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The Effects of Melatonin on Cisplatin-Induced Renal Cortical Cell Injury in Rabbits

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Melatonin, a pineal gland hormone, is believed to act as an antioxidant via the stimulation of radical detoxifying enzymes and scavenging of free radicals. In this study, effects of *in vitro* and *in vivo* treatments of melatonin on the cisplatin-induced lipid peroxidation, LDH release and plasma creatinine were determined in rabbit renal cortical cells. The level of malondialdehyde (MDA) was assayed as an index of lipid peroxidation and the level of LDH release as an indicator of cellular damage. In *in vitro* studies, cisplatin increased the levels of MDA and LDH release in a concentration-and time-dependent manner. Melatonin inhibited the cisplatin-induced lipid peroxidation and LDH release in a concentration-dependent manner. The minimal effective concentration of melatonin that significantly reduced the 300 μM cisplatin-induced lipid peroxidation and LDH release was 1 mM. In *in vivo* studies, the levels of lipid peroxidation and LDH release in renal cortical cells increased significantly 24 or 48 hours after a single injection of cisplatin (6 mg/kg). When the cisplatin-injected rabbits were pretreated with 10 mg/kg of melatonin, a significant reduction in both lipid peroxidation and LDH release was observed. The plasma creatinine level increased from 0.87 ± 0.07 mg/dl in control to 6.33 ± 0.54 mg/dl in cisplatin-injected rabbits ($P < 0.05$). Melatonin partially prevented the increase in serum creatinine level (1.98 ± 0.11 mg/dl) by cisplatin ($P < 0.05$). In the proximal tubules from cisplatin-treated group, tubular cells had microvilli of variable heights. Necrotic debris was seen in tubular lumens. In most of cells, the mitochondria and lysosomes were increased in frequency. The endocytic vacuoles were not prominent and distribution of the brush border was irregular and shortened. These cisplatin-induced morphological changes were moderate in the melatonin-pretreated group. These results suggest that the toxicity of cisplatin is associated with the generation of reactive oxygen free radicals and that melatonin is a powerful antioxidant, which prevents some of the adverse effects of cisplatin.

Key Words: Melatonin, Renal cortical cell, Cisplatin, Lipid peroxidation, LDH

INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum II) is being used as a potent anticancer drug against a variety of solid tumor (Borch, 1987). Higher doses of cisplatin are more effective for the treatment of cancer. However, they produce various side effects, including nephrotoxicity (Garnick et al, 1988). It is well known that cisplatin generates active oxygen free radicals (Masuda et al, 1994) and that oxygen free radicals induce renal dysfunction (Kato et al, 1994). The free radical scavengers are reported to provide protections against cisplatin-induced renal injury (Dobyan et al, 1986), suggesting that oxygen free radicals may play a central role in cisplatin-induced nephrotoxicity.

Biological membranes contain a large amount of polyunsaturated fatty acids, which are susceptible to peroxidative attacks by oxygen free radicals, resulting in lipid peroxidation. Therefore, lipid peroxidation of the cell membrane has been considered to be an

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evidence of oxygen free radical-induced injury (Janero, 1990; Ohtake et al, 1997). However, the peroxidation of membrane lipid could be a result of cell death rather than a cause of irreversible cell injury induced by oxygen free radicals. The cell injury by cisplatin would involve alterations in physical and functional integrity of the cell membrane. In fact, it has been reported that cisplatin causes an increase in a LDH release, an indication of irreversible lethal cell injury in renal proximal tubular cells (Kim et al, 1997). If indeed lipid peroxidation is a cause of cell injury, then antioxidants should prevent the cell death.

Melatonin, a hormone produced and secreted by the pineal gland, behaves as an oxygen free radical scavenger and reduces oxidative stress by stimulating antioxidant enzymes (Antolin et al, 1996; Reiter et al, 1997). Therefore, it seems worthwhile to investigate whether the administration of melatonin could prevent the nephrotoxicity induced by cisplatin. To our knowledge, there have been no reports on the effect of melatonin on the cisplatin-induced oxidative damage in renal cortical cells.

