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Melatonin attenuates radiation-induced learning deficit and brain oxidative stress in mice

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Abstract. Oxidative stress has been implicated in cognitive impairment in both experimental animals and humans. This implication has led to the notion that antioxidant defence mechanisms in the brain are not sufficient to prevent oxidative damage, and that dietary intake of a variety of antioxidants might be beneficial for preserving brain function. The present study, therefore, aimed to investigate the protective effect of melatonin against radiation-induced impairment in the learning ability of mice. Twenty days oral administration of melatonin (0.1 mg/kg b.w.), followed by an acute exposure to ϒ-radiation (6 Gy), inhibited the radiation-induced decline in learning ability. Biochemical estimation of brain protein carbonyls, malondialdehide (MDA) and reduced glutathione (GSH) in these mice indicated that radiation-induced augmentation of protein oxidation and lipid peroxidation had been significantly ameliorated in melatonin treated, irradiated mice. Radiation-induced deficit of glutathione was also normalized by melatonin administration, as there was no statistical difference from normal at $P<0.001$. Results indicate the antioxidative as well as neuroprotective properties of melatonin against the radiation. These findings support results showing melatonin as a free radical scavenger.

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Key words: radiation, melatonin, learning, oxidative stress, antioxidative

INTRODUCTION

Radiation induced oxidative stress has been implicated in cognitive impairment in both experimental animals and aged humans. Exposing animals to radiation produces changes similar to those seen in aged animals (Joseph et al. 1998). The aging mouse brain becomes highly enriched with polyunsaturated fatty acids that render it susceptible to oxidative damage by free radicals. Brain is highly susceptible to the oxidative damage due to its high utilization of oxygen and rather poorly developed antioxidative defense.

Exposure to ionizing radiation greatly augments formation of the free radicals, which may disrupt functioning of the dopaminergic system and behaviors mediated by this system, such as motor performance and amphetamine-induced conditioned taste aversion. Radiation induced oxidative stress is known to play a major role in the destruction of the dopaminergic neurons. It increases concentration of the Fe^{+3} ions in the substantia nigra, and since hydrogen peroxide is produced during dopamine metabolism in the dopaminergic neurons, there is the possibility of Fe^{+3} catalyzed production of hydroxyl radicals that may induce significant damage to these neurons. Hydroxyl radical production is also increased in the mitochondrial respiratory chain dysfunctions, as has been found in diverse tissues of Parkinson's patients (Pollack and Leeuwenburgh 1999).

This implication has led to the notion that antioxidant defense mechanisms in the brain are not sufficient to prevent abnormally high levels of free radicals present during the oxidative stress. Therefore, the dietary intake of a variety of antioxidants might be beneficial for preserving neuronal function.

Melatonin, the major secretory product of the pineal gland, has been shown to participate in a number of physiological processes, such as regulation of reproduction (Reiter 1998), sleep (Waldhauzer et al. 1998), mood and behavior (Zhdanova et al. 1998) and circadian rhythms (Yu et al. 1993). Many studies show that melatonin inhibits tumor growth both *in vivo* and *in vitro* (Anisimov et al. 1997, Blask 1993). As age advances, the nocturnal production of melatonin decreases in various animal species and humans (Reiter et al. 2001, Waldhauzer et al. 1998).

Melatonin is also now known to be an antioxidant. It detoxifies a variety of free radicals and reactive oxygen intermediates, including the hydroxyl radical, peroxynitrite anion, singlet oxygen and nitric oxide (Tan et al. 1993, 2000). Melatonin crosses all morphophysiological barriers, e.g., the blood-brain barrier, placenta, and distributes throughout the cell; these features increase the efficacy of melatonin as an antioxidant (Reiter 1995). The interest in melatonin, both *in vitro* and *in vivo*, was significantly increased after the discovery of the antioxidant potential of the molecule (Allegra et al. 2003, Reiter et al. 2001, Tan et al. 1993). Since melatonin has demonstrated excellent antioxidative properties, the possibility of its neuroprotective action cannot be ruled out.

