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Melatonin and Oxidative Damage in Mice Liver Induced by the Prooxidant Antitumor Drug, Adriamycin

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Abstract. *Antioxidant properties have been attributed to melatonin; it seemed therefore worthwhile to determine its effects in relation to the prooxidant action of adriamycin, which contributes to its toxic and therapeutic effects. Melatonin effectively acts as a direct free radical scavenger in the concentration range of 20-100 μM as determined in vitro, using Fenton reaction as a source of free radicals that were determined by EPR using spin trapping method. Following the administration of a single i.v. dose of 28 mg/Kg or of 3 repeated i. p. doses of 5 mg/Kg adriamycin to CBA mice, glutathione levels in the liver cells were significantly reduced. When the treatment with adriamycin was preceded by the s.c. administration of 2 mg/Kg melatonin, the decrease in total and reduced glutathione concentrations was significantly prevented. A significant increase in lipid peroxidation was observed in liver cells after a single administration of adriamycin which was not attenuated by pretreatment with melatonin. These results indicate that further examination of the possible protective action of melatonin on the toxic effects of prooxidant antitumor drugs on normal and neoplastic tissues would be of interest also in relation to their chronotoxicological properties.*

The role of melatonin, the indolic hormone produced by pineal gland, in physiology and pathology has been extensively examined in the last decades (1). Experimental evidence is available indicating that melatonin is involved in tumor progression, and melatonin was shown to rescue bone marrow from the toxic action of the antitumor drugs cyclophosphamide and etoposide (2).

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Evidence has been provided showing that melatonin causes direct antioxidant effects, being a potent free radical scavenger effective on hydroxyl (3,4) and peroxyl radicals (5). Additionally, melatonin can also act indirectly, upregulating endogenous antioxidant defenses such as glutathione peroxidase activity in the brain (6) and in the liver (7).

Numerous antitumor drugs have been shown to possess a prooxidant action, which appears to contribute to their toxic and/or therapeutic effects, and among them, anthracyclines such as adriamycin and daunorubicin are highly effective antineoplastic agents. Cancer patients are frequently treated with these drugs or with combinations of them (8). These treatment have proved to be effective against hematological and solid malignancies (9). However, the major problem encountered during long-term treatment with anthracyclines, besides the development of tumor resistance, is the severe cardiotoxicity of these drugs. In this connection, the toxicity for normal tissues of the host caused by anthracyclines has been largely attributed to lipid peroxidation via generation of free radicals (10,11).

It thus appeared worthwhile to examine the possible effects of melatonin on the oxidative damage caused by the anthracycline, adriamycin, in CBA mice. Since the formation of free radicals of adriamycin should be highest in the liver because of its high content of iron and higher activity of microsomal enzymes in comparison with other tissues (12), the antioxidant effects of melatonin have been examined in the liver. Free radical scavenger activity of melatonin was preliminary evaluated in a model *in vitro* system, using the Fenton reaction as a source of free radicals. These short lived free radicals were determined at different concentrations of melatonin, by EPR using the spin trapping method. In order to examine the mechanism of melatonin antioxidant action *in vivo*, the levels of hepatic glutathione were measured in relation to lipid peroxidation expressed as MDA (malondialdehyde) concentrations, after the treatment of mice with adriamycin. The results obtained are reported herein.

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Materials and Methods

Chemicals. Melatonin was a kind gift of Prof. Fraschini, University of Milano, Italy; Adriamycin was purchased from Pharmacia S.p.A., Milano, Italy; the other reagents used were purchased from Sigma-Aldrich S.r.l., Milano, Italy.

Treatment with adriamycin and melatonin. The animals used were male CBA mice, bred locallis. The animals, weighing 25-28 g, were provided with food and water *ad libitum*, and were kept in a 12/12 light/dark cycle (lights on from 7 a.m. to 7 p.m.). Adriamycin and melatonin were dissolved in physiological NaCl (0.9% w/v) solution. Adriamycin was administered as a single dose (28 mg/Kg i.v. in 0.05 ml/10 g of body weight), or once every week (5 mg/Kg i.p. in 0.1 ml/10 g of body weight) for 3 weeks, as indicated in the Tables. Melatonin (2 mg/kg s.c. in 0.05 ml/10 g of body weight) was administered at 7 p.m. (light off), followed 1 hour later by the administration of adriamycin. After sacrifice, the livers were removed, frozen and kept at -80°C for subsequent MDA and GSH analysis.