In the present study we have examined possible cisplatin-induced oxidative damages in rabbit renal cortex using the level of malondialdehyde (MDA) as an index of lipid peroxidation, the LDH release as an index of irreversible cell damage, the plasma creatinine level as an indication of renal dysfunction, and electron micrography for morphologic alterations.

METHODS

Chemicals

All chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). The chemicals were freshly prepared immediately before use. Melatonin was dissolved in DMSO and dispensed to the culture media at the final concentrations required by the various protocols. DMSO in culture media never exceeded 1%. Cisplatin was dissolved in distilled sterile water and added to the culture media at the desired final concentrations.

Renal cortical cell preparation

Renal cortical cells were isolated from rabbit kidneys by enzymatic dissociation. Briefly, New Zealand white male rabbits weighing 1.5~2 kg were sacrificed after adequate anesthesia. The kidneys were dissected out and immediately perfused through the renal artery with ice-cold isotonic saline solution containing 140 mM NaCl and 10 mM KCl to remove as much blood as possible. The kidneys were then perfused with a normally Ca^{2+}-free Tyrode solution for 5 min, followed by Ca^{2+}-free Tyrode solution containing 0.01% collagenase (5 mg/50 cc, Yakult, Japan). After 15~25 min of enzymatic treatment, Kraft's Brhe (KB) solution was perfused. After 5 min of perfusion of KB solution, the kidneys were removed from the cannula, and four to six pieces of renal cortex were prepared using a Stadie-Riggs microtome. They were gently agitated in a small beaker with KB solution and were stored in an ice-cold modified Cross-Taggart medium containing 130 mM NaCl, 10 mM KCl, 1.5 mM $CaCl_2$, 5 mM glucose, and 20 mM Tris-HCl (pH 7.4).

Induction of oxidant injury in in vitro study

Chemical cell injury was induced by incubating the renal cortical cells in a dulbecco's modified eagle medium (Cat. No 11965-092, GIBCOBRL, Grand Island, N.Y. USA) with CO_2 and without Ca^{2+} in the presence of 300 μM cisplatin for 40 min at 37°C.

Experimental design for in vivo study

Thirty-two male New Zealand white rabbits, initially weighing 1.5 to 2 kg, had free access to standard rabbit chow and water. The rabbits were assigned to four groups as follows: group 1, vehicle (saline containing 1% DMSO)-treated controls (n=8); group 2, cisplatin-treated rabbits (n=8); group 3, rabbits treated with cisplatin plus 5 mg/kg body weight melatonin (n=8); group 4, rabbits treated with cisplatin plus 10 mg/kg body weight melatonin (n=8).

Cisplatin was intraperitoneally injected in a dose of 6 mg/kg body weight. Melatonin was subcutaneously injected in a dose of 5 (group 3) or 10 (group 4) mg/kg body weight 60 min before the injection of cisplatin.

Measurement of cell injury in renal cortical cells

For the measurement of LDH release, renal cortical cells were centrifuged at 1000 rpm for 5 min. The pellet was discarded and the supernatant was saved. LDH activity was determined in the supernatant and incubation medium using a LDH assay kit (Asan

Pharm. Co., LTD., Kyunggee-do, Korea). Final values were expressed as the percent of the control value.

Lipid peroxidation assay

Lipid peroxidation was estimated by measuring the content of malondialdehyde (MDA) according to the method of Okawa et al (1979). Renal cortical cells were homogenized in an ice-cold 1.15% KCl (5% wt/vol). A 0.2 ml aliquot of homogenate was mixed with 50 μl of 8.1% sodium dodecyl sulfate, and incubated for 10 min at room temperature. Acetic acid (375 μl, 20%, pH 3.5) and 375 μl of thiobarbituric acid (0.6%) were added. The mixture was heated for 60 min in a boiling water bath. The samples were allowed to cool at room temperature. After addition of *n*-butanol and pyridine (15 : 1) (1.25 ml), the contents were vigorously vortexed and centrifuged at 1000 rpm for 5 min. The absorbance of the upper, colored layer was measured at 535 nm and 520 nm with a spectrophotometer (Hitach, U-2000) and compared with freshly prepared 1,1,3,3-tetraethoxypropane standards. Final values were expressed as the percent of the control group value.

