Melatonin treatment for prevention of oxidative stress: involving histopathological changes

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Abstract. This study was undertaken to test the effect of irradiation on the histopathology of the hypothalamus and cerebral cortex. In addition, the probable effects of radiotherapy on the activities of antioxidant enzymes and levels of nitric oxide (NO) in the plasma were investigated as well. The effects of melatonin treatment on radiotherapy-based central nervous system (CNS) damage were also studied. For this purpose, the rats were randomized into four groups. The first group was the control group (sham-exposed group), the second group received only melatonin, the third group was irradiated and the fourth group received both melatonin and irradiation. Plasma samples of rats were collected for measuring the activities of superoxide dismutase (SOD), catalase (CAT) and the levels of NO. 24 h after the interventions, tissue samples were obtained from the hypothalamus and the cerebral cortex for the light microscobic investigations. These tissues were mostly affected by radiation. The results indicated that the application of radiation significantly enhanced the levels of plasma SOD and NO. On the other hand, melatonin pretreatment prevented the decrease in plasma CAT activity induced by irradiation. It was found that the application of melatonin could significantly prevent the irradiation-induced damages. Light microscopic results revealed that the damage of the CNS by radiation was prevented by the application of melatonin.

Key words: Irradiation — Central nervous system — Melatonin — Light microscopy — Antioxidative enzymes

Introduction

Radiotherapy has an indispensable role in the treatment of central nervous system (CNS) tumors and other organ malignancies. However, the severity of its side effects and its harmful effects on normal brain tissues makes this treatment difficult and results in dose restriction (Yoneoka et al. 1999). In the past, the belief was that only high doses of radiotherapy produced side effects in CNS, yet the data that has accumulated within the last 10 years demonstrated that even very low-doses of radiation could result in cellular damage of CNS. Data obtained from post-mortem histopathological studies showed that, following the radiotherapy, 40–50% of the patients have cerebral demyelinization, atrophy, apoptosis and necrosis of the neurons (Mildenberger et al. 1990; Fike et al. 1995; Schultheiss et al. 1995; Siegal et al. 1996; Belka et al. 2001). Today, in order to reduce radiotherapy-induced CNS damage, several attempts are being made. One of such approaches is to apply the total dose in fractions and locally in order to preserve healthy neural tissue. Moreover, searches for new treatment opportunities to prevent radiation damage are continuing, as well. Some of these attempts are based on prevention from oxidative

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damage. According to this hypothesis, oxidative damage is the main factor responsible for radiation-induced damage (that is observed *via* neuronal death and demyelinization). By preventing oxidative damage, the CNS can also be protected from radiation injury (Matsubara 1988; Dansette et al. 1990; Blickenstaff et al. 1994; De Laurenzi et al. 1995; Badr et al. 1999; Deng et al. 1999; Belka et al. 2001; Turner et al. 2002; Kaptanoglu et al. 2003). With the aim of protection against the side effects of radiotherapy, antioxidants have been used in several experimental studies and shown to have partial or complete success in protection. During recent years, strong endogenous antioxidant effects of melatonin have been the subject matter of several research studies. Melatonin can inhibit DNA and lipid peroxidation that occurs *via* oxygen free radicals and can decrease the amount of tissue damage in the pathologies in which oxidative damage is the main factor (Marchall et al. 1996; Moore et al. 2002). Melatonin is reported to have protective and neuronal damage reducing effects in different pathologies characterized by neuronal loss (Marchall et al. 1996; Moore et al. 2002; Duan et al. 2006)**.** In this study, we aimed at investigating whether the pre-administration of melatonin to rats would be effective in preventing the CNS damage induced by 50% lethal dose of irradiation and to observe whether it would be influential on the changes in the plasma levels of nitric oxide (NO) and activities of antioxidant enzymes.

Materials and Methods

Animals

Wistar rats (250–300 g) were used for this experimental study. Animals were housed in cages with free access to food and water. All animals were maintained under constant laboratory conditions 18–21°C (room temperature) kept under 12 : 12 h light-dark cycle.

Group Design

Forty Wistar rats were randomly divided in four groups with 10 animals in each group: sham-exposed control group (CG), melatonin administered group (MG), whole-body irradiated group (RG), melatonin administered and irradiated group (MRG).

Experimental procedure

All of the rats were anesthetized with 15 mg/kg intramuscular ketamine. Before the irradiation, the position was planned under the simulator machine. The field size of rats was measured for total body irradiation from anterior-posterior opposite fields at source axis distance: 80 cm; this was confirmed with an X-ray film. Then the rats were placed in the same position and the same field size was opened under the treatment machine; a total (central axis absorbed) dose of 675 cGy (lethal dose, LD_{50}) was administered with a Co-60 gamma-ray machine. The same procedure was repeated for each group. Rats in CG underwent the same procedure except for irradiation and were given 50% saline and 50% ethanol mixture (1 ml/kg) intraperitoneally half an hour before sham exposure.

Melatonin (10 mg) (Sigma, St. Louis, MO) was dissolved in absolute ethanol (0.5 ml) and then diluted the same amount of saline (0.5 ml) were applied in the rats of the MG (1 mg/kg body weight) before sham exposure. In the rats of RG (irradiated by 675 cGy, one dose), equal amount of saline and ethanol mixture (1 ml/kg) were given intraperitoneally before irradiation. In MRG, melatonin was administered to the rats 30 min before irradiation. At 24 h following exposure, all animals were sacrificed in deep anesthesia. Then, after opening the skull, the cerebral cortex and hypothalamus were dissected immediately. There were no mortality in irradiated rats since animals were sacrified during the early period of applications.

Light microscopy

Cerebrum cortex and hypothalamus were dissected from the rats. The cerebral tissue samples were fixed in 10% buffered paraformaldehyde. Tissue specimens were prepared with an autotechnicon (tissue processor) model HMP 300 (Carl Zeiss Corporation, Oberkochen, Germany) and then embedded in paraffin blocks. Five slices of 5-μm thickness were prepared for each tissue using a microtome and stained with hematoxylin-eosin. The specimens were observed and photographed using a photomicroscope (Axiolab model, Carl Zeiss Corporation). The specimens were masked to prevent bias during their examination.

Blood obtaining procedure and measurement of blood parameters

Blood samples (5 ml from each animal) were collected by intracardiac puncture into the heparinized tubes following first and second anesthesias. Plasma was separated by centrifuging of blood at 4000 rpm for 15 min. Plasma samples were frozen and stored at –80°C until measurement of plasma superoxide dismutase (SOD), catalase (CAT) activities and NO levels.

CuZn-SOD activity was assayed by the method of Kostyuk and Potapovich (1989). A simple assay system was based on the inhibitory effects of SOD on the spontaneous oxidation of quercetin. 1.0 U was defined as that amount of enzyme causing half maximal inhibition of quercetin auto-

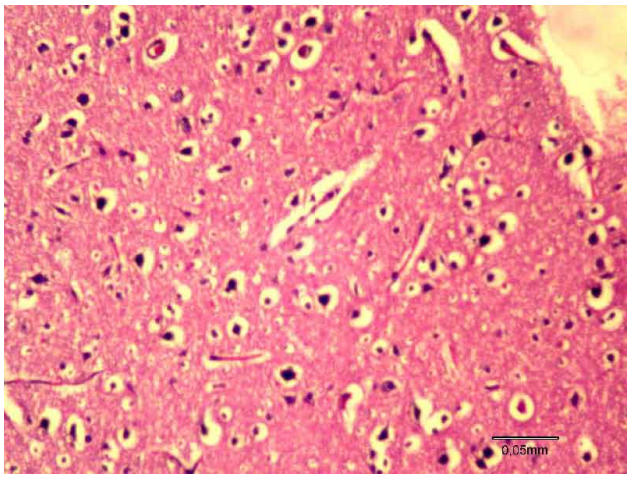

Figure 1. RG of cortex. Light microscopic examination in cerebral cortical cells of RG is shown that more intense swelling in cortical ganglions, glial cells augmentation and separation in neural prolongations.

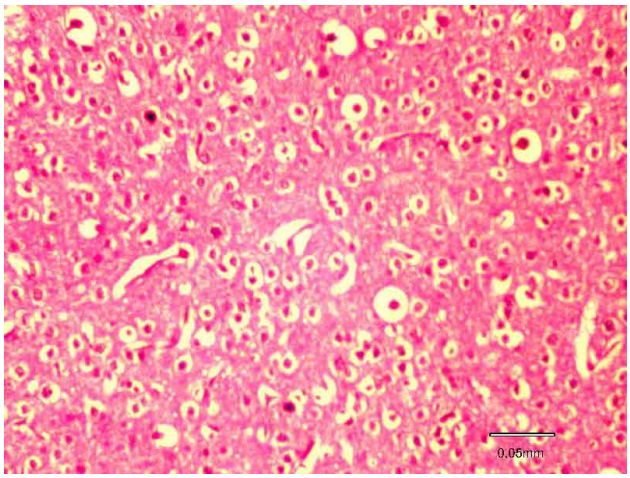

Figure 2. Melatonin preadministered plus RG of cortex. There is less swelling of cortical ganglions, separation in neuronal prolongation and increase in glial cells in MRG.