Lipid peroxidation assay. This assay consisted of hydrolysis of liver lipoperoxides, to form MDA. Reaction of MDA with thiobarbituric acid (TBA) was performed in order to obtain a red MDA-TBA adduct. It was measured spectrophotometrically, after extraction with butanol, following the method of Uchiyama and Mihara (13). Briefly, after thawing, the liver of each mouse was homogenized with a potter teflon pestle glass homogenizer (1g liver in 5 ml of 1.15% KCI) at 0°C. To 0.5 ml of liver homogenate, 3 ml of 0.5% TCA and 1 ml of 0.6% TBA aqueous solution were added. The mixture was heated for 45 minutes in a boiling water bath; after cooling, 4 ml of n-butanol were added and mixed vigorously. The butanol phase was separated by centrifugation (4000 rpm × 10 minutes) and optical absorbance was measured at 532 nm. Liver lipoperoxide concentrations were computed with respect to a calibration curve prepared with tetraethoxypropane (TEP), which undergoes hydrolysis to liberate stoichiometric amounts of MDA (14). MDA concentration was expressed as nmol/mg of protein, which was assayed by the method of Lowry *et al* (15).

Glutathione assay. The reduced (GSH) and the oxidized (GSSG) glutathione levels in liver were detected by a high-performance liquid chromatography (HPLC) technique. Each liver was homogenized as already described, and was processed following the method of Reed *et al* (16). Briefly, to 1 ml of liver homogenate (20-30 mg/ml protein) 0.05 ml of 70% perchloric acid was added. After protein precipitation, 0.5 ml of the supernatant was treated immediately with 50 µl of a fresh aqueous solution of iodacetic acid (0.08 M), and then neutralized with an excess of $NaHCO_3$. After 60 minutes in the dark at room temperature, 0.5 ml of an alcoholic solution of 1-fluoro-2,4-dinitrobenzene (1.5 ml/ 98.5 ml of absolute ethanol) was added, and the reaction allowed to proceed for 4 hours in the dark. The samples were then chromatographed using a reverse-phase ion exchange column microbondapak NH2 3.9×300 mm (Waters). Glutathione levels were related to protein content in the sample, which was determined by the method of Lowry *et al* (15).

Spin trapping experiment. Free radical scavenging activity of melatonin was measured in phosphate buffered saline containing 0.1 mM EDTA (pH 7, 320 mosmol). The buffer was supplemented with the spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 1 mM), 0.02 mM $FeSO_4.7H_2O$ and 0.01 % (w/v) of H_2O_2 (final concentrations). The intensity of the EPR spectra of the DMPO-OH adduct was measured over time, in presence of different concentrations of melatonin (17).

Statistical analysis. Tabled values are group means±SD. Data were subjected to the appropriate factorial ANOVAs to assessing significance against an a alpha-level $p < 0.05$. When the individual effects of the treatments were significant and the interaction between the independent variables in a 2× 2 ANOVA was significant, the data were subjected to *post hoc* Tukey test for significance of the differences in the mean values. All analyses were performed using standard procedures implemented in the Systat package.

Results

The effects of melatonin administration on oxidative damage induced by adriamycin treatment in liver of CBA mice were studied by monitoring biochemical parameters which are related to cellular oxidative stress, i.e. glutathione levels and total lipid peroxidation, whereas direct free radical scavenger activity of melatonin was investigated *in vitro.*

Free radical scavenger activity of melatonin. The free radical scavenger activity of melatonin was detected in spin trapping experiments using Fenton reaction as a model for production of hydroxyl radicals, in which DMPO-OH adducts were detected by EPR. Only a very slow decrease of this adduct with time was observed (Figure 1). When Fenton reaction was initiated in presence of melatonin, the intensity of EPR spectra was decreased, indicating an effective scavenger activity of melatonin, which prevented binding of OH radical to DMPO. The intensity of EPR spectra decreased with increasing concentration of melatonin, and a significant decrease to about 30% of the basal value was observed in the presence of 50 µ M melatonin (Figure 2).