Determination of serum creatinine

In all groups of rabbits, plasma creatinine was estimated calorimetrically at 500 nm by a spectrophotometer using a creatinine standard from a Sigma diagnostic kit.

Morphologic study

Morphologic alterations in the kidney were determined in the rabbits 48 hours after a single injection of cisplatin (6 mg/kg). Kidneys were removed on the second day after cisplatin or saline (control) treatment and fixed by perfusing through the renal artery for 10 min with 0.1 M phosphate buffer containing 1.7% glutaraldehyde and 1.6% paraformaldehyde at a pressure of 120 mmHg. The kidneys were then excised and stored in fixative. Random samples of cortical tissues were postfixed in Epon 812. Ultra-thin sections (50~70 nm) were obtained, stained with uranyl acetate and lead nitrate, and viewed in an electron microscope (Jeol 1200 EX II, Japan).

Statistical analysis

All values are expressed as mean±SE. The differences between mean values were analyzed by one-way analysis of variance followed by Tukey post hoc test. $P<0.05$ was considered statistically significant.

RESULTS

In vitro studies

The effects of cisplatin on the lethal cell injury and lipid peroxidation were examined over the concentration range of 100~500 μM in renal cortical cells

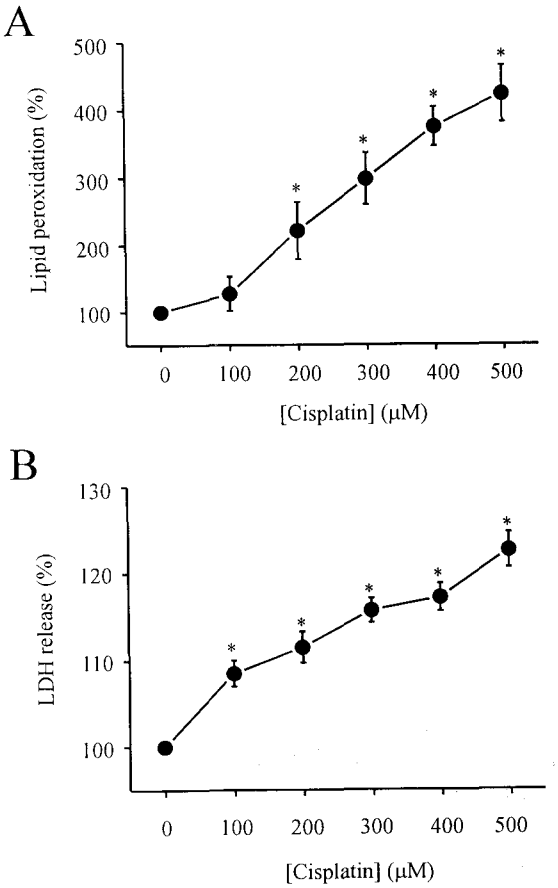

Fig. 1. Quantitative comparison of data showing the effect of increasing concentrations of cisplatin on the levels of MDA (A) and LDH release (B) in renal cortical cells. Renal cortical cells were treated with various concentration of cisplatin for 60 min at 37°C. Data are mean±SE of sixteen experiments. *$P<0.05$ compared with the control (in the absence of cisplatin).

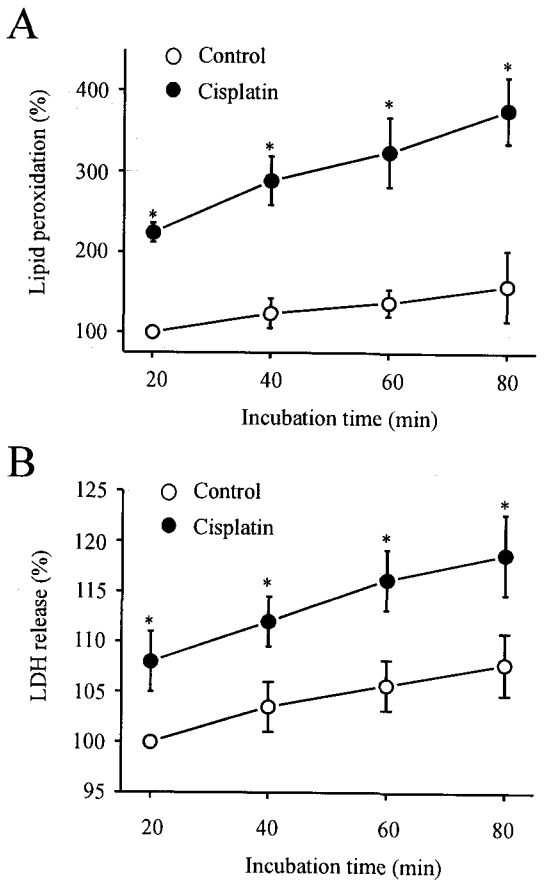

Fig. 2. Time course of cisplatin-induced lipid peroxidation (A) and LDH release (B) in renal cortical cells. The concentration of cisplatin was 300 μM. Data are given as mean SE of fourteen experiments. *P < 0.05 compared with the control (in the absence of cisplatin).

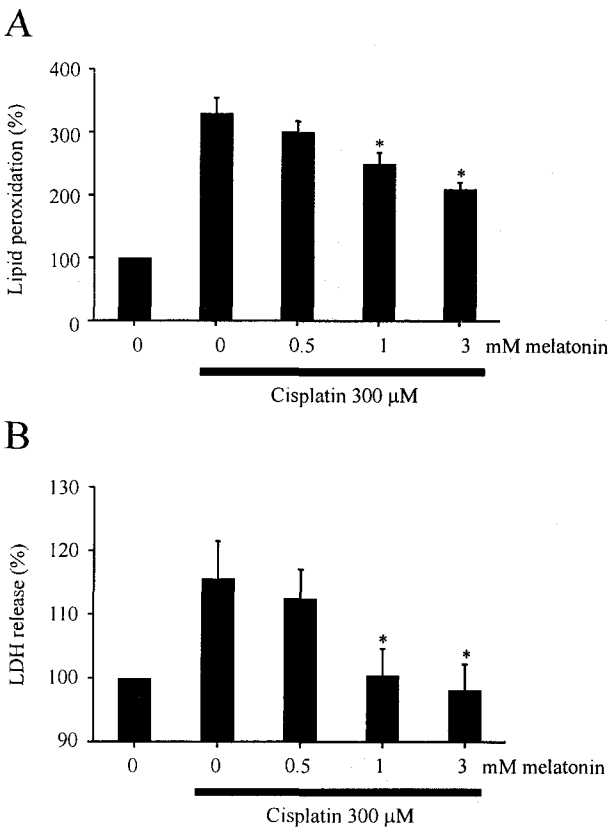

Fig. 3. Effect of different concentrations of melatonin on cisplatin (300 μM)-induced lipid peroxidation and LDH release in renal cortical cells. The incubation time was 60 min. Data are mean ± SE of twelve experiments. *P < 0.05 compared with cisplatin only treatment group.

incubated for 60 min at 37°C. As shown in Fig. 1A, cisplatin above 200 μM caused a significant increase in MDA level, the effect being proportional to the concentration of cisplatin. Likewise, cisplatin increased the LDH release in a concentration-dependent manner at concentrations above 100 μM (Fig. 1B). For subsequent studies, a 300 μM concentration of cisplatin was used, as this concentration of cisplatin yielded highly significant lipid peroxidation and LDH release.

It was observed that lipid peroxidation (Fig. 2A) and LDH release (Fig. 2B) increased linearly with the incubation time when 300 μM cisplatin was used. The incubation time of 40 min was selected for the subsequent experiments, as it is sufficient for the determination of lipid peroxidation and LDH release.

Melatonin inhibited cisplatin-induced lipid peroxidation (Fig. 3A) and LDH release (Fig. 3B) in a concentration-dependent manner. The minimal effective concentration of melatonin that for both lipid peroxidation and LDH release induced by 300 μM cisplatin was 1 mM ($P < 0.05$). When renal cortical cells were exposed to 300 μM cisplatin in the presence of 1 mM melatonin, the lipid peroxidation and LDH release were not different from the control (data not shown).

In vivo study

The level of lipid peroxidation in renal cortical cells increased significantly 24 hours (Fig. 4A) or 48 hours (Fig. 4B) after a single injection of cisplatin (6 mg/kg). These effects of cisplatin were significantly blunted by pretreatment of animals with melatonin 5 and 10 mg/kg.

The plasma creatinine level increased from a con-

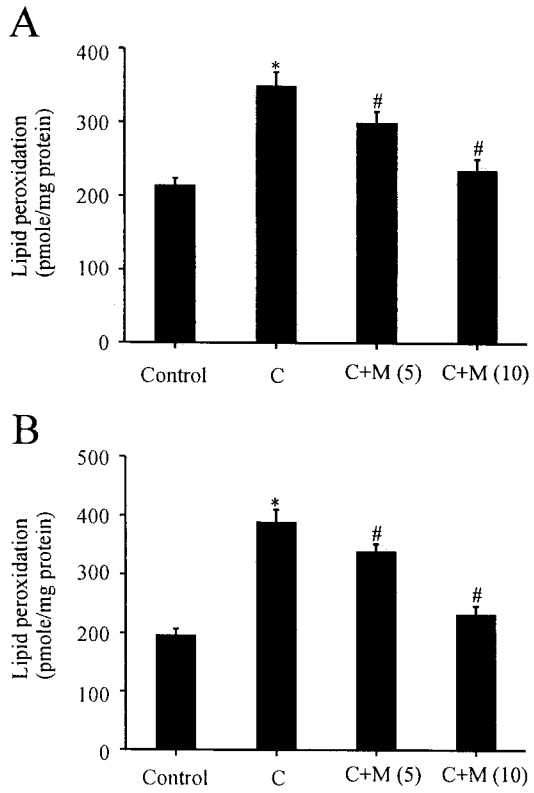

Fig. 4. Effect of melatonin on lipid peroxidation in cisplatin-injected rabbits. Rabbits were sacrificed one day (A) or two days (B) after injection of cisplatin with melatonin. Data are mean±SE of eight experiments. *P <0.05 compared with control group. #P <0.05 compared with cisplatin treatment group. C, 6 mg/kg cisplatin; M(5), 5 mg/kg melatonin; and M (10), 10 mg/kg melatonin.

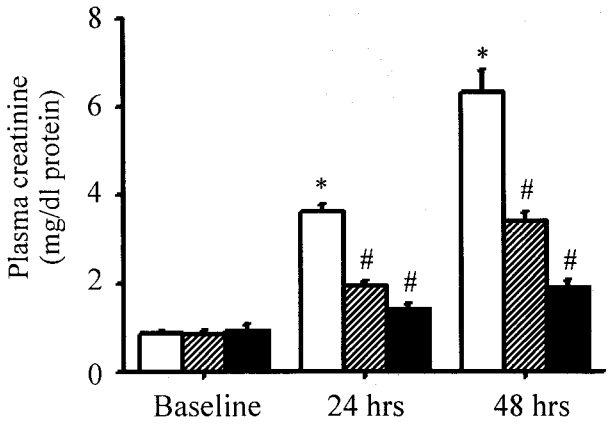

Fig. 5. Effect of melatonin on plasma creatinine concentrations in cisplatin-injected rabbits. Data are mean±SE of six different animals per group. Each assay was run in triplicate. *P <0.05 compared with baseline control group. #P <0.05 compared with cisplatin treatment group. Cisplatin-treated (open bars) and cisplatin plus melatonin rabbits: 5 mg/kg melatonin (hatched bars), 10 mg/kg melatonin (filled bars). The concentration of cisplatin to be used was 6 mg/kg body weight.

trol 0.87±0.07 mg/dl to 6.33±0.54 mg/dl in cisplatin-injected rabbits ($P<0.05$). This change was partially prevented by melatonin (Fig. 5).

Morphologic study

Morphologic alterations observed in proximal tubular cells after cisplatin treatment were similar to those described previously (Kim et al, 1995). In brief, a focal loss of the microvillous brush border was evident and the cytoplasm of many cells appeared condensed (Fig. 6). In proximal tubules of cisplatin-treated animals (Fig. 6B), some tubular cells had microvilli of variable heights. Necrotic debris was seen in many tubular lumens. In most of cells, mitochondria and lysosomes were increased. The endocytic vacuoles were not prominent and the brush border was irregular and microvilli were shortened. In proximal tubules of cisplatin plus melatonin-treated animals (Fig. 6C), the cells recovered with more or less increased height. In addition, there were many autolysosomes and elongated microvilli in the apical part of tubular cells. The number of small vesicles increased under microvilli. The microvilli of the irregular brush border were frequently more elongated than these in the cisplatin-treated group.

DISCUSSION

Cisplatin has also been reported to generate superoxide anion and hydroxyl radical (Masuda et al, 1994) and to increase renal lipid peroxidation (Nakano et al, 1989). Since the oxygen free radical scavengers superoxide dismutase (SOD) and *o*-(β-hydroxyethyl)-rutoside provided protections against cisplatin-induced structural and functional alterations (McGinness et al, 1978; Dobyan et al, 1986), it was suggested that oxygen free radicals play a central role in cisplatin-induced renal injury.

Previous studies have shown that melatonin, *in vivo*, protects the kidney from free radical-induced damages caused by either adriamycin or δ-aminolevulinic acid, respectively (Montilla et al, 1998;

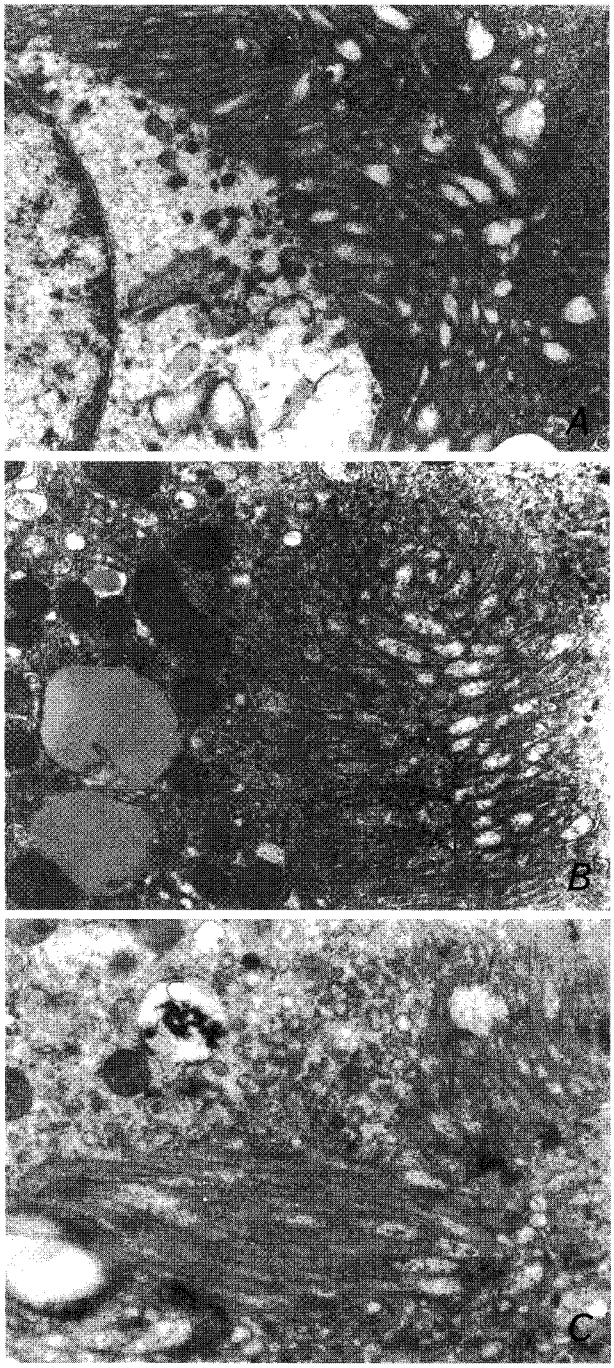

Fig. 6. Electron micrograph of the proximal tubule at the second days after cisplatin injection in control (A), cisplatin-treated (B), and cisplatin plus melatonin (10 mg/kg)-treated rabbits (C). The concentration of cisplatin to be used was 6 mg/kg body weight. Magnification, 12000.