oxidation. The assay mixture contained 0.9 ml of 16 mmol/l (pH 9.2) phosphate buffer including 0.890 mmol/l TMEDA (N,N,N',N'-tetramethylethylenediamine) and 0.0890 mmol/l EDTA, 0.05 ml of 20.000 g supernatant (or water) and 0.05 ml of 0.3 mmol/l quercetin in a final volume of 1.0 ml. Quercetin was added immediately and after rapid mixing, the decrease in absorbance at 406 nm was followed for 20 min.

CAT activity was measured by the method of Aebi (1987). In this method enzyme catalyzed decomposition of H_2O_2 was measured spectrophotometrically at 240 nm. Plasma CAT activitiy was assayed by incubating with Triton X-100 (10 : 1, v/v) for 20 min at 4°C to increase the observable enzyme level. From this solution, 100 μl was diluted to 10 ml with 50 mmol/l phosphate buffer, pH 7.0. In a 1-ml cuvette, 500 μl of diluted solution was added and the reaction was started by adding 250 μl of freshly prepared 30 mmol/l H_2O_2. The rate of decomposition of H_2O_2 was determined by the absorbance changes at 240 nm. The results are expressed in terms of first order rate constant k *per* milligram protein.

NO, as an unstable molecule, that reacts with oxygen and biological molecules to form several end products (e.g. nitrite, nitrate and S-nitrosothiols) that can be determined by different methods. The most preferred method is based on Griess reaction which can be easily applied in the laboratory. The plasma sample (0.5 ml) was incubated with nitrate reductase (EC 1.6.6.2) from *Aspergillus sp*. (50 mU/100 μl of sample) with NADPH (final concentration = 80 μmol/l) diluted in 20 mmol/l tris buffer (pH 7.6) for 30 min at room temperature for nitrate reduction. The control sample was analyzed daily with an exogeneous standard (sodium nitrate, 50 μmol/l). After the reduction, 5% (w/v) $ZnSO_4$ was added for deproteinization. Then this mixture was centrifuged at 5000 × *g* for 10 min. The supernatant's nitrite levels were measured by the Griess reaction. Tissue nitrite levels were expressed as μmol/mg protein (Green et al. 1982).

Protein content was measured by the procedure of Lowry et al. (1951) with the Folin reagent using bovine serum albumin as a standard.

Statistical analysis

The results were expressed by means ± standard deviation (SD). Mann–Whitney U test was used for statistical analyses. Statistical significance was $p < 0.05$.

Results

Light microscopic examination in cerebral cortical cells of RG showed intense swelling in cortical ganglions, glial cells augmentation and separation in neural prolongations indicated the harmful effects of irradiation on CNS (Figure 1). However, there was less swelling of cortical ganglions, separation in neuronal prolongation and less increase in glial cells in MRG (Figure 2). There were no pathological appearances in MG and CG cortical structures. Additionally, in light microscopy of hypothalamus, prominent endothelial injury in RG (Figure 3), whereas less injury of endothelial layer in MRG were observed (Figure 4). No histopathological data were obtained in MG and CG.

SOD and CAT activities and NO levels of plasma are shown in Table 1 for all groups. Plasma SOD activity

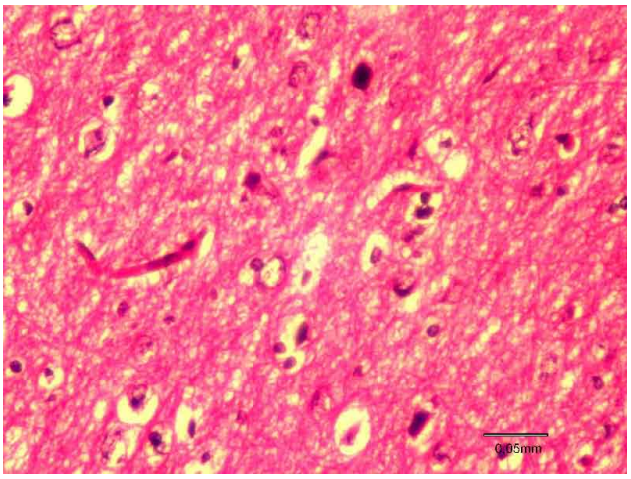

Figure 3. Light microscopy of hypothalamus, prominent endothelial proliferations in RG (RG of endothelial layer).

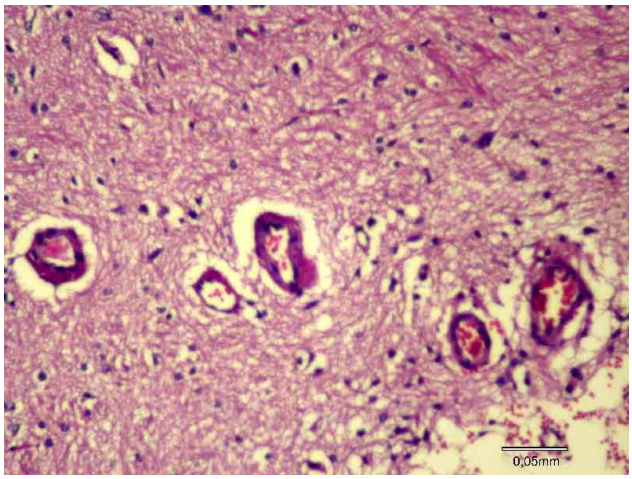

Figure 4. Light microscopy of MRG. Less injury of endothelial layer in MRG were existed (MRG of endothelial layer).

was found to be significantly high in RG compared to CG. Melatonin pretreatment prevented the increase in plasma SOD activity induced by irradiation. Melatonin administration alone caused significant increases in plasma SOD activity in sham-irradiated control rats. But this augmentation was significantly below that of the rats receiving both melatonin and irradiation. Plasma CAT activity was found to be significantly low in RG compared to CG. Melatonin pretreatment prevented the decrease in plasma CAT activity induced by irradiation. Melatonin administration also prevent more increase in plasma NO levels of irradiated rats. Irradiation resulted in significant increases in plasma NO levels. On the contrary, melatonin administration significantly decreased plasma NO levels in irradiated rats.

Discussion

Histopathological data demonstrated that the post-irradiation damage of the CNS, first ultrastructural changes related to the damage at the level of organelles developed within minutes, followed by the appearance of apoptotic cells and necrotic fields in the following days (Mitsuhashi et al. 1998; Hall 2000; Somosy 2000). Kubato et al. (2000) stated that within minutes following ionizing irradiation, neuronal damage started. The damage that starts after the administration of radiation increases in severity within a week or two reflected by an increase in the numbers of apoptotic and necrotic cells and reaches its maximum levels within four weeks. Radiation injury is not limited to the neurons of the CNS, glial cells and the cells of the vascular bed are also affected (Sims et al. 1985; Chiang et al. 1993; Pellmar and Lepiniski 1993). With the damage of astrocytes and vascular endothelial cells, the integrity of the blood-brain barrier is lost, thereby increasing microvascular permeability and resulting in interstitial edema. The disappearance of the blood brain barrier following irradiation seems to be an important pathophysiological mechanism in the evolution of the CNS damage (Schultheiss et al. 1995; Mitsuhashi et al. 1998; Belka et al. 2001).

In our study, light microscopic examinations demonstrated the presence of damage in the neuronal and vascular structures of the hypothalamus in the rats of RG (Figure 3). Additionally, light microscopic examinations showed similar pathologic features in hypothalamus of RG (Figure 3). In the MRG receiving radiation together with melatonin, the radiation injury of the CNS neurons and endothelial cells was very mild (Figure 2 and 4).

Today, several factors are claimed to be stimulating the development of radiation damage. These factors are the stimulation of inflammatory response, increase in the production of free radicals and decrease in the degree of effectiveness of the free radicals scavengers. Oxidative damage seems to be a very important etiological factor in radiation injury as well. Exposure of CNS to ionizing radiation results in abrupt increases in the levels of free radicals (Rabin 1996; Lenton and Greenstock 1999). Free radicals disrupt the chemical structures of the DNA, lipids and proteins in the cells resulting in biological damage of various degrees and cellular death following the advancement of this damage (Anderson 1996; Fang et al. 2002). NO that has been studied extensively in recent years is reported to have a significant role in the development of CNS damage. It has been demonstrated that similar to the effects of oxygen radicals, NO might induce necrosis and

Table 1. Plasma superoxide dismutase (SOD) (U·mg^{-1} protein), catalase (CAT) (k·mg^{-1} protein) and nitrite levels (μmol·mg^{-1} protein) in control group (CG), melatonin applied group (MG), irradiated group (RG), irradiated plus melatonin applied group (MRG)

	SOD activity	CAT activity	nitrite level
CG	96.8 ± 25.5 (n = 10)	5.32 ± 0.80 (n = 8)	6.05 ± 1.98† (n = 10)
MG	147.2 ± 20.2*† (n = 10)	5.03 ± 0.18 (n = 7)	32.81 ± 4.3*† (n = 10)
RG	479.0 ± 162.9*† (n = 10)	3.77 ± 1.43*† (n = 7)	42.3 ± 11.62*† (n = 10)
MRG	117.1 ± 54.9 (n = 10)	5.81 ± 1.46 (n = 8)	10.95 ± 3.44* (n = 10)

Results are expressed as means ± SD (2 to 4 independent experiments). p values are shown as * $p < 0.05$ *versus* respective CG; † $p < 0.05$ *versus* MRG. Other details are given in Materials and Methods.

apoptosis in the neurons and result in cell death as well (Lautenschlager et al. 2000).

Following the radiotherapy, in red blood cell, malondialdeyde level was increased and in contrast antioxidant enzymes such as SOD and CAT were decreased.

Irradiation-induced oxidant stress plays a pivotal role in the inactivation of antioxidant enzymes by free radical attack (Sabitha and Shyamaladevi 1999). In addition, antioxidant defense mechanisms of the CNS are comparatively weak. The brain contains low levels of glutathione and vitamin E and low activity of glutathione peroxidase (GPx) (Gorman et al. 1996). The brain also contains only low to moderate activities of SOD, CAT and GPx (Dringen 2000). Combined with this, the relatively high oxygen consumption required by the brain makes neurons particularly vulnerable to oxidative damage. All of these influences render the CNS sensitive to oxidative damage and to damage induced by ionizing radiation. Several studies indicated that the administration of antioxidant treatments prevented radiation injury (De Laurenzi et al. 1995; Pocernich et al. 2000). Reiter et al. (2002) demonstrated that melatonin could strongly inhibit the severe inflammatory response in rats. Melatonin inhibited NO synthase and decreased the production of NO from L-arginine, thereby decreasing the production of peroxynitrite (Lautenschlager et al. 2000). This effect of melatonin is realized in a dose-dependent manner (Reiter et al. 2002). Besides its direct scavenging actions, melatonin stimulates several anti-oxidative enzyme activities including SOD, GPx and glutathione reductase (Reiter 1998; Reiter et al. 2002).

The microscopic findings in this study demonstrated that whole body radiation results in damage of CNS and that melatonin can partially reduce such damage. With the administration of melatonin, significant increases in the level of NO and activity of SOD caused by radiation could be prevented, as well (Table 1). Observed data makes us think that the increases in the levels of plasma NO could be increasing radiation-induced damage. The source of increase in the plasma NO levels is unclear. However, it is thought that the changes in NO levels proceed from tissues affected from ionizing irradiation. One of the source can be cerebrum. The increases in the levels of SOD might be related to the leak of this intracellular enzyme to the extracellular environment because of cellular injury. The increase in plasma SOD activities can also be source from tissues damaged from irradiation. More definitive data would have been reached by means of the planing study interested in local cranial irradiation. Having low levels of another antioxidant enzyme, CAT, in the group that received radiation contradicts with the increases in the levels of SOD. The administration of melatonin might prevent the decrease in the levels of CAT that is observed after radiation. One of the reasons behind this contradiction might be the functioning of GPx in addition to CAT in the detoxification of H_2O_2. Another reason might be having two distinct types of SOD, mitochondrial and cytoplasmic, whereas CAT only has a single type. However, we certainly need more comprehensive data in order to explain this conflict. Irradiation may reveal remarkable cytostatic and cytotoxic effects in which assumably oxidative stress does participate. Antioxidant may diminish the oxidative injury and minimize the damage to healthy tissues. On the other hand, the protective effect of antioxidants from the radiation injury can decrease the cytotoxic effects of irradiation on tumor tissue. For this resason, the appearing of the effects of antioxidants on the course of the therapy of cerebral tumors needs additional researchs.

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