Effects of a single administration of adriamycin and melatonin on glutathione levels and lipid peroxidation. When the animals are treated with a single dose of 28 mg/Kg adriamycin i.v. and with a single dose of 2 mg/Kg. melatonin s.c. and their livers are examined, separate 2 × 2 ANOVAs indicate a significant effect of adriamycin, melatonin and of the interaction of both treatments on total glutathione ($F_{(1,22)} = 134.1$, $p < 0$ 0001, $F_{(1,22)} = 80.2$, $p < 0.0001$ and $F_{(1,22)} = 101.4$, $p < 0.0001$, respectively), on reduced glutathione ($F_{(1,22)} = 143.5$, $p < 0.0001$, $F_{(1,22)} = 87.4$, $p < 0.0001$ and $F_{(1,22)} = 107.3$, $p < 0.0001$, respectively), and on GSH/GSSG ratio ($F_{(1,22)} = 25.4$, $p < 0.0001$, $F_{(1,22)} = 33.4$, $p < 0.0001$ and $F_{(1,22)} = 15.2$, $p = 0.001$, respectively). With respect to oxidized glutathione, a significant effect is observed for the treatment with adriamycin and for the treatment with both substances ($F_{(1,22)} = 13.9$, $p = 0.001$, and $F_{(1,22)} = 15.1$, $p = 0.001$, respectively). With respect to MDA, only the effect of adriamycin is significant ($F_{(1,23)} = 22.9$, $p < 0.0001$). Tukey test indicates that the reduction of glutathione concentration levels (GSH, GSSG, tGSH and GSH/GSSG) caused by adriamycin is significantly reverted by the concurrent administration of melatonin (Table I).

Effect of repeated administrations of melatonin and adriamycin on glutathione levels and lipid peroxidation. When the animals are treated with 5mg/Kg adriamycin i.p. and with 2 mg/Kg melatonin s.c. once a week for three weeks and their livers are examined, separate 2 × 2 ANOVAs indicate a significant

Table I. *Effects of a single administration of adriamycin and melatonin on lipid peroxidation and glutathione levels in the liver of CBA mice.*

ADRIA	MELA	MDA*	GSH*	GSSG*	GSH\GSSG*	tGSH*
-	-	$0.38\pm0.12^{a,b}$	56.42 ± 2.46^{a}	2.79 ± 0.15^{a}	20.25 ± 0.31^{a}	59.21 ± 2.61^{a}
-	+	$0.45\pm0.13^{c,d}$	55.08 ± 3.77^{b}	2.56 ± 0.32^{b}	21.64 ± 1.56^{b}	57.64 ± 4.07^{b}
+	-	$1.51\pm0.85^{a,c}$	$26.76\pm4.42^{a,b,c}$	$1.96\pm0\ 24^{a,b,c}$	$13.63\pm1.52^{a,b,c}$	$28.72\pm4.59^{a,b,c}$
+	+	$1.58\pm0.60^{b,d}$	52.93 ± 1.74^{c}	2.58 ± 0.32^{c}	20.79 ± 2.61^{c}	55.51 ± 1.99^{c}

*: nmol/mg protein

Groups of 5-9 CBA male mice were treated at 7.00 p.m. with melatonin (MELA; 2 mg/Kg s.c.), followed at 8.00 p.m. by adriamycin (ADRIA; 28 mg/Kg i.v.). 4 days after the treatment, the animals were sacrificed, and MDA and GSH concentrations were determined in the liver.

Each value is the mean ± SD; means marked with the same letter are statistically different, Tukey test ($p<0.05$).

