Int. J. Radiat. Biol., Vol. 82, No. 6, June 2006, pp. 411 – 419

Taylor & Francis
Taylor & Francis Group

Melatonin prevents X-ray irradiation induced oxidative damage in peripheral blood and spleen of the seasonally breeding rodent, *Funambulus pennanti* during reproductively active phase

SHWETA SHARMA & CHANDANA HALDAR

Pineal Research Laboratory, Department of Zoology, Banaras Hindu University, Varanasi, India

(Received 18 January 2006; revised 20 April 2006; accepted 21 April 2006)

Abstract

Purpose: Biological effects of X-ray irradiation and protection by melatonin on the immune status of a tropical rodent, *Funambulus pennanti*, was examined by estimating oxidative damage of peripheral blood and spleen and protection by melatonin treatment.

Material and methods: Seventy squirrels were divided into 4 sets of 25, 25, 10 and 10 having subdivisions into a total of eight groups. Squirrels of set 1 (groups A and B) received only normal saline, set 2 (groups C and D) received 25 μg/100 g body weight (bwt) melatonin and set 3 (groups E and F) received 25 μg/100 g bwt vitamin E for four weeks. Groups G and H of set 4 received high doses of melatonin (0.5 mg/100 g bwt) 30 min prior to and 30 min after X-ray irradiation, respectively, and were sacrificed 1 h after irradiation. Groups B and D were sacrificed after 4 h, 24 h, 48 h and 72 h of irradiation for total leukocyte count (TLC) in peripheral blood, percent apoptotic cells and lipid peroxidation (LPO) in spleen while group F was sacrificed after 4 h of irradiation to measure LPO.

Results: Pre-melatonin treatment (25 μg/100 g bwt) restored TLC, percent apoptotic cells and LPO levels of X-ray exposed squirrels. High dose pre-melatonin treatment (0.5 mg/100 g bwt) restored the above conditions significantly while post treatment did not. Vitamin E reduced elevated LPO level in irradiated tissue, but the effect of melatonin was more potent.

Conclusion: Melatonin administration prior to X-ray irradiation prevented radiation-induced oxidative damage during the reproductively active phase of the seasonally breeding rodent suggesting a high protective role of melatonin following X-ray irradiation.

Keywords: *X-ray irradiation, melatonin, total leukocyte count, apoptosis, lipid peroxidation, reproductive active phase*

Introduction

The biological effects of ionizing radiation on cells are quite similar to those produced by endogenous stress. Indeed, ionizing radiation normally interacts with materials indirectly, via the formation of radical species. Much evidence has been gathered showing that enhanced free radical levels and an impaired antioxidant pools are important factors underlying the pathophysiological mechanisms in a variety of diseases including neurodegenerative diseases (Mates et al. 1999, Parthasarathy et al. 1999).

The hematopoietic system is known to be radiosensitive and its damage may be critical for survival. The cell membrane has been known for some time to be a secondary target for ionizing radiation. The chain reaction initiated by ionizing radiation leads to the formation of a variety of degradation products in biological membranes including products of lipid breakdown. Radiation induced lipid peroxidation is a free-radical process (Sitasawad & Kale 1994) which involves oxidative conversion of polyunsaturated fatty acids (PUFA) to several products including malondialdehyde (MDA) and lipid peroxides. MDA serves as an index of oxidative damage (Bakan et al. 2002). MDA also promotes tumor growth and act as a co-carcinogenic agent because of its high cytotoxicity and inhibitory actions on protective enzymes (Taysi et al. 2003). Thiobarbituric acid reactive substances (TBARS) are the commonly measured parameter of lipid damage after ionizing radiation exposure (Karbownik & Reiter 2000) leading to cell death via necrosis and apoptosis.

Correspondence: Prof. C. Haldar, Pineal Research Lab., Department of Zoology, Banaras Hindu University, Varanasi - 221 005, India.
E-mail: chaldar@bhu.ac.in, chaldar2001@yahoo.com

ISSN 0955-3002 print/ISSN 1362-3095 online © 2006 Taylor & Francis
DOI: 10.1080/09553000600774105

Some chemicals, acting as free radical scavengers, such as vitamin E, vitamin C, vitamin A, beta-carotene, melatonin, 5-aminosalicylic acid, alpha-tocopherol monoglucoside (alpha-TMG) and flavanoids can provide protection against radiation injury to living cells (Sudheer et al. 2003, Satyamitra et al. 2001, Ganasoundari et al. 1997). Melatonin, chemically known as N-acetyl-5-methoxytryptamine, is a product of the vertebrate pineal gland, as well as other selected organs, and is a potent free radical scavenger (Badr et al. 1999, Tan et al. 2002, Hardeland et al. 1993, Allegra et al. 2003) and an indirect antioxidant (Rodriguez et al. 2004, Reiter & Tan 2003, Mayo et al. 2002, 2003, Tan et al. 1993). Vijayalaxmi et al. (1995, 1995a, 1998) reported that melatonin pre treated human peripheral blood lymphocytes, when exposed to gamma radiation *in vitro,* exhibited a significantly reduced (60–70%) incidence of chromosomal aberrations, micronuclei and primary deoxyribonucleic acid (DNA) damage as compared with irradiated cells not pretreated with melatonin. Koc and his co-workers (2002) reported the ability of melatonin to act as a radical scavenger in peripheral blood cells during total body irradiation in rats. X-rays produced DNA damage and a marked decrease in antioxidants in the bone marrow of mice (Umegaki & Ishikava 1994). A literature search suggests that the effects of X-ray irradiation on the immune system have never been studied in seasonally breeding mammals. However, X-ray irradiation-induced DNA damage has been extensively reported in laboratory animals such as rats and mice. Since, wild, seasonally breeding animals are frequently exposed to many natural forms of radiation that may cause unknown influences at different physiological levels including immune status, it is worthwhile to study the experimental effects of X-ray radiation effect on the immune status of a common tropical rodent *Funambulus pennanti*. Furthermore no report was found showing a protective effect of melatonin on peripheral blood cells in irradiated seasonally breeding rodents. Therefore, the antioxidative efficacy of melatonin during oxidative stress was assessed by comparing melatonin with another antioxidant, vitamin E or alpha-tocopherol. The radioprotective role of vitamin E against X-ray and gamma irradiation has already been established (Sarma & Kesavan 1993, Konapacka et al. 1998).

To clarify the role of melatonin as a radioprotective agent in seasonally breeding rodents, we investigated the effects of X-ray irradiation and exogenous melatonin on the hematopoietic status of peripheral blood and apoptosis and lipid peroxidation in the spleen of *F. pennanti* during its reproductively active phase. During this phase endogenous levels of melatonin are low due to its inverse relation with gonadal steroid. The efficacy of melatonin was compared with vitamin E for effects on LPO.

Materials and methods

All the experiments were conducted in accordance with institutional practice and within the framework of the revised animals (Scientific Procedures) Act of 2002 of the Government of India on Animal Welfare.

Animal care and maintenance

Seventy young adult male squirrels (*Funambulus pennanti*), weighing 110–130 g were obtained locally (Varanasi; Lat. 25°18′N; Long. 83°0′E), as they are easily available in this vicinity. All the squirrels were kept in a ventilated animal room with room temperature maintained at $25 \pm 2^{\circ}$C and photoperiod of 12-h light and 12-h dark cycle with lights on from 06:00–18:00 h. The squirrels were fed soaked gram seed (*Cicer arietinum*) and seasonal nuts and grains along with water *ad libitum*.

Experimental design

All the chemicals used were purchased from Sigma-Aldrich Chemicals, St Louis, Missouri, USA. After acclimatization for one week to laboratory conditions, the squirrels were divided into four sets namely set 1, set 2, set 3 and set 4. Squirrels of set 1, set 2 and set 3 were subdivided into six groups namely A, B, C, D, E and F. Set 1 with 5 squirrels in group A and 20 squirrels in group B were given only normal saline treatment, groups C (5 squirrels) and D (20 squirrels) of set 2 were subcutaneously (abdominal) injected with 25 μg melatonin/100 g body weight and groups E and F of set 3, each having 5 squirrels were injected with 25 μg vitamin E/100 g bwt daily for four weeks at 18:00 h when lights were turned off. Set 4 of 10 squirrels was divided into two groups G and H, each having 5 squirrels. Group G was pretreated with high dose of melatonin, i.e., 0.5 mg/100 g bwt subcutaneously just half an hour prior to X-ray exposure (pre-treatment) while group H was injected with same high dose of melatonin a half an hour after X-ray exposure (post-treatment). Melatonin for injection was prepared freshly every 7 days by dissolving it in trace amounts of absolute ethanol and then diluting it in 0.9% normal saline (NaCl). Alpha-tocopherol (vitamin E) was prepared in 0.9% NaCl and 20% ethanol. All injections were given subcutaneously during evening hours. Animals in groups B, D, F, G and H were anesthetized with 1.75 mg/kg of thiopental sodium injected subcutaneously. Some 55 anesthetized squirrels received X-ray irradiation at a source to

skin (focused on the abdomen, near the splenic region) distance of 10 cm for 60 sec with the absorbed dose at 2.06 Gy. The dental X-ray machine (1070D, Picks, Mumbai, India) was operated at 70 kV potential with 1.5 mm aluminium filter. All radiations were done during morning hours at 11:00 h. Squirrels of control group A, melatonin-treated group C and alpha-tocopherol-treated group E were anesthetized, but were not irradiated. After the exposure, groups B and D were again divided into subgroups of four for different time scheduling of sacrifice at 4 h, 24 h, 48 h and 72 h. Subgroups B1 and D1 sacrificed after 4 h, B2 and D2 sacrificed after 24 h, B3 and D3 sacrificed after 48 h and B4 and D4 sacrificed after 72 h. Pre-melatonin-treated group G and post-melatonin-treated group H were sacrificed 1 h after X-ray exposure.

Parameters assayed

Total leukocyte count. After sacrificing the squirrels, blood was collected in heparinized tubes and processed for total leukocyte count (TLC) in Neubauer's counting chamber (Paul Marienfeld GmBH & Co. KG, Lauda-Königshofen, Germany) under the microscope.

Morphological analysis of apoptotic cells. Observation of splenocytes for apoptosis was performed following Acridine orange (AO) staining. 0.4 ml of acid-detergent solution (0.1% Triton X-100, 0.08 N Hydrochloric acid, 0.15 M NaCl, pH 1.2) was admixed gently with 0.2 ml of the diluted sample (1×10^6 cells/ml in phosphate buffered saline). The samples were stained by addition of 1.2 ml of AO (Sigma-Aldrich Chemicals, St Louis, Missouri, USA) in 0.001 M ethylene diamine tetra-acetic acid (EDTA) on ice for 15 min. A drop of this mixture was placed on a clean slide and covered with a cover glass and observed immediately using a fluorescence microscope with photoautomat (Leitz MP3, Wetzlar, Hesse, Germany) using 440 nm excitation and measuring emission at 520 nm.

Malondialdehyde level estimation. All squirrels were sacrificed by decapitation and spleens were excised and weighed. The tissues were homogenized in 20 mM tris HCl buffer (pH 7.4) and 10% homogenates, tissue/buffer (w/v) were centrifuged for 15 min at 3000 ***g*** at 4°C. The supernatant was collected and then subjected to the thiobarbituric acid (TBA) assay based on the method of Ohkawa et al. (1978). Total thiobarbituric acid reactive substances (TBARS) were expressed as MDA as nmol/mg, taking 1,1,1,1-tetraethoxy propane (TEP) as standard. The standard curve was calibrated using a 10 nM concentration of TEP.

Statistical analysis

Using linear graph paper, the mean MDA for each standard used on the X-axis vs. the corresponding colorimeter reading on the Y-axis was plotted and MDA concentration was determined in samples by interpolation from the standard curve. All the data were expressed as mean $\pm$ SEM. The statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Student Newman-Keuls' Multiple Range tests. The differences were considered significant for both F and p was at 5% ($F/p < 0.05$).

Results

Total leukocyte count in spleen

Radiation significantly lowered the peripheral total leukocyte count as measured 4 h after irradiation. This was seen to a lesser degree in the leukocyte counts in X-ray-exposed squirrels kept under continuous melatonin treatment. The X-ray-exposed squirrels showed a sharp decline in peripheral blood leukocyte count when compared with the control group while the melatonin-treated plus irradiation group had a significantly increased total leukocyte count ($p < 0.01$) compared to the control (Figure 1a). A reduction in the peripheral leukocyte counts at 4 h and 24 h was noted when compared with the control group, while from 48 h to 72 h a drastic recovery (approximately 98%) was observed in the irradiated group when compared with melatonin plus irradiated group (Figure 1b).

Morphological observation of apoptotic splenocytes

X-ray exposure caused an increase of 18% in apoptosis of splenocytes compared with control cells from 40 – 58% 4 h after radiation. In the melatonin (physiological dose) plus irradiation group the percentage apoptosis of splenocytes was significantly lower (20%) compared with the radiation only group from 58 – 38% ($p < 0.01$). The percentage of apoptosis was also significantly lower in the melatonin-treated (physiological dose) no irradiation group compared to the control (Figure 2a). The increase in apoptosis caused by radiation alone decreased to control or sub control levels by 48 h and 72 h, respectively. Melatonin treatment (physiological dose) much reduced the radiation induced increase in apoptosis and kept apoptosis at or below the melatonin alone group levels 24, 48 and 72 h after irradiation (Figure 2b). Melatonin treatment, both physiological treatment and high dose treatment, before radiation reduced radiation induced apoptosis but high dose treatment after irradiation did not (Figure 2c).

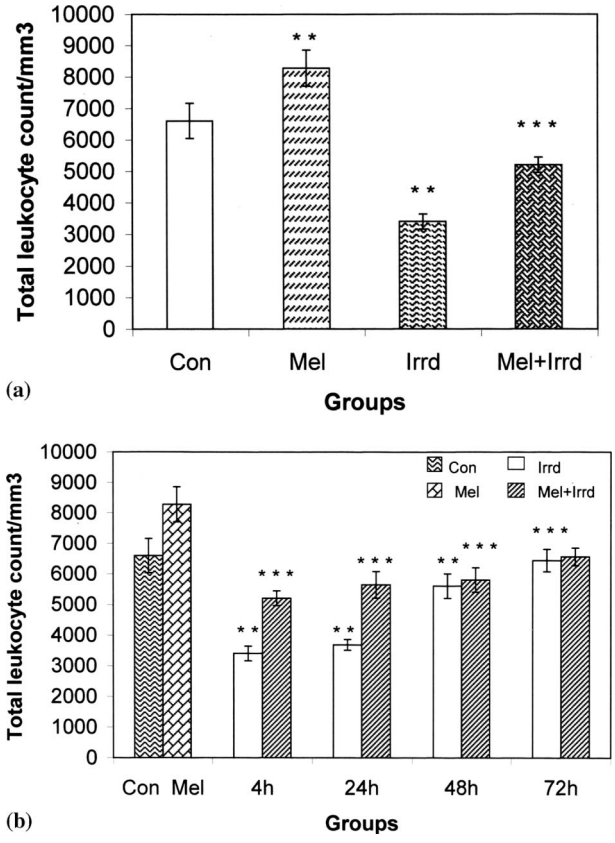

Figure 1. (a) Effect of pre-treatment with a physiological dose of melatonin (25 μg/100 g bwt) on total leukocyte count of the Indian palm squirrel, *Funambulus pennanti* during their reproductively active phase (March to June) after 2.06 Gy of X-ray irradiation. Histograms represent Mean $\pm$ SEM, $n = 5$ for each group. Con, Control; Mel, Melatonin only; Irrd, Irradiation only; Mel + Irrd, Melatonin treatment and irradiation; $^{**}p < 0.01$ when compared with control; $^{***}p < 0.01$ when compared with the irradiated group. (b) Effect of pre-treatment with a physiological dose of melatonin (25 μg/100 g bwt) on total leukocyte count of the Indian palm squirrel, *F. pennanti* during their reproductively active phase (March to June) after 2.06 Gy of X-ray irradiation. Squirrels were sacrificed at different time intervals after irradiation of 4, 24, 48 and 72 h. Histograms represent Mean $\pm$ SEM, $n = 5$ for each group. Con, Control; Mel, Melatonin only; Irrd, Irradiation only; Mel + Irrd, Melatonin treatment and irradiation; $^{**}p < 0.01$ when compared with control; $^{***}p < 0.01$ when compared with melatonin-treated group.

Lipid peroxidation in the spleen

MDA levels were significantly higher in the spleen tissue of the irradiation alone group when compared with the controls 4 h after irradiation ($p < 0.01$) (Figure 3a). Continuous melatonin at a dose of 25 μg/100 g bwt significantly ($p < 0.01$) reduced MDA levels in the spleen of squirrels subjected to X-ray exposure compared to the irradiation alone group. Vitamin E treatment significantly ($p < 0.05$) reduced the MDA level in its irradiated group compared to the radiation alone group but was comparatively higher ($p < 0.01$) than melatonin-treated plus irradiation group (Figure 3a). The higher MDA level noted in the irradiated group at 4 h had declined by 24 h onwards when compared with control group (Figure 3b). Pretreatment with both physiological dose melatonin and high dose melatonin showed significant protection ($p < 0.01$) against radiation induced MDA production but high-dose melatonin treatment post melatonin treatment showed no decrease in MDA level when compared with irradiated group only (Figure 3c).

Discussion

Proliferating cells are highly sensitive to irradiation. Recently, Hussein et al. (2005) have reported the radioprotective role of melatonin against X-ray-induced skin damage in albino rats suggesting a need for the identification and development of non-toxic and effective radioprotective compounds that can reduce the effect of radiation. A single whole body exposure of mammals to ionizing radiation results in a complex set of symptoms whose onset, nature and severity are a function of both total radiation dose and radiation quality.

The results of the present study demonstrate that X-ray irradiation in squirrels causes tissue damage in the spleen, as assessed by increased lipid peroxidation level, percent apoptosis and in peripheral blood as assessed by decreased total leukocyte count, as these cells are highly radiosensitive. Similarly, Koc et al. (2002) confirmed the marked effects of radiation on the stem cells of the leukocytes, lymphocytes and platelets. It has been suggested that the survival after irradiation is really a result of recovery of target tissues. In our study, we examined the effect of melatonin on recovery of peripheral blood cell counts in squirrels that received X-ray irradiation. During the reproductively active phase, exogenous melatonin supplementation can restore blood parameters up to control level in pinealectomized squirrels (Rai & Haldar 2003). We considered that inhibition of free radical generation or intensified scavenging capacity, enhancement of DNA and membrane repair, replenishment of dead hematopoietic and other cells and stimulation of immune cell activity by melatonin are important areas for radioprotection.

Several reports have suggested that melatonin has the ability to act as an immunomodulator (Maestroni 1993). In our present study, melatonin treatment prevented induced lipid peroxidation and apoptosis in a seasonal breeder verifying the protective effect of melatonin against oxidative injury by X-ray irradiation up to 72 h. Ionizing radiation is a potent carcinogen due to resulting oxidative damage and the molecule most often reported to be damaged by this physical agent is DNA (Daly et al. 1999, Toule 1987). The ability of melatonin to enter all cells

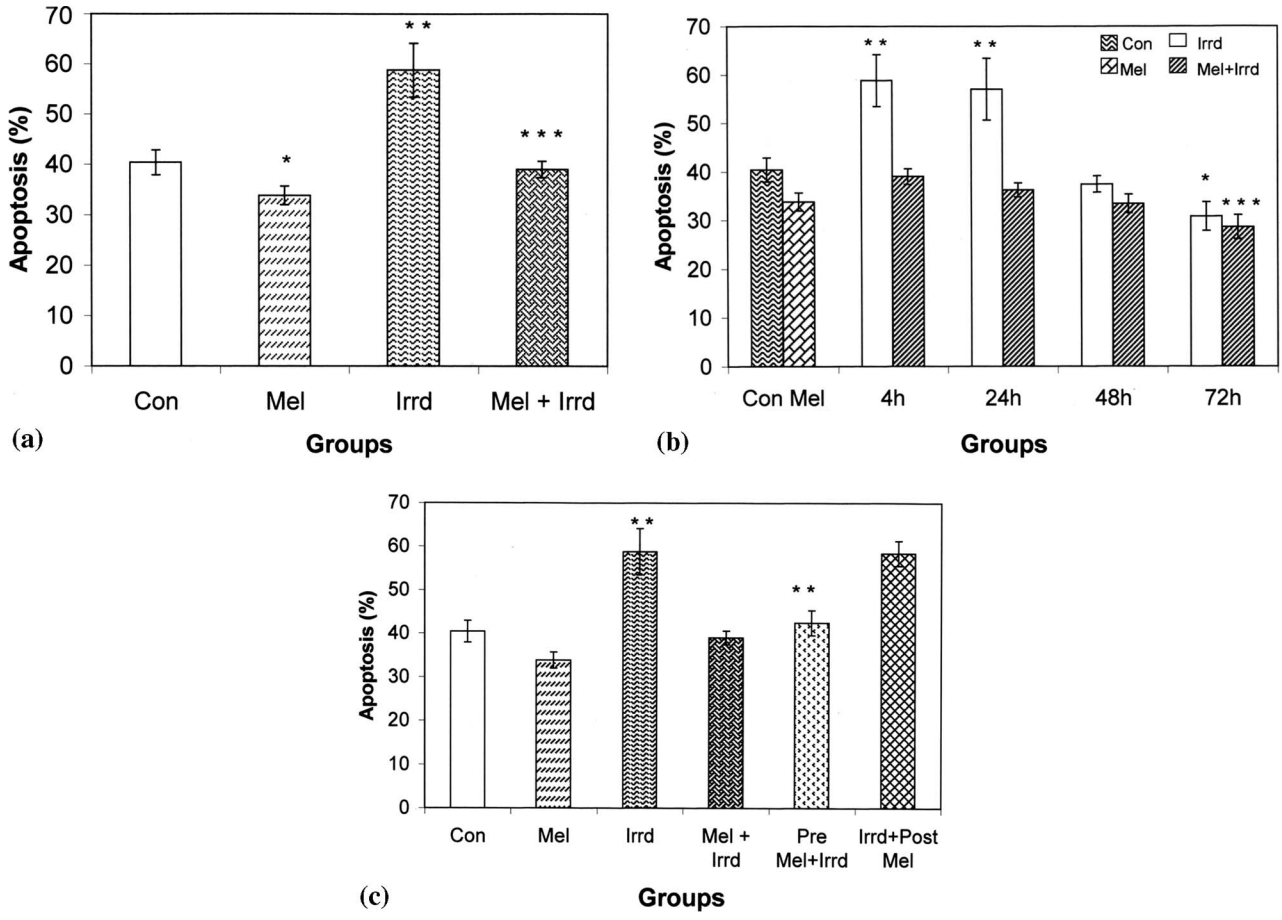

Figure 2. (a) Inhibitory effect of pre-treatment with a physiological dose of melatonin (25 μg/100 g bwt) treatment on apoptosis of splenocytes of the Indian palm squirrel, *Funambulus pennanti* during their reproductively active phase (March to June) after 2.06 Gy of X-ray irradiation. Histograms represent Mean $\pm$ SEM, $n = 5$ for each group. Con, Control; Mel, Melatonin only; Irrd, Irradiation only; Mel + Irrd, Melatonin treatment and irradiation; $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with control, $^{***}p < 0.01$ when compared with irradiated group. (b) Inhibitory effect of pre-treatment with physiological dose of melatonin (25 μg/100 g bwt) on apoptosis of splenocytes of the Indian palm squirrel, *F. pennanti* during their reproductively active phase (March to June) following 2.06 Gy of X-ray irradiation at different time intervals of 4, 24, 48 and 72 h. Histograms represent Mean $\pm$ SEM, $n = 5$ for each group. Con, Control; Mel, Melatonin only; Irrd, Irradiation only; Mel + Irrd, Melatonin treatment and irradiation; $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with control, $^{***}p < 0.01$ when compared with melatonin-treated group. (c) Inhibitory effect of pre treatment with physiological (25 μg/100 g bwt) and pre- and post-treatment with high (0.5 mg/100 g bwt) melatonin doses on apoptosis of splenocytes of the Indian palm squirrel, *F. pennanti*, during their reproductively active phase (March to June) following 2.06 Gy of X-ray irradiation. Histograms represent Mean $\pm$ SEM, $n = 5$ for each group. Con, Control; Mel, 25 μg/100 g bwt melatonin only; Irrd, Irradiation only; Mel + Irrd, 25 μg/100 g bwt melatonin treatment and irradiation; Pre-Mel + Irrd, 0.5 mg/100 g bwt melatonin prior to irradiation; Irrd + Post-Mel, 0.5 mg/100 g bwt melatonin after irradiation; $^{**}p < 0.01$ when compared with control, and $^{***}p < 0.01$ when compared with irradiated group.

in the organism and all cellular compartments relates to its physical and chemical properties. Within cell membranes, melatonin may align itself in a position to scavenge free radicals in both the lipid and aqueous environments (Costa et al. 1997, Shida et al. 1994) of the membrane.

In spite of having an indirect contribution to defence against oxidative stress, the immune system influences the function of other systems such as the reproductive, neuroendocrine, nervous and endocrine systems (Maestroni 1993, Haldar & Singh 2001) and antioxidant status (Rodriguez et al. 1999). The antioxidant effects of melatonