demonstrates that subchronic melatonin injection to bank voles has similar effect on the tissue accumulation and toxicity of Cd to that produced by a short photoperiod. The Cd-induced toxicity in the liver and kidneys of melatonin co-treated animals appears to be due to increased Cd accumulation and decreased synthesis of MT.

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