Table II. *Effects of repeated administrations of adriamycin and melatonin on lipid peroxidation and glutathione levels in liver of CBA mice.*

ADRIA	MELA	MDA*	GSH*	GSSG*	GSH/GSSG*	tGSH*
-	-	0.76 ± 0.25	65.85 ± 6.38^{a}	$3.20\pm0.36^{a,b}$	20.74 ± 3.09	69.06 ± 6.41^{a}
-	+	0.73 ± 0.09	68.76 ± 7.44^{b}	2.93 ± 0.36^{c}	23.53 ± 0.36	71.69 ± 7.78^{b}
+	-	0.43 ± 0.13	$41.87\pm2.10^{a,b,c}$	$1.55\pm0.06^{a,c,d}$	27.01 ± 2.36	$43.43\pm2.04^{a,b,c}$
+	+	0.68 ± 0.09	68.54 ± 3.48^{c}	$2.41\pm0.38^{b,d}$	29.01 ± 5.57	70.95 ± 3.16^{c}

*: nmol/mg protein

Groups of 6 CBA male mice were treated at 7.00 p.m. with melatonin (MELA; 2 mg/Kg s.c), followed at 8.00 p.m. by adriamycin (ADRIA; 5 mg/Kg i.p); the treatment was repeated every 7 days for a total of 3 administrations. 3 days after the last treatment, the animals were sacrificed, and MDA and GSH concentrations were determined in the liver.

Each value is the mean ± SD; means marked with the same letter are statistically different, Tukey test ($p<0.05$).

effect of adriamycin, melatonin and of the interaction of both treatments on total glutathione ($F_{(1,8)} = 18.5$, $p = 0.003$, $F_{(1,8)} = 24.2$, $p = 0.001$ and $F_{(1,8)} = 16.5$, $p = 0.004$, respectively) and on reduced glutathione ($F_{(1,8)} = 15.7$, $p = 0.004$, $F_{(1,8)} = 23.5$, $p = 0.001$ and $F_{(1,8)} = 15.2$, $p = 0.005$, respectively). A significant effect of adriamycin and of the interaction of the treatments with both substances is observed on oxidized glutathione ($F_{(1,8)} = 32.4$, $p < 0.0001$, $F_{(1,8)} = 8.9$, $p = 0.018$), whereas on GSH/GSSG ratio only the effect of adriamycin is significant ($F_{(1,8)} = 7.6$, $p = 0.024$). With respect to MDA, the effects of the treatment with adriamycin are significant ($F_{(1,18)} = 7.4$, $p = 0.014$), whereas the interaction of the treatments with adriamycin and melatonin is marginally significant ($F_{(1,18)} = 4.1$, $p = 0.058$). Tukey test indicates that the reduction of glutathione levels (GSH, GSSG and tGSH) caused by adriamycin is significantly reverted by the concurrent administration of melatonin (Table II).

Discussion

The antitumor drug adriamycin has been shown to be effective in the treatment of a broad spectrum of neoplastic diseases. Its clinical use, however, is accompanied by toxic effects common to antineoplastic chemotherapy; in experimental animals, renal and peripheral nervous system toxicity was also observed (18). Moreover, adriamycin displays a characteristic organ toxicity consisting of a dose-limiting, cumulative cardiomyopathy (19). Cardiotoxicity has been attributed to the prooxidant action of the drug and to the consequent damage associated to lipid peroxidation of the cell membrane of heart cells (10). The formation of an adriamycin semiquinone, either by an enzimatically catalyzed or by a spontaneous reaction (20), and its subsequent redox cycling to generate reactive oxygen species, could account for the above mentioned lipid peroxidation (21,22). Moreover, a decrease in intracellular glutathione levels was observed after adriamycin treatment in the heart, as well as in liver cells (23), which has been considered as a further factor contributing to the cellular toxicity of adriamycin (24). These findings suggest that the free radical challenge induced by the drug may overwhelm the antioxidant protective systems of the cells, including the glutathione redox cycle, and contributing in this way to the toxic action of adriamycin on normal tissues (25).

