Preserving effects of melatonin on the levels of glutathione and malondialdehyde in rats exposed to irradiation

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Abstract. In this study we investigated whether pretreatment with melatonin was protective against the injury of the central nervous system (CNS) in rats receiving LD_{50} whole body irradiation. The wistar rats were randomized into four groups: i) the control group (CG), ii) melatonin-administered group (MG; 1 mg/kg body weight), iii) irradiated group (RG; 6.75 Gy, one dose), and iv) melatonin-administered and irradiated group (MRG). Blood samples were drawn from the rats 24 h after the treatment and plasma glutathione levels were assayed. Plasma glutathione level was significantly higher in RG than CG. The melatonin pretreatment prevented GSH increase induced by irradiation. Lipid peroxidation and glutathione levels of rat cerebral cortex were determined in all groups after 24 h. Cortical malondialdehyde (MDA) was significantly higher in the RG. The melatonin pretreatment prevented cortical MDA increase induced by irradiation. Cortical GSH was significantly lower in RG than the CG. The melatonin pretreatment prevented cortical GSH decrease induced by irradiation. Tissue samples were obtained from cerebral cortex and hypothalamus which also were affected by ionizing irradiation in the CNS and were evaluated with electron microscopy. Histopathological findings showed that LD_{50} whole body irradiation resulted in damage of the neuronal cells of CNS. The results obtained from this study demonstrated that pretreatment with melatonin prevented the damage that develops in CNS following irradiation. The beneficial effect of melatonin can be related to protection of the CNS from oxidative injury and preventing the decrease in the level of cortical glutathione.

Key words: Melatonin — Irradiation — Glutathione — Oxidative stress — Rat

Introduction

Ionizing radiation is frequently utilized in the treatment of cancer. On the other hand radiotherapy can induce significant health problems due to its side effects. Until recently, several studies indicated that damage of the central nervous system (CNS) resulted from very high doses of irradiation. However, during the last two decades it was demonstrated that even very small doses of radiation can adversely affect the CNS (Koropatnick et al. 1989). In a human study, it was reported that a dose of 1 Gy resulted in fatigue and loss of strength, while a dose of 5 Gy resulted in inadequate orientation. In an experimental study, doses lower than 5 Gy were shown to decrease the learning abilities of mice (Koropatnick et al. 1989). For the purpose of reducing the radiation-induced CNS damage and protecting the healthy regions of the CNS from the unfavorable effects of irradiation, several alternatives are being investigated. Oxidative damage is the main cause of radiation-induced neuronal injury and antioxidants have been used in experimental studies with the aim of protection from harmful effects of

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radiation. Partial or significant protective effects have been observed (Shiraishi et al. 1986; Matsubara 1988; Deng et al. 1999; Gordon 2000; Moore et al. 2002). Ionizing radiation exerts toxic effects both on neuroglial and vascular structures (Pellmar and Lipinski 1993; Schultheiss et al. 1995). After brain irradiation, acute inflammatory response was determined within one week (Fike et al. 1995). Inflammatory response has been shown to play a major role in the pathogenesis of neuronal injury. Radiation-induced endothelial damage can breakdown the blood brain barrier and the genesis of interstitial edema by increasing microvessel permeability (Mildenberger et al. 1990; Chiang et al. 1993; Mitsuhashi et al. 1998; Belka et al. 2001). Enhanced vascular permeability also plays an important role in the progression of inflammatory response. 1–2 weeks following the radiation, coagulation necrosis has been shown to develop in the irradiated sites of the brain. Necrotic areas progressively enlarge, reaching their maximum size 4 weeks after irradiation (Fike 1995). Diffuse white matter injury can be detected in the late phase of irradiation (Sims et al. 1985). Degenerative changes in some axis cylinders (e.g. swollen axons) have been shown 4 weeks after irradiation (Fike 1995). Neuronal death also stimulates the migration of non-neuronal cells such as microglial cells and astrocytes towards the lesions and the proliferation of these cells (gliosis) (Belka et al. 2001). Proliferated astrocytes and microglias play a role in clarifying and repairing injured regions of brain (Stollg and Jander 1999).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a hormone secreted primarily by the pineal gland situated in the brain. In recent years, potent antioxidant effects of melatonin have been mentioned in several studies (Mahal et al. 1999; Reiter et al. 2002; Vijayalaxmi et al. 2004). It reduces oxidative damage and has an anti-aging effect. The increases in the life span have been explained by the oxidative damage reducing and aging retarding effects of melatonin (Reiter et al. 2002). Oxygen radical toxicity can play a pivotal role in the etiopathogenesis of several pathologies that cause neuronal loss. Therefore, administration of melatonin may prevent and/or decrease the neuronal damage in toxic and/or degenerative conditions of the CNS. In this study, we investigated the preventative effect of melatonin on irradiation-induced cerebral damage and evaluated whether the changes in the level of glutathione could play a role in this possible effect.

Materials and Methods

Animals

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

Group design

Forty two Wistar rats were randomly divided in four groups and in each group: sham exposed controls (CG, $n = 10$), melatonin-administered group (MG, $n = 10$), whole-body irradiated group (RG, LD_{50}: 6.75 Gy, $n = 11$), melatonin-administered and irradiated group (MRG, $n = 11$).

Experimental procedure

All of the rats were anesthetized with 15 mg/kg intramuscular ketamine. 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. The melatonin (10 mg) (Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol at the beginning, and then diluted the same amount of saline. An equal volume of melatonin, disolved in ethanol, was added to saline as indicated in Koc et al. (2003). The melatonin was applied in the rats of the MG and MRG (1 mg/kg body weight) before sham exposure. The rats in RG were irradiated from postero-anterior and antero-posterior paralel fields using Co-60 treatment machine, Theratron 780C (Theratronics, Canada) by SAD: 80 cm technique. Whole body irradiation was administered to each rat seperately. The total body dose including the head was 6.75 Gy. Since 50% lethal dose for the rats is 6.75 Gy, this dose was administered in our study (Hall 2000). In the rats of RG were administered equal amounts of saline and ethanol mixture (1 ml/kg) before irradiation (6.75 Gy, one dose). Melatonin was administered to the rats in MRG 30 min before irradiation. At 24 h following exposure, all animals were sacrificed. Before sacrification, all animals were anesthetized by intramuscular ketamine injection (Yarom et al. 1993). At the end of the experiment skulls were opened, cerebral cortex and hypothalamus was dissected immediately. Tissue samples were used for biochemical analysis. There were no mortality in irradiated rats since animals were sacrified during the early period following applications.

Transmission electron microscopy

One animal of RG and one animal of MRG were used for transmission electron microscopy (TEM) examination. The materials of two rats used for TEM were obtained after transcardiac perfusion of the rats with phosphate buffered 2.5% gluteraldehyde / 2% paraformaldehyde solution. Hypothalamic samples were post fixed with phosphate buffered 2% osmium tetroxide for 1 h. After buffering they were treated with increasing degrees of alcohol. Later, the tissues

were blocked with araldite CY212 and 1–2 μ thick semi-thin sections were obtained by LKB NOVA ultra-microtom and stained with toluidine blue. Then the microtom was used to obtain 60–90 nm thick thin sections which were contrasted with uranyl acetate and lead citrate and pictured with JEOL JEM 1200 electron microscope.

Blood obtaining procedure

Blood samples (5 ml from each animal) were obtained from intracardiac puncture into the heparinized tubes following anesthesia. 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 total glutathione levels.

The measurement of brain malondialdehyde and glutathione levels

The concentration of malondialdehyde (MDA) was measured as an index of lipid peroxidation by the method of Uchiyama and Mihara (1978) based on the reaction with thiobarbituric acid and were expressed as nmol/g wet tissue. Brain samples were homogenized in 1 : 10 (w : v) potassium phosphate buffer (50 mmol/l, pH 7.4) by use of a teflon headed homogenizer. Homogenate (0.5 ml) was mixed with 3 ml of 1% phosphoric acid and 1 ml of 0.67% TBA (thiobarbituric acid) was added. Tubes were placed into boiling water for 45 min. After cooling the tubes, TBARS (thiobarbituric acid-reactive substance) were extracted into n-butanol and absorbance was read at 532 nm ($\varepsilon = 1.56 \times 10^5$ mol·l^{-1}·cm^{-1}).

Reduced glutathione (GSH) was measured through total sulfhydryl groups using Ellman´s reagent (DTNB; 5,5'-dithio-bis(2-nitrobenzoic acid)). Tissue homogenate was deproteinezed and neutralized by using 0.7 mol/l K_3PO_4. The resulting precipitate was removed by centrifugation and the supernatant was used for GSH determination as described Tietz (1969). GSH levels were calculated as millimole *per* gram wet tissue, millimole *per* milliliter for serum samples.

Statistical analysis

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

Table 1. Plasma reduced glutathione (GSH) levels (mmol/ml) of control group (CG), melatonin-administered group (MG), irradiated group (RG) and melatonin-administered and irradiated group (MRG)

	GSH levels
CG ($n = 10$)	4.50 ± 1.49
MG ($n = 10$)	4.32 ± 1.13
RG ($n = 10$)	12.41 ± 4.80*, **
MRG ($n = 10$)	5.77 ± 2.92

MG and MRG rats received 10 mg/kg melatonin. Rats in the third and fourth groups were exposed to 6.75 Gy gamma irradiation. Values are means ± SD of ten rats. Significance of difference was assessed by Mann–Whitney U test; p values are shown as * $p < 0.05$ *vs.* CG; ** $p < 0.05$ *vs.* melatonin-treated groups (this text is valid also for Tab. 2).

Table 2. Cortical malondialdehyde (MDA) and reduced glutathione (GSH) levels of control group (CG), melatonin-administered group (MG), irradiated group (RG) and melatonin-administered and irradiated group (MRG)

Cortex	MDA (nmol/g wet weight)	GSH (mmol/g wet weight)
CG	94.94 ± 21.94	1.65 ± 0.16
MG	99.96 ± 18.04	1.78 ± 0.06*
RG	112.76 ± 23.90*, **	1.28 ± 0.34*, **
MRG	95.14 ± 22.61	1.58 ± 0.33

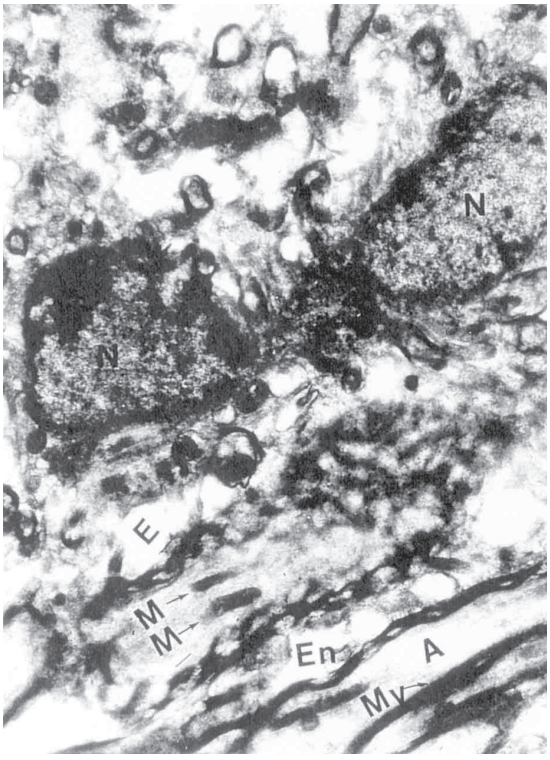

Figure 1. Electron micrograph of the hypothalamic samples of the irradiated group (RG). A, axon; E, edema; En, endothelium; M, mitochondria; My, myelin sheath; N, nucleus.

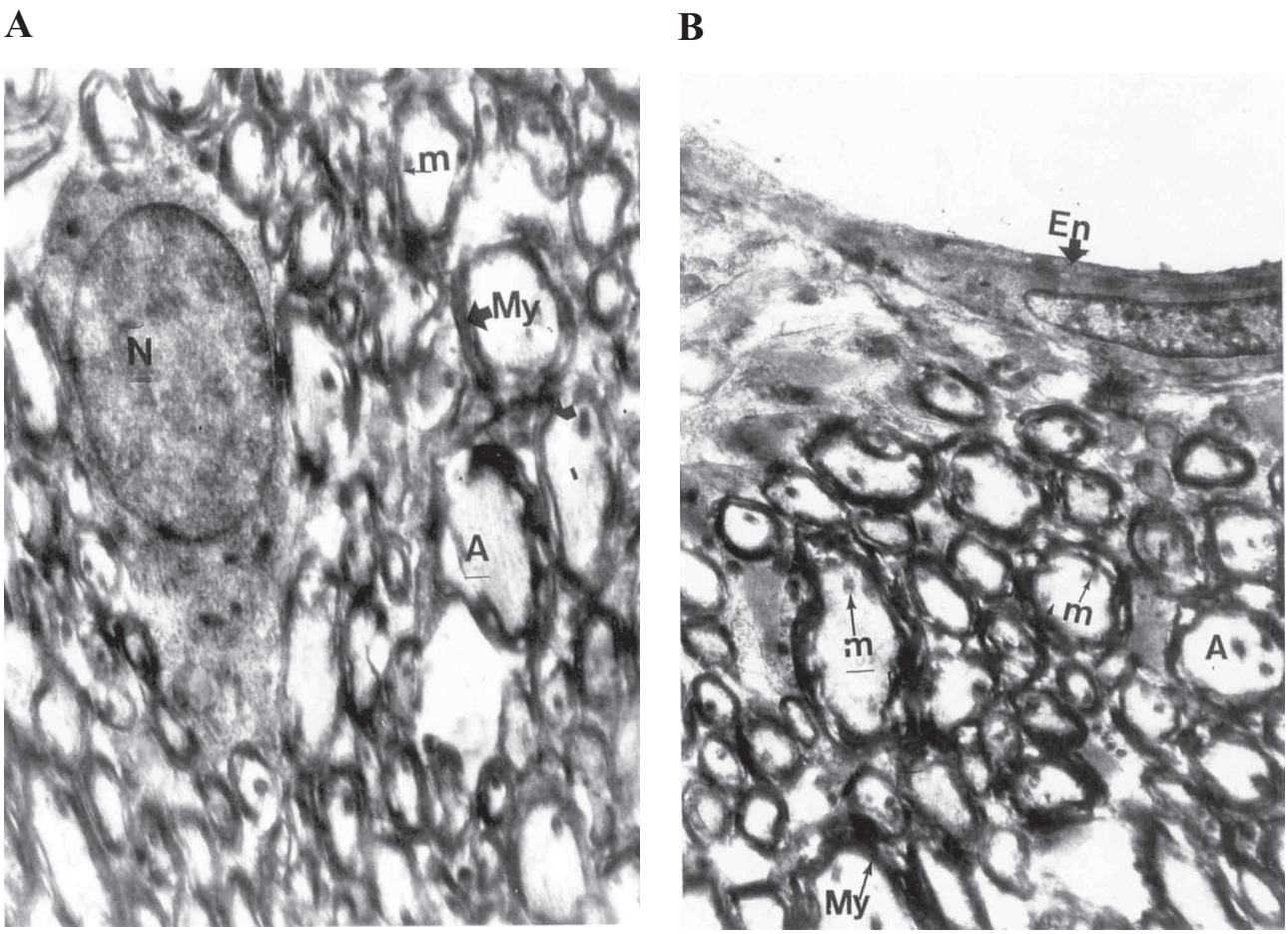

Figure 2 (A, B). Electron micrographs of the hypothalamic samples of the melatonin-administered and irradiated group (MRG). A, axon; En, endothelium; m, mitochondria; My, myelin sheath; N, nucleus.

Results

Plasma glutathione levels of the CG, MG, RG and MRG results are shown in Table 1. It can be seen that the plasma glutathione level was significantly higher in the irradiated group than the control group. The melatonin pretreatment prevented GSH increase induced by irradiation.

Cortical MDA and GSH levels in the CG, MG, RG and MRG results are shown in Table 2. Cortical MDA was significantly higher in the irradiated group than the control group. The melatonin pretreatment prevented cortical MDA increase induced by irradiation. Table 2 also shows that cortical GSH was statistically lower in the irradiated group than in the controls. The melatonin pretreatment prevented cortical GSH decrease induced by irradiation.

In electron microscopic examination of the hypothalamic samples of the RG, destruction of the white matter was observed. There were edematous spaces in the cytoplasm and between myelinated axons. The myelin sheath lamella was separated and axonal injury was prominent. The mitochondria were scarce and swollen. Some glial nuclear destruction was also present (Figure 1).

In the samples of MRG, axonal myelin sheaths, axonal structures, glial organelles were intact. There were numerous mitochondria with normal ultrastructure. The nuclear injury was somewhat, and the axonal injury was greatly recovered compared to the hypothalamic samples of RG (Figure 2).

Discussion

High oxygen consumption which is the indicator for high metabolic activity in the CNS results in production of free oxygen radicals in generous amounts. High production of free radicals renders CNS neurons sensitive to oxidative damage and increases the importance of antioxidant systems for CNS which detoxify oxygen radicals. Neuronal membranes are

rich in lipids, especially unsaturated fatty acids, and are very sensitive to peroxidative damage mediated by free radicals (Dringen 2000). It is currently accepted that increases in the generation of free radicals play an effective role in both the etiopathogenesis and the progression of several neurological diseases that are characterized with functional and/or cellular loss in neurons (De Laurenzi et al. 1995; Anderson 1996; Gorman et al. 1996; Pocernich et al. 2000).