In our previous studies we reported that melatonin afforded significant protection against aging, cyclophosphamide and radiation-induced oxidative stress in mice, measured in the terms of lipid peroxidation and glutathione (Bhatia and Manda 2004, Manda and Bhatia 2003a,b,c). Therefore, in the present study we investigated the protective effect of melatonin against gamma radiation-induced impairment of learning in mice. We wanted to validate the hitherto debated role of melatonin against radiation-induced behavioral changes.

METHODS

Animals

Male Swiss albino mice, 6–8 weeks of age, were selected from an inbred colony. Mice were maintained under controlled conditions of temperature (22 ± 1) and light (12L:12D) and provided standard mouse feed (Procured from Hindustan Lever Ltd. Delhi) and water *ad libitum*. The research was conducted with the approval of the institution's ethical committee for animal use.

Chemicals

Melatonin was purchased from Aristo Pharmaceuticals Ltd, India. Other chemicals were purchased from Sigma, unless indicated otherwise in the text.

Irradiation

The cobalt teletherapy unit (ATC-C9) at the Cancer Treatment Centre, Radiotherapy Department, SMS Medical College and Hospital, Jaipur, India, was used for irradiation. Unanesthetized animals were restrained in well-ventilated wooden boxes and exposed to

whole-body gamma irradiation at a distance (SSD) of 71.3 cm from the source delivering the irradiation at the dose rate of 1.17 Gy/min.

Dose and administration of melatonin

Melatonin was dissolved in 70% ethanol (3 mg/ml) and such stock solution was further diluted with double distilled water to obtain the desired concentration. Each mouse was administered melatonin orally at a daily dose of 0.1 mg/kg body weight for 20 consecutive days. A specially designed needle with a rounded silver head was used for oral administration of the drug. The amount of alcohol was not more than 1 ml per dose. Melatonin was administered every day 10 min prior to the beginning of the dark period (7:00 P.M.). Dose selection was based on our previous study (Bhatia and Manda 2004).

Experimental design

The mice were divided into four groups (10 animals in each group). The first group (vehicle treatment) served as control (normal). The second and third groups were administered melatonin (0.1 mg/kg body weight), orally, for 20 days (once a day). The third group was exposed to an acute dose of gamma radiation (6 Gy/mice) at the dose rate of 1.17 Gy/min) on the 20th day. The fourth group was treated with vehicle and then exposed to radiation on the 20th day. All these animals were initially trained in the Hebb-William's Maze, model D (Brown 2006) for 10 days (along with melatonin treatment). After radiation exposure, learning ability was recorded continuously for 30 days, i.e., from days 21 to 50. In the learning experiment mice deprived of food for 12 hours navigated the path in a closed maze to explore for food. At day 51 (i.e., 30 days after exposure) mice were autopsied after cervical dislocation and the whole brain was removed for biochemical assay.

Assays for lipid peroxidation

The levels of lipid peroxidation were quantified by the thiobarbituric acid-reactive substances (TBARS) assay as previously described (Ohkawa et al. 1979) with minor modifications. The tissue was homogenized in 2.5% SDS containing 6.25 μM deferoxamine and 12.5 μM probucol (to prevent further oxidation). Four hundred microliters of homogenate was added to an aqueous solution consisting of 375 μl of 20% acetic acid solution (pH 3.5) and 225 μl of 1.33% thiobarbituric acid, and the mixture was heated at 95°C for 1 h. Then one ml of a 5:1 1-butanol-pyridine solution was added and TBARS were extracted into the organic layer by centrifugation at 4 000 × g for 10 min. The amounts of TBARS were determined by spectrophotometry at 532 nm and were calculated as ng malondialdehyden (MDA) equivalent per mg of protein according to a standard curve prepared from malonaldehyde bis (dimethyl acetal).

Assays for protein oxidation

Protein carbonyl content, as an index of protein oxidation, was measured by a modification of a described technique (Levine et al 1994). Briefly, sample tissues were homogenized in 50 mM phosphate buffer at pH 7.4 (10% wt vol) and centrifuged at 11 000 × g for 15 min to sediment insoluble materials. The resulting supernatants containing 2–5 mg of soluble proteins were used for reaction with 2,4-dinitrophenylhydrazine (DNPH). For each sample, the supernatants were divided into two equal volumes. Four volumes of 10 mM DNPH in 2 M HCl were added to one of the sample pair, and four volumes of 2 M HCl alone were added to the other one (for reagent blank assay).