Considering the available evidence from literature,

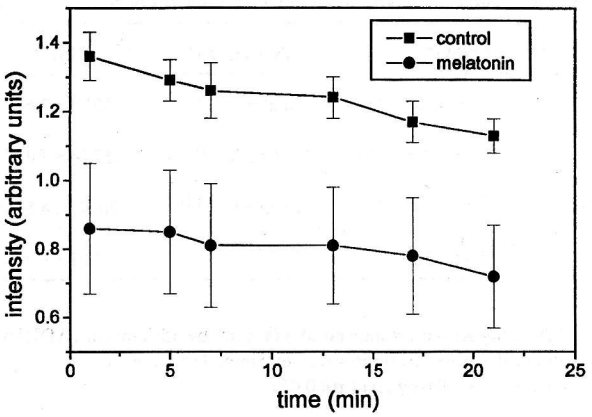

Figure 1. *EPR spectra intensity decrease with time of DMPO-OH adduct produced in Fenton reaction in presence and absence of melatonin. Final concentrations: 1 mM DMPO, 50 μM melatonin, 0.02 mM Fe^{2+}, 0.01% H_2O_2 in PBS (pH 7) with 1 mM EDTA. Each point is mean value of 5 measurements, bars indicate standard deviations.*

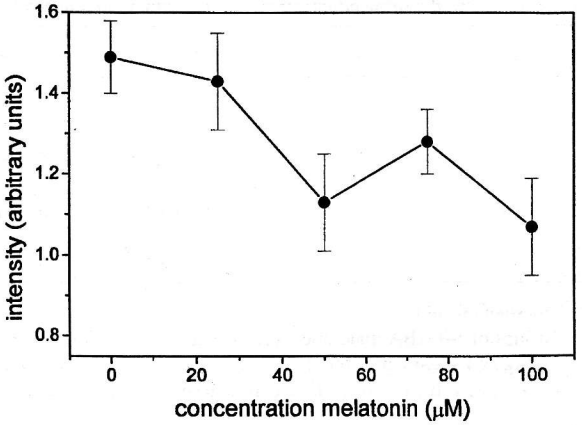

Figure 2. *Influence of melatonin concentration on the production of DMPO-OH adducts during Fenton reaction. Final concentrations: 1mM DMPO, 0.02 mM Fe^{2+}, 0.01% H_2O_2 in PBS (pH 7) with 1 mM EDTA. Each point is mean value of 5 measurements, bars indicate standard deviations.*

showing the antioxidant properties of melatonin, the aim of this investigation was to examine the possible protection caused by melatonin on the oxidative damage induced by adriamycin. A first series of experiments was performed in order to examine the possible action of melatonin as a direct free radical scavanger. The results obtained using the Fenton reaction followed by EPR spectroscopy indicate that melatonin effectively acts as a hydroxyl radical scavenger. The effects of melatonin occur in a dose dependent way in a concentration ranging from 20 to 100 μM. This result is in agreement with the findings reported by Tan *et al* (3), and more recently by Matsuzak *et al* (26).

A further series of experiments was aimed to determine the effects of melatonin on cellular antioxidant defenses in relation to the damage caused by adriamycin. The study has been carried out examining the effects of the *in vivo* treatments on liver cells, since the formation of free radical from adriamycin is higher in the liver than in other organs, including heart (12). An increase in lipid peroxidation, as indicated by elevated MDA levels, is caused by the treatment with a single dose of adriamycin (28 mg/Kg), as already reported by other authors (27). On the other hand, when the treatment was performed using 3 weekly administrations of the drug (5 mg/Kg), MDA levels were not significantly different from those measured in drug untreated controls, as already reported by Lazzarino *et al* (28). These effects of adriamycin may be interpreted by considering the action of this drug on the glutathione redox cycle. Indeed, after a single administration of the drug, the GSH/GSSG ratio was significantly reduced in comparison with that observed in the drug untreated control group, this reduced ratio being an accepted index of oxidative stress (29). It is thus reasonable to assume that adriamycin redox cycling transiently overwhelms the capacity of the liver cells to eliminate peroxides, in this way enabling the production of hydroxyl radicals *via* iron-catalyzed Haber-Weis reaction (30). This hypothesized sequence of events is supported by available evidence, showing that purified glutathione peroxidase can be inhibited by the drug after a single acute treatment (31). The breakdown of the glutathione redox cycle caused by decreased levels of reduced glutathione, of glutathione peroxidase, or of glutathione reductase would be responsible for tissue injury by an adriamycin-derived oxygen radical flux.

In the case of repeated treatments with adriamycin, even if the drug significantly lowers the levels of reduced and oxidized glutathione, the treated cells seem to be capable of maintaining the equilibrium of intracellular peroxide homeostasis. The fact that GSH/GSSG ratio does not differ in the treated *versus* the control group after repeated administrations of adriamycin may be interpreted assuming the development of an efficient balance system shuttling oxidized and reduced glutathione through GSH peroxidase and GSSG reductase, as a response to a chronic oxidative damage. Moreover, a substantial decrease in intracellular GSH content seems to be required to have a significant increase in cytotoxicity (30). This latter observation suggests that few glutathione molecules can be efficiently recycled to reduce hydrogen peroxide or lipid hydroperoxides by means of oxidation and reduction cycles. The absolute intracellular concentration of GSH must be markedly depleted before hydroperoxide metabolism is adversely affected (30), as presently observed following the treatment with a single adriamycin administration.

In the experiment with a single dose of adriamycin, the administration of melatonin 2 mg/Kg s.c. had no protective effect on lipid peroxidation in liver cells, as determined by

MDA measurements. A similar result has been obtained in rats treated with carbon tetrachloride (CCl_4), a prooxidative substance commonly used to experimentally induce hepatic oxidative damage. The *in vivo* treatment with CCl_4 caused an increase of lipid peroxidation products in the liver, which was not prevented by melatonin 10 mg/Kg, although melatonin was effective in experiments *in vitro.* These authors assumed that since CCl_4 is primarily a hepatotoxin, it may accumulate to considerable amounts in the liver. Concentrations of melatonin higher than those achieved with the *in vivo* administered dose of 10 mg/Kg, also considering its rapid metabolism in the liver, would be required to obtain an adequate protection against CCl_4 induced oxidative damage (32). These results seem to rule out a significant role of melatonin on hepatic lipid peroxidation in a range of concentrations close to physiological ones, obtained by the administration of 2 mg/Kg. Indeed, after the administration of 10-20 mg/Kg s.c., a significant reduction in MDA levels after adriamycin treatment was observed (unreported results). However, in order to administer to mice dosages of melatonin greater than 2 mg/Kg, ethanol has to be used as a solubilizer, and ethanol itself may display either antioxidant or prooxidant effects, which complicate the interpretation of the results (33).

The role of melatonin in the protection against the oxidative damage caused by adriamycin is evident when glutathione redox cycle is examined. Melatonin has been shown to be one of the most effective peroxyl radical scavenger, acting either directly (5) or indirectly increasing glutathione peroxidase (6,7). According to Pierrefiche *et al* (34), melatonin also stimulates hepatic and cerebral glucose-6-phosphate dehydrogenase (G6PDH) activity in mice, thereby increasing NADPH levels. In this way, melatonin promotes the enzymatic conversion of GSSG to GSH by glutathione reductase. The results of this work are in agreement with the results reported above, since total intracellular glutathione is decreased by adriamycin with both treatment schedules used, and since the observed reduction is prevented when adriamycin treatment is preceded by melatonin administration.

In conclusion, the indices of oxidative stress, GSSG and the GSH/GSSH ratio, indicate that melatonin protects the liver from the oxidative damage caused by the treatment with a single administration of adriamycin. This finding suggests further examinination of the protective action of melatonin on the cytotoxic effects of anthracyclines and other prooxidant or alkylating antitumor drugs in normal and neoplastic tissues, and investigation of the mechanism of its protective action considering, the available chrono-toxicological data.

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