Ionizing irradiation results in the generation of excessive amounts of free radicals in the CNS (Siegal et al. 1996; Lenton and Greenstock 1999; Rabin et al. 2005). MDA levels significantly increase following radiotherapy. This proves that radiation leads to oxidative injury in neurons and especially in their lipid rich membranes. The abundantly generated free radicals not only affect lipids, but also destruct the chemical structures of other cellular biomolecules such as DNA, proteins and enzymes (Rejholcova and Wilhelm 1989; Somosy 2000). Additionally, the decreases in the activities of antioxidant enzymes following radiotherapy contribute to the progression of oxidative injury induced by radiation (Sabitha and Shyamaladevi 1999).

The tripeptide glutathione is a thiol containing compound present as a physiological antioxidant in the defense against free radical toxicity (Dringen 2000). In addition to the enzymic scavenging of H_2O_2, GSH is effective in non-enzymic detoxification of $O_2^{\bullet -}$, $NO^{\bullet}$ and $^{\bullet}OH$. Thus it has an indispensable role in antioxidant efficiency. Glutathione consumption that occurs during the scavenging of oxygen radicals decreases the levels of cellular glutathione. For this reason, low levels of neuronal glutathione should be accepted as an early indicator for oxidative stress. While the levels of intracellular glutathione decrease due to oxidative damage, increase in the level of extracellular glutathione have been documented. Glutathione which crosses to the extracellular environment due to the damage of cellular membranes seems to be mainly responsible for the increase in the level of extracellular glutathione. It has been put forward that the increases in the concentration of GSH under neurotoxic circumstances should be accepted as an indicator for neuronal injury (Dringen 2000). In this study, plasma levels of glutathione were found to be higher in the group receiving radiation when compared to the control group and the administration of melatonin resulted in attenuation in the levels of glutathione. GSH levels of the cerebral cortex were lower in the irradiated group when compared to the control group and this decrease was also reversed with melatonin. These two findings are in correlation with each other indicating that consumption of cerebral glutathione increased due to radiation induced cellular injury. The glutathione enhancement might occur by glutathione transfer from the intracellular environment to the extracellular fluid. On the other hand, in the group treated with melatonin, cortical glutathione levels were replenished and plasma levels were unaffected as well. This demonstrated that melatonin was successful in preserving the glutathione levels in CNS.

In this study, the levels of MDA after irradiation in the cerebral cortex were found to be higher when compared to the control group and this increase was partly prevented with pretreatment of melatonin. Cortical MDA levels of the rats pretreated with melatonin were relatively less affected; it is suggested that oxidative injury induced by radiation in CNS could partly be prevented with pretreatment of melatonin. When the changes in cortical and plasma levels of glutathione and the changes in the cortical levels of MDA are evaluated together, it is understood that melatonin is protective against the oxidative damage induced by irradiation. This hypothesis is further validated when the biochemical data indicating the protective effects of melatonin from radiation-induced oxidative injury is supported with histopathological findings.

Electron microscopic examinations showed that neuronal injury in CNS has been started within minutes after the application of radiation. In several studies, following exposure to radiation neuronal injury develops in very short term in CNS and results in neuronal death in middle and long term (Kubato et al. 2000). Neuronal injury criteria such as swelling and fragmentation of the nuclei, some kind of DNA lesions such as cross-links and strand breaks in chromosomes, dissolution of nuclear laminae, disruption of mitochondrial membranes, swelling and disappearance of the mitochondria cristae, vacuolization and fragmentation of golgi complex, dilatation of the endoplasmic reticulum, rupture of lysosomal membranes, disorganization of gap junctions and cytoskeletal system are observed following irradiation (Belka et al. 2001).

In our study, the electron microscopic examination showed that a damage affecting neuronal cells has developed in the hypothalamic areas of the rats exposed to radiation. The biochemical and histopathological findings obtained from this study indicate the protective effects of melatonin pretreatment from radiation injury of CNS partially although not completely at this dose. Further studies are needed to improve the effect of melatonin on the treatment of radiation injury.

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Final version accepted: November 19, 2007