Samples were then incubated for 1 h at room temperature in the dark with continuous stirring and were precipitated with an equal volume of 20% trichloroacetic acid (TCA). After 10 min on ice, samples were centrifuged at 3 000 × g for 5 min and supernatants were discarded. Protein pellets were washed in 10% TCA once and in ethanol-ethyl acetate (1:1) three times to remove free DNPH and additional lipid contaminants. Final protein precipitates were dissolved in 6 M guanidine hydrochloride solution. The differences (D) in absorbance between the DNPH-treated and the HCl-treated samples were determined by spectrophotometry at 375 nm, and the amount of carbonyl contents (C) was calculated by using a molar extinction coefficient of e of 22 000^{-1} cm^{-1} Data were expressed as mM carbonyl per mg of soluble extracted protein.

Assays for reduced glutathione (GSH)

GSH was measured as described by Ellman (1959) using 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent. Briefly, the small amount of DTNB (0.01–0.05) was added to a large volume of buffered

(pH 8.0) solution of brain homogenate. The color developed rapidly (2 min), and the absorbance was recorded at 412 nm. The amount of GSH was calculated by using a molar extinction coefficient e of 13 600^{-1} cm^{-1}. Data were expressed as nM of GSH/g tissue.

Statistical analysis

The difference between various groups in the terms of learning ability was analyzed by ANOVA and difference in the level of MDA, protein carbonyls and GSH was estimated by Student's *t*-test.

RESULTS

After exposure to radiation, there was a decline in the learning ability, which was measured in terms of time taken to reach the goal (access interval, AI). AI in the irradiated group continuously increased in comparison to the groups treated only with vehicle or melatonin (Fig. 1). The irradiated group pretreated with melatonin showed a significant ($F_{1,58}$=238.352, P<0.001) protection against the impairment in leaning ability, which was reflected by a constant decline in the AI. However, it is noted that up to the day 7 post irradiation the difference between the two groups was non-significant, with a clear-cut and continuous increase in the learning ability thereafter. After the day 18 post exposure there was no significant difference in the learning pattern between melatonin treated irradiated and normal groups. Therefore, it may be concluded that melatonin treated irradiated mice attained near normal condition after the interval. Melatonin treated (sham irradiated) mice took slightly less time to reach

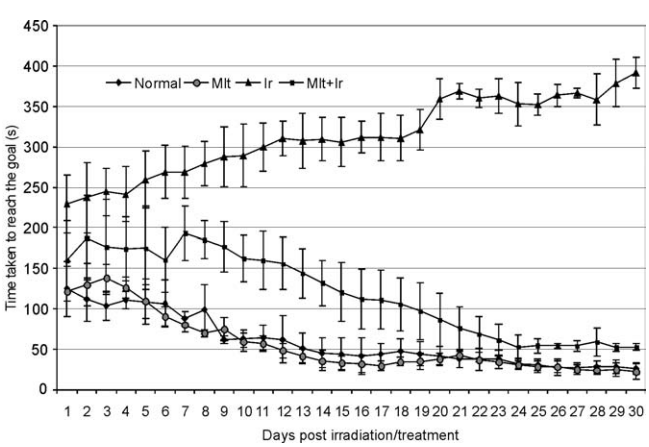

Fig. 1. Variation in the learning ability of mice after gamma irradiation with and without melatonin (Values ± SD)

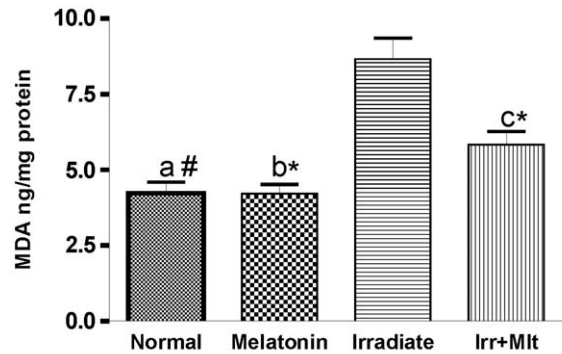

Fig. 2 Difference in the values of MDA content after radiation exposure, with and without melatonin administration. (a) Statistical difference with irradiated; (b) statistical difference with Normal; (c) statistical difference with irradiated. (*) Statistically significant at P<0.001; (#) statistically insignificant.Y error bar indicate SEM.

their goals in comparison with normal mice. However, the difference between these two groups was not significant even at P<0.1 ($F_{1,58}$=0.1341). In addition, the analysis of variance among all the four groups indicated a significant difference ($F_{3,116}$=250.0664, P<0.001).

Level of lipid peroxidation as indicated by MDA equivalents in untreated irradiated mice brain was significantly (P<0.001) higher in comparison to the melatonin pretreated irradiated group (Fig. 2). Similarly, protein carbonyl content was also found significantly (P<0.001) lower in the melatonin pretreated irradiated group in comparison to the untreated irradiated group (Fig. 3). Radiation-induced depletion of GSH level in brain was also ameliorated significantly (P<0.001)

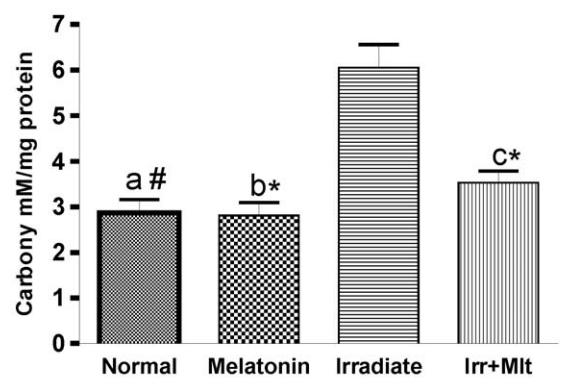

Fig. 3. Difference in the values of protein carbonyl content after radiation exposure, with and without melatonin administration. (a) Statistical difference with irradiated; (b) statistical difference with Normal; (c) statistical difference with irradiated. (*) Statistically significant at P<0.001; (#) statistically insignificant. Y error bar indicate SEM.

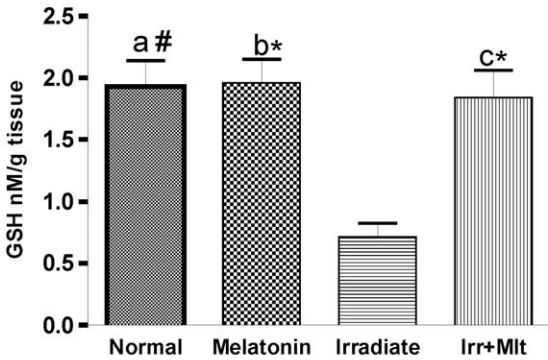

Fig. 4. Difference in the values of GSH content after radiation exposure, with and without melatonin administration. (a) Statistical difference with irradiated; (b) statistical difference with Normal; (c) statistical difference with irradiated. (*) Statistically significant at $P<0.001$; (#) statistically insignificant. Y error bar indicate SEM.

in the melatonin pretreated irradiated group (Fig. 4). Interestingly, level of GSH content in the melatonin pretreated irradiated group attained normal value. No statistically significant difference in the level of lipid peroxidation, protein carbonyl and GSH was observed between the melatonin treated sham irradiated and normal groups.

DISCUSSION

Melatonin has been reported to have antioxidant properties in addition to its known hormonal activities. However, reports on low-level chronic administration are scanty. The present study used low-dose, long term chronic administration of melatonin. Results indicate that pre-administration of melatonin can correct and normalize the radiation-induced impairment in learning ability through the inhibition of oxidative stress in the brain. Radiation-induced oxidative stress has been evaluated by two independent approaches; lipid peroxidation and protein oxidation.

In the present study we found that melatonin markedly inhibited lipid proxidation in the brain. This result agrees with our previous studies (Bhatia and Manda 2004, Manda and Bhatia 2003 a,b,c). Lipid peroxidation is a fundamentally deleterious reaction and is implicated in various types of neurodegenerative disorders. The products of lipid peroxidation such as MDA and 4-hydroxynonenal are toxic to cells (Raleigh 1985). Lipid peroxidation within the membrane has a devastating effect on the functional state of the membrane because it alters membrane fluidity, typically decreasing it and thereby allowing ions such as Ca^{++} to leak into the cell. The peroxyl radical formed from lipid peroxidation attacks membrane protein and enzymes and reinitiates lipid peroxidation. The ameliorating action of melatonin against radiation-induced lipid peroxidation is due to its free-radical scavenging and chain breaking properties (Karbownik and Reiter 2000, Tan et al. 1993, Vijayalaxmi et al. 2004).

Radiation-induced decrease in GSH level was also significantly ameliorated by melatonin pretreatment, which further validates the hypothesis that melatonin may scavenge the free radicals formed during oxidative stress. Previous findings also suggest that depletion of glutathione results in enhanced lipid peroxidation (Manda and Bhatia 2003d). Excessive lipid peroxidation can cause increased glutathione consumption (Comporti 1987) as observed in the present study. GSH, with its sulfhydryl group, functions in the maintenance of sulfhydryl groups of other molecules (especially proteins), as a catalyst for disulfide exchange reactions, and in the detoxification of foreign compounds, hydrogen peroxide and free radicals. When GSH acts as a reducing agent, it's SH becomes oxidized and forms a disulfide link with other molecules of GSH. (Gul et al. 2000).

We also showed marked antioxidant activity of melatonin in protein oxidation. Many studies suggest that oxidative stress is responsible for age associated pathologies such as arteriosclerosis and neurodegenerative disorders (Butterfield and Kanski 2001, Goto et al. 1999, Yan et al. 1996). Oxidation of proteins modifies the side chains of methionine, histidine and tyrosine, forming cysteine disulfide bonds (Stadtman 1993). Metal catalyzed oxidation of proteins introduces carbonyl groups (aldehydes and ketones) in lysine, arginine, proline or threonine residues in a site-specific manner (Stadtman 1993). Many investigators have demonstrated that the hydroxyl radical causes oxidative modification of amino acid residues of proteins and that cross-linking and fragmentation of the proteins results in loss of function and increased susceptibility to proteases (Davies and Delsignore 1987, Rivett 1985, Wolff and Dean 1986). However, there have been very few reports showing inhibitory effects of melatonin on protein oxidation in the brain. The inhibitory effect of melatonin on protein oxidation is not clear, but it may inhibit production of the hydroxyl

radical. Our findings suggest that melatonin may protect against cellular and extracellular protein damage from oxidative stress and therefore permit maintenance of the neurophysiological functions. Results of the current study also corroborate the previous findings suggesting the neuroprotective role of melatonin against lipid peroxidation, protein and DNA damage (Lima et al. 2003, Undeger et al. 2004, Zavodnik et al. 2004).

Our finding that melatonin ameliorates radiation-induced learning deficit in mice corroborates the findings of Gonenc and coauthors (2005), who investigated the protective effect of melatonin against ethanol-induced oxidative stress and spatial memory impairment in the rat. In addition, the role of melatonin against learning and memory deficits in rats induced by chronic exposure to thinner, a neurotoxic mixture that is widely used as an aromatic industrial solvent, has also been evaluated (Baydas et al. 2005).

Liu and others (2003) suggested that decline in learning and memory is associated with a very significant increase in two parameters of oxidative stress in the brain, levels of lipid peroxidation and of protein oxidation. These results support the hypothesis that oxidative stress contributes to radiation-related impairment in learning. The protective effect of melatonin against radiation-induced oxidative stress might be due to free-radical scavenging and the singlet oxygen quenching ability of melatonin. If melatonin is to produce an antioxidative effect, it must be absorbed by the body and available in the tissues exposed to oxidative stress. Melatonin crosses all morphophysiological barriers, which increase the efficacy of melatonin as an antioxidant (Reiter 1995).

Besides being an antioxidant, melatonin is also involved in the regulation of neural cell adhesion molecules (NCAMs). NCAMS are members of the immunoglobulin superfamily and are involved in synaptic rearrangements in the mature brain. There are three major NCAM forms: NCAM 180, NCAM 140 and NCAM 120. It has been proposed that NCAMs mediate synaptic plasticity during learning and memory formation (Baydas et al. 2002).

CONCLUSION

In conclusion, melatonin plausibly exerts its neuroprotective effect on the whole body gamma irradiated mice through inhibiting the radiation induced GSH depletion and ameliorating lipid peroxidation and protein oxidation.

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