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Effect of Melatonin on Cadmium-Induced Hepatotoxicity in Male Sprague-Dawley Rats

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KIM, C.-Y., LEE, M.-J., LEE, S.M., LEE, W.C. and KIM, J.S. *Effect of Melatonin on Cadmium-Induced Hepatotoxicity in Male Sprague-Dawley Rats.* Tohoku J. Exp. Med., 1998, **186**(3), 205-213 —— Effect of melatonin on toxicity of cadmium (Cd) was studied in male SD rats co-administered daily Cd (1 mg/kg b.w., s.c.) with melatonin (10 mg/kg b.w., i.p.) for 15 days. Cd alone injection decreased GSH concentrations in the liver and RBC by 35% and 43% compared with those in saline-treatment group, but not in the kidney and whole brain. The activity of GSSG-reductase was significantly decreased in the liver of Cd alone injected rats, while melatonin given in combination with Cd failed to prevent the Cd-induced decreased activity of hepatic GSSG-reductase. However, the hepatic GSH concentration decreased by Cd alone was restored by melatonin treatment, and the melatonin also ameliorated Cd-induced histopathological changes in the liver. Therefore, data indicate that melatonin restores the reduction of hepatic GSH level induced with Cd regardless of GSSG-reductase activity, and suggests that melatonin may ameliorate Cd-induced hepatotoxicity. ———— cadmium; melatonin; hepatotoxicity; glutathione

Cadmium (Cd), an environmental pollutant, has a strong affinity for thiol groups and is hepatotoxic after acute Cd exposure (Dudley and Klaassen 1984). After parenteral administration of a soluble Cd salt, the metal rapidly accumulates in the liver (WHO 1992) and subsequently produces hepatic necrosis and lipid peroxidation (Dudley et al. 1982; Stacy and Kappus 1982; Dudley and Klaassen 1984; Hussain et al. 1987; Anderson and Anderson 1988). In particular, previous studies reported a common toxic effect that glutathione (GSH) levels were lowered in the liver of Cd-treated animals (Kawata and Suzuki 1983; Chung and Maines 1987; Shukla et al. 1988), possibly due to the interaction of Cd^{2+} with the residual thiol group of GSH.

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It was reported that the Cd-induced hepatotoxicity was largely modified by hepatic GSH concentration (Shinhal et al. 1987). Moreover, in animals exposed to Cd, GSH depletion increased the frequency of mortality and hepatotoxicity (Dudley et al. 1982; Shinhal et al. 1987). Presumably GSH, usually the most abundant cellular thiol, may partly protect essential cellular constituents by binding Cd^{2+}, thus decreasing its availability for toxic interactions, i.e., Cd-complexation with essential cellular constituents (e.g., thiol-containing enzymes) (Vallee and Ulmer 1972; Vallee 1979). In other words, depletion of GSH in cells can impair the cell's defense against various toxic actions and may lead to cell injury and death (Meister and Anderson 1983; Reed 1990; Deleve and Kaplowitz 1991).

Melatonin, the pineal hormone, was found to have potent antioxidative activity (Poeggler et al. 1993; Reiter et al. 1995). It has been demonstrated that the cataract formation due to GSH depletion in lens can be inhibited by melatonin administration, suggesting that melatonin is capable of stimulatory effect on GSH production (Abe et al. 1994). Therefore, it is possible that melatonin via GSH synthesis may modify toxic effects of Cd such as a decrease in GSH level.

To test this possibility, male SD rats were exposed to Cd with or without melatonin. The alteration of GSH level was investigated as an indication of Cd toxicity. The activity of GSSG-reductase, capable of reducing oxidized GSH (GSSG) to GSH, was also determined in various tissues.

Materials and Methods

Chemicals

All reagents were of the highest quality available. Melatonin, cadmium chloride ($CdCl_2$), reduced GSH, GSSG, GSSG-reductase (yeast), NADPH, *o*-phthaldehyde (OPT), methanol, 3, 3′-diaminobenzidine, and EDTA were purchased from the Sigma Chemical Co. (St. Lous, MO, USA). DAKO ABC kit was obtained from DAKO Company (Tokyo).

Animal grouping and treatment

Male SD rats weighing 55 g approximately (4 weeks old) were purchased from Dae Han Experimental Animal Co. (Seoul, Korea). They were housed in plastic rat cages with *ad libitum* access to feed (Rodent Pellet Chow, Chunhajeil Co., Seoul, Korea) and water. The light cycle was 12 hours : 12 hours (light period, 08 : 00–20 : 00), and the room temperature was maintained at 21–23°C. Twenty-four rats were allotted to 3 groups, depending on the treatment regimen as follows; 8 Cd-treated group (Cd, 1 mg • kg^{-1} • day^{-1}, s.c.), 8 Cd and melatonin-treated group (Cd, 1 mg • kg^{-1} • day^{-1}, s.c.; melatonin, 10 mg • kg^{-1} • day^{-1}, i.p.), and 8 saline-treated (control) group for 15 days. Melatonin was dissolved in absolute ethanol (the alcohol concentration in the final solution: 1%). Control rats

received an equivalent volume of saline. At 24 hours after last injection, the liver, kidney, whole brain, and red blood cells (RBC) were collected under ether anesthesia for assays and thereafter rats were sacrificed by cervical dislocation.

Assay of GSH concentration and enzyme activity

The concentration of GSH was determined by the method of Cohn and Lyle (1966) using OPT as slightly modified by Kim et al. (1995). The tissues (liver, kidney, and whole brain) were homogenized in an extraction mixture of 5% trichloroacetic acid containing 10 mM EDTA. The homogenates, consisting of 150 mg liver in 3 ml, 200 mg kidney in 3 ml, 200 mg whole brain in 3 ml and 300 mg RBC in 3 ml of the extraction mixture, were centrifuged at $800 \times g$ for 10 minutes to remove proteins, and then the supernatant was stored at $-80°C$ until use. Five ml of 0.4 M Tris-HCl buffer (pH 8.9) containing 10 mM EDTA and 250 μl of OPT in methanol (1 mg/ml) were added to 100 μl of the sample supernatant. After a 5 minutes reaction period, the GSH content was determined with a fluorometer (Jusco, Model FP-750, Tokyo).

For activities of the GSSG-reductase, the tissues (liver, kidney, and whole brain) were weighed and homogenized in 20 vols. Of 0.25 M sucrose. The supernatant fraction was prepared by centrifugation at $105\,000 \times g$ for 1 hour, and then the supernatant was stored at $-80°C$ until use. The activities of the GSSG-reductase assay (Worthington and Rosemeyer 1976) was determined by measuring the disappearance of NADPH using spectrophotometer (Uvikon 860, Kontron, Switzerland) at 340 nm. The assay medium (1 ml) contained enzyme source, 100 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 20 mM potassium chloride, 0.1 mM NADPH, and 1 mM GSSG. The reaction was started by the addition of GSSG. The blank assay system did not contain GSSG. Protein was determined by the procedure of Lowry et al. (1951) using bovine serum albumin as a standard.

Determination of cadimium

The tissues (liver, kidney, RBC) were weighed and wet-ashed with a mixture of nitrate : sulfate : Perchloric acid (1 : 4 : 1) at 140–160°C for 30 minutes using a thermo-aluminum bath (Iwaki). Cd concentration was analyzed using AAS (Shimadzu AA-680, Tokyo) with a deuterium background correction.

Histopathological examination of the liver

The liver was fixed in 10% phosphate-buffered (pH 7.0) formalin, dehydrated in ethanol and xylene, embedded in paraffin, cut into 4- to 5-μm sections, and stained with hematoxylin-eosin for microscopic examination. In addition, sections were immunohistochemically stained for transforming growth factor-α (TGF-α) as markers of hepatocytes undergoing rapid compensatory liver growth (liver regeneration) (Mead and Fausto 1989; Tamano et al. 1994), using a rat

monoclonal antibody (at 1 : 100 dilution) obtained from DAKO Company. DAKO ABC kit (DAKO Co., Tokyo) was used for performance of ABC immunohistochemistry with 3, 3′-diaminobenzidine as the chromogen and hematoxilin for counterstaining. The numbers of positive cells per approximately 2000 liver cell were counted with the microscope for randomly-selected areas.

Statistical analysis

All data are presented as the mean±S.D. Statistical analyses were performed by Student's t-test or by Duncan's multiple-range test.

Results

The highest concentration of Cd was found in the livers of both groups (Cd alone and melatonin combined with Cd), and their Cd concentrations were twice

Table 1. *Cd concentration in various tissues after Cd alone or Cd and melatonin combined treatment*

	Cd alone (μg/g tissue)	Cd+Melatonin (μg/g tissue)
Liver	68.5 ±6.8	74.0±14.8
Kidney	33.5 ±2.8	37.9± 8.5
RBC	0.95±0.1	1.0± 0.1

Values represent the mean±S.D. ($n=8$).

Cadmium (1 mg•kg^{-1}•day^{-1}, s.c.) and melatonin (10 mg•kg^{-1}•day^{-1}, i.p.) were injected for 15 days.

No significant difference was formed by Student's t-test.

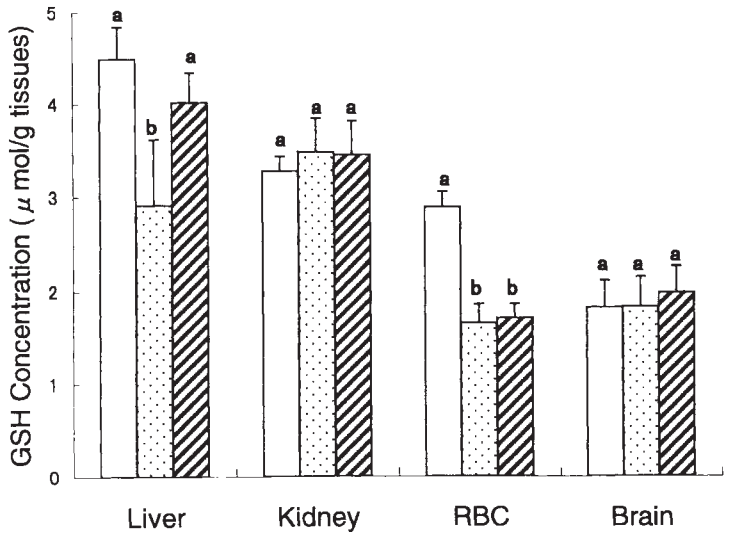

Fig. 1. The changes of the level of GSH in Cd alone or Cd and melatonin combined treatmemt. Each column represents mean±S.D. The same letter do not differ from each other ($p<0.05$). □, Saline; ⬚, Cd; ▨, Cd+Mel.

TABLE 2. *The effect of melatonin on the activity of GSSG-reductase in Cd alone or Cd and melatonin combined-treated rats. (unit: mU/mg protein)*

	Liver	Kidney	Brain
Saline	80.6± 8.3[a]	127.8±12.6	33.7±10.4
Cd alone	59.5±10.5[b]	120.2±10.6	33.5± 9.7
Cd+Melatonin	61.7±16.4[b]	118.9± 6.8	32.0± 9.6

Values represent the mean±S.D. ($n=8$).

Cadmium (1 mg•kg^{-1}•day^{-1}, s.c.) and melatonin (10 mg•kg^{-1}•day^{-1}, i.p.) were administered for 15 days.

Means with the same row followed by the same superscript letter do not differ from each other ($p<0.05$); Duncan's multiple-range test.

as high as those of the kidney (Table 1).

Cd alone treatment decreased GSH concentrations in the liver and RBC by 35% and 43% compared with those in control group, respectively, but not in the kidney and whole brain (Fig. 1). At the same time, the activity of GSSG-reductase activity was significantly decreased in the liver, while no change was observed in the kidney and whole brain tissue (Table 2). As shown in Fig. 1, the reduction of hepatic GSH concentration was restored by melatonin given in combination with Cd to the same extent as that of control group, while melatonin failed to prevent the Cd-induced decrease in GSSG-reductase activity of the liver (Table 2).

Melatonin treatment alleviated histopathological alterations of the liver induced wit Cd exposure such as single, focal necrosis and necrotic areas (Figs. 2a and b). In addition, as markers of hepatocytes undergoing rapid compensatory liver growth (liver regeneration) (Mead and Fausto 1989; Tamano et al. 1994), TGF-α labeling index values were 24.1±6.5 (mean±S.D.) for Cd alone injection and 16.9±8.3 (mean±S.D.) for Cd combined with melatonin treatment ($p<0.05$, data not shown), respectively (Figs. 2d and e).

DISCUSSION

In this study, the highest concentration of Cd was found in the livers of both groups, Cd alone and Cd combined with melatonin treatment. Cd alone injection decreased GSH concentrations in the liver and RBC, an indication of Cd toxicity. It was reported that alteration of GSH levels may be useful as a biomarker for toxicity (Uhlig and Wendel 1992; Jones et al. 1995).

Previous studies have reported Cd toxicity, showing a similar effect being a decrease of GSH level in the liver, testes, and brain of Cd-exposed animals (Kawata and Suzuki 1983; Chung and Maines 1987; Shukla et al. 1988). In the present study, a decrease in hepatic GSH level was produced by Cd alone treatment, consistent with those of the previous studies, and was accompanied by a

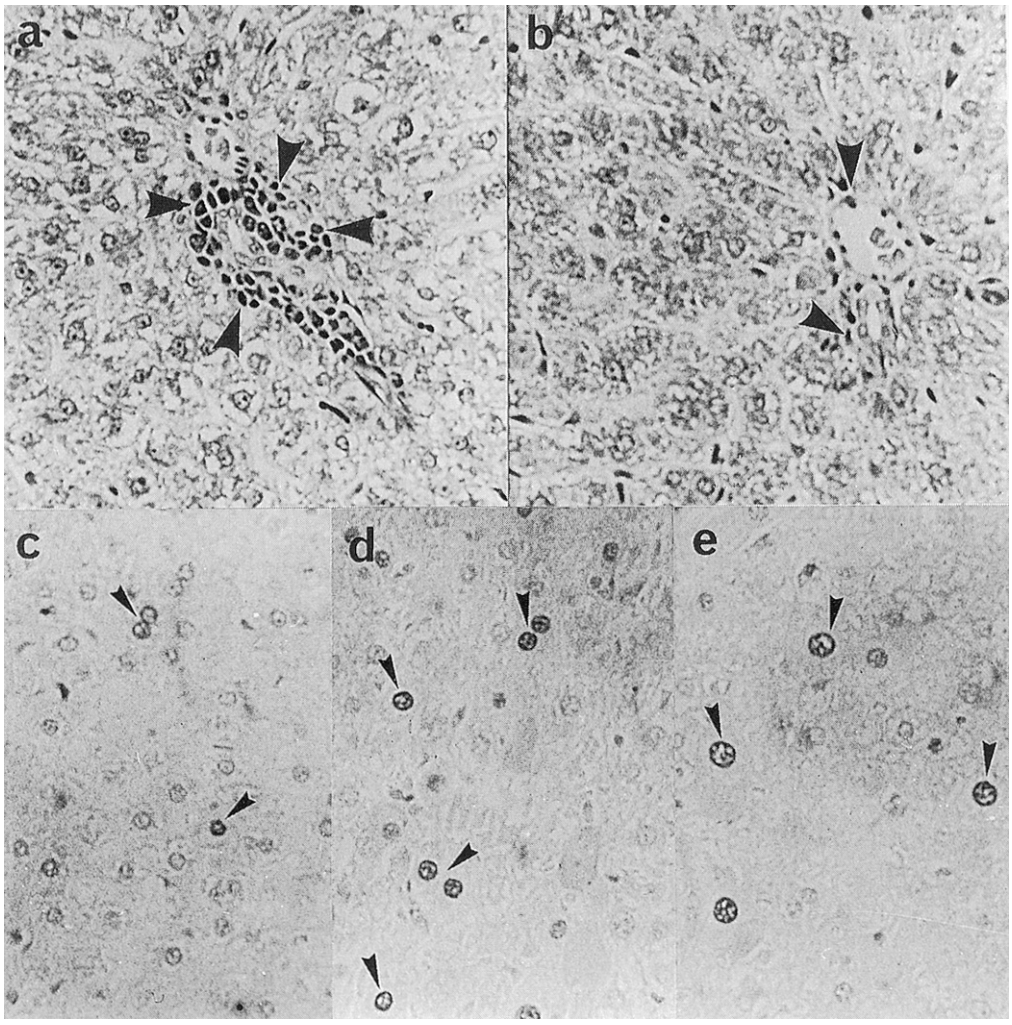

Fig. 2. Photomicrograph of liver after administration of $CdCl_2$ (1 mg•kg^{-1}•day^{-1}, s.c.) with or without melatonin (10 mg•kg^{-1}•day^{-1}, i.p.) during 15 days. Approximate magnification 220X. Top photo (H.E staining), a) Cd alone group (arrows point to large necrotic area); b) Cd combined with melatonin group (arrows point to decreased necrotic area). Bottom (TGF-α staining), c) control group (shows normal condition); d) Cd alone group (shows increased TGF-α positive cells); e) melatonin combined with Cd group (shows decreased TGF-α positive cells).

decrease in the activity of hepatic GSSG-reductase which is capable of reducing GSSG to GSH.

Under physiological conditions, GSSG-reductase will reduce any GSSG to GSH at the expense of NADPH, so that more than 98% of intracellular GSH is the reduced GSH form (Deleve and Kaplowitz 1991). However, conditions of marked toxicity or oxidative stress elevate intracellular levels of GSSG, which brings GSSG-reductase into play to reduce GSSG to GSH (Plummer et al. 1981). Other studies with isolated hepatocytes reported that Cd might itself diminish cellular GSH (Stacy and Kappus 1982; Muller 1986) presumably by inhibition of GSSG-reductase (Muller 1986). Therefore, the finding of this study, a decrease of hepatic GSH concentration produced by Cd alone may be due to 1) the activity

of GSSG-reductase depends upon the intactness of enzymatic thiol groups (Muller 1986), and thus its inhibition, i.e., Cd-complexation with GSSG-reductase may malfunction in reduction of GSSG to GSH, 2) the utilization of GSH to directly interact with Cd^{2+} or to lower the level of toxic substances (presumably, free radicals) induced by Cd^{2+}.

As shown in Fig. 1, the decrease in hepatic GSH concentration was restored by melatonin given in combination with Cd to the same extent as that of saline-treated control, while melatonin failed to prevent a decreased activity of GSSG-reductase in the liver (Table 2). Abe et al. (1994) demonstrated that the cataract formation due to GSH depletion in lens can be inhibited by melatonin administration, suggesting that melatonin is capable of stimulatory effect on GSH production. Melatonin administration was reported to restore the lowered hepatic GSH level due to paraquat injection (Melchiorri et al. 1995). Therefore, in the present study, it is possible that a decrease in hepatic GSH concentration is restored by the preventive effect of melatonin via GSH synthesis. In addition, the fact that melatonin failed to prevent the decrease in GSSG-reductase activity (Table 2) also suggests that such restoration of hepatic GSH level was not carried out by GSSG-reductase activity capable of reducing GSSG to GSH.

Histopathological examination showed that melatonin administration decreased single, focal necrosis and necrotic areas induced with Cd toxicity (Fig. 2a), which was consistent with the report of hepatic necrosis induced by Cd exposure (Sudo et al. 1996). Probably, such protection against the development of histopathological changes might be attributed partly to the antitoxic action of hepatic GSH restored by melatonin, because GSH has multiple functions in detoxification, and its decrease has been associated with an increased risk of chemical toxicity (Meister and Anderson 1983; Reed 1990). In addition, as markers of hepatocytes undergoing rapid compensatory liver growth (liver regeneration) (Mead and Fausto 1989; Tamano et al. 1994), the result of TGF-α immunohistochemistry (Figs. 2c, d and e) correlated with that of HE staining, showing that the extent of hepatic injury by Cd alone was ameliorated by the combined treatment of melatonin with Cd; TGF-α labeling index values (data not shown), Cd alone injection (24.1 ± 6.5) vs. melatonin treatment (16.9 ± 8.3, $p < 0.05$).

In recent studies, melatonin was shown to be an efficient free radical scavenger against the toxic actions of the extremely reactive hydroxyl radical which abstracts a hydrogen atom, i.e., initiates lipid peroxidation, from cellular membrane (Poeggler et al. 1993; Reiter et al. 1995). Although the exact mechanism(s) by which Cd^{2+} produces toxicity are not yet known, it was suggested that antioxidative defense system and membrane lipid were impired by Cd oxidative damage (Stacy and Kappus 1982; Hussain et al. 1987; Sarkar et al. 1995). Thus, further study is required to investigate whether melatonin possibly contributes to the preventive effects against oxidative damages induced by Cd.

In the present study, data show that melatonin restored a decreased GSH concentration and thereby ameliorated histopathological changes in the liver induced by Cd toxicity. Such preventive effect of melatonin at least may contribute to detoxification capacity of the liver that is vulnerable to various hepatotoxicants including Cd.

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References

1) Abe, M., Reiter, R.J., Orhii, P.B., Hara, M. & Poeggeler, B. (1994) Inhibitory effect of melatonin on cataract formation in newborn rats: Evidence for an antioxidative role for melatonin. *J. Pineal Res.*, **17**, 94–100.
2) Anderson, H.R. & Anderson, O. (1988) Effect of cadmium on hepatic lipid peroxidation in mice. *Pharmacol. Toxicol.*, **63**, 173–177.
3) Chung, A.S. & Maines, M.D. (1987) Differential effect of cadmium on GSH-peroxidase activity in the Leydig and Sertoli cells of rat testes. Suppression by selenium and the possible relationship to heme concentration. *Biochem. Pharmacol.*, **36**, 1367–1372.
4) Cohn, V.H. & Lyle, J. (1966) A fluorometric assay for glutathione. *Anal. Biochem.*, **14**, 434–440.
5) Deleve, L.D. & Kaplowitz, N. (1991) Glutathione metabolism and its role in hepatotoxicity. *Pharmacol. Ther.*, **52**, 287–305.
6) Dudley, R.E., Svoboda, D.J. & Klaassen, C.D. (1982) Acute exposure to cadmium causes severe liver injury in rats. *Toxicol. Appl. Pharmacol.*, **65**, 302–313.
7) Dudley, R.E. & Klaassen, C.D. (1984) Changes in hepatic glutathione concentration modify cadmium-induced hepatotoxicity. *Toxicol. Appl. Pharmacol.*, **72**, 530–538.
8) Hussain, T., Shukla, G.S. & Chandra, S.V. (1987) Effects of cadmium on superoxide dismutase and lipid peroxidation in liver and kidney of growing rats: In vivo and in vitro studies. *Pharmacol. Toxicol.*, **60**, 355–358.
9) Jones, D.P., Brown, L.A.S. & Sternberg, P. (1995) Variability in glutathione-dependent detoxication in vivo and its relevance to detoxication of chemical mixtures. *Toxicology*, **105**, 267–274.
10) Kawata, M. & Suzuki, K.T. (1983) Relation between metal and glutathione concentrations in mouse liver after cadmium, zinc or copper loading. *Toxicol. Lett.*, **15**, 131–137.
11) Kim, C.-Y., Watanabe, C. & Satoh, H. (1995) Effects of buthionine sulfoximine (BSO) on mercury distribution after Hg° exposure. *Toxicology*, **98**, 67–72.
12) Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
13) Mead, J.E. & Fausto, N. (1989) Transforming growth factor-α may be a physiological regulator of liver regeneration by means of an autocrine mechanism. *Proc. Natl. Sci. USA*, **86**, 1558–1562.
14) Meister, A. & Anderson, M.E. (1983) Glutathione. *Annu. Rev. Biochem.*, **52**, 711–760.
15) Melchiorri, D., Reiter, R.J., Attia, A.M., Hara, M., Burgos, A. & Nistico, G. (1995) Potent protective effect of melatonin on in vivo paraquat-induced oxidative damage in rats. *Life Sci.*, **56**, 83–89.

16) Muller, L. (1986) Consequences of cadmium toxicity in rat hepatocytes: Effects of cadmium on the glutathione-peroxidase system. *Toxicol. Lett.*, **30**, 259–265.
17) Plummer, J.L., Smith, B.R., Sies, H. & Bend, J.R. (1981) Chemical depletion of glutathione in vivo. *Meth. Enzymol.*, **77**, 50–59.
18) Poeggler, B., Reiter, R.J., Tan, D.-X., Chen, L.-D. & Manchester, L.C. (1993) Melatonin, hydroxyl radical-mediated oxidative damage, and aging: A hypothesis. *J. Pineal Res.*, **14**, 151–168.
19) Reiter, R.J., Melchiorri, D., Sewerynek, E., Poeggler, B., Barlow-Walden, L.R., Chuang, J., Ortiz, G.G. & Acuna-Castroviejo, D. (1995) A review of the evidence supporting melatonin's role as an antioxidant. *J. Pineal Res.*, **18**, 1–11.
20) Reed, D.J. (1990) Glutathione: Toxicological implications. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 603–631.
21) Sarkar, S., Yadav, P., Trivedi, R., Bansal, A.K. & Bhatnagar, D. (1995) Cadmium-induced lipid peroxidation and status of the antioxidant system in rat tissues. *J. Trace Elem. Med. Biol.*, **9**, 144–149.
22) Shukla, G.S., Srivastava, R.S. & Chandra, S.V. (1988) Prevention of cadmium-induced effects on regional glutathione status of rat brain by vitamin E. *J. Appl. Toxicol.*, **8**, 355–359.
23) Shinhal, R.K., Anderson, M.E. & Meister, A. (1987) Glutathione, a first line of defense against cadmium toxicity. *FASEB J.*, **1**, 220–223.
24) Stacy, N.H. & Kappus, H. (1982) Comparison of methods of assessment of metal-induced lipid peroxidation in isolated rat hepatocytes. *J. Toxicol. Environ. Health*, **9**, 277–285.
25) Sudo, J.-I., Hayashi, T., Kimura, S.-I., Kakuno, K., Terui, J., Takashima, K. & Soyama, M. (1996) Mechanism of nephrotoxicity induced by repeated administration of cadmium chloride in rats. *J. Toxicol. Environ. Health*, **48**, 333–348.
26) Tamano, S., Merlino, G.T. & Ward, J.M. (1994) Rapid development of hepatic tumors in transforming growth factor α transgenic mice associated with increased cell proliferation in precancerous hepatocellular lesions initiated by N-nitrosodithylamine and promoted byphenobarbital. *Carcinogenesis*, **15**, 1791–1798.
27) Uhlig, S. & Wendel, A. (1992) The physiological consequences of glutathione variations. *Life Sci.*, **51**, 1083–1094.
28) Vallee, B.L. & Ulmer, D.D. (1972) Biochemical effects of mercury, cadmium, and lead. *Annu. Rev. Biochem.*, **41**, 91–128.
29) Vallee, B.L. (1979) Metallothionein: Historical review and perspective. *EXS (BASEL)*, **34**, 19–40.
30) World Health Organization (1992) Environmental Health Criteria 134. Cadmium, Geneva, Switzerland.
31) Worthington, D.J. & Rosemeyer, M.A. (1976) Glutathione reductase from human erythrocytes: Catalytic properties and aggregation. *Eur. J. Biochem.*, **67**, 213–238.