Reiter, 1998), suggesting that melatonin is taken up by the kidney in sufficiently large quantities to protect it from highly damaging agents.

In preliminary experiments, we observed that cisplatin caused nephrotoxicity at a dose of 5 mg/kg or above in rabbits (data not shown). Therefore, we conducted nephrotoxicity experiments in rabbits utilizing a dose of 6 mg/kg. This dose corresponds to the dose of cisplatin currently being used in clinical treatment protocols, which produced ototoxicity in rats (Ravi et al, 1995). Previous study have shown that other toxic effects, such as neurotoxicity, bone marrow toxicity, and gastrointestinal toxicity of cisplatin, are also markedly increased with higher doses of cisplatin (Baldew et al, 1991; Hamers et al, 1993). Chemoprotection by various agents is being evaluated against the dose of cisplatin in clinical treatment protocols (Cozzaglio et al, 1990; Fontanelli et al, 1992). Thus, this study evaluated the protective effects of melatonin against the toxic renal effects that occur with cisplatin in rabbits.

In this study, the *in vitro* and *in vivo* effects of melatonin on cisplatin-induced lipid peroxidation, LDH release and serum creatinine levels were determined in renal cortical cells of the rabbit. We observed that both *in vitro* and *in vivo* studies the level of lipid peroxidation and LDH release in renal cortical cells induced by cisplatin was markedly reduced by melatonin. In *in vitro* studies, melatonin inhibited the cisplatin-induced lipid peroxidation and LDH release in a concentration-dependent manner. In addition, in *in vivo* studies, 10 mg/kg melatonin reduced lipid peroxidation, LDH release and the increase in serum creatinine level caused by cisplatin (6 mg/kg body weight). Morphologic observations showed that cisplatin caused a focal loss of the microvilli brush border. Our results suggest that the initiation of lipid peroxidation may contribute to the cisplatin-induced nephrotoxicity, in agreement with previous studies (Husain et al, 1998; Rao et al, 1999) and that melatonin attenuates cisplatin-induced renal damage through diminished tubular damage.

The protective effect against cisplatin-induced nephrotoxicity as observed with melatonin administration in this study was similar to those observed in rabbits with other chemoprotectants (Kim et al, 1997). Our findings are also consistent with previous studies in which melatonin protected against oxidative damages by ferric nitrilotriacetate in the rat kidney (Qi et al, 1999) and cisplatin in the mice kidney (Sener et al, 2000). It has been suggested that the protective effect of melatonin may be related to the action of indoleamine. Melatonin has been known to be a direct free radical scavenger of the highly toxic hydroxyl

radical (Stasica et al, 1998). Melatonin can also neutralize several other reactive oxygen free radicals as an endogenous antioxidant (Pieri et al, 1994; Cuzzocrea et al, 1997; King & Scaiano, 1997; Noda et al, 1999). In addition to its direct free radical scavenging effect, melatonin plays a role as an indirect antioxidant by stimulating the mRNA level and the activities of superoxide dismutase (Kotler et al, 1998), glutathione peroxidase and reductase (Pablos et al, 1998). Since melatonin is highly lipophlic (Costa et al, 1994) as well as somewhat hydrophilic (Shida et al, 1994), it easily enters cells and subcellular compartments where it prevents oxidative damage to a variety of molecules (Reiter, 1998). These abilities of melatonin may contribute to reduce cisplatin-induced oxidative damage both in vitro and in vivo in the rabbit kidney.

In summary, oxygen free radicals contribute to the development of cisplatin-induced renal toxicity, which is reduced by melatonin as observed by a reduction of plasma creatinine, renal lipid peroxidation, and LDH release. Changes in plasma creatinine reflect changes in glomerular function, whereas changes in lipid peroxidation and LDH release represent tubular cell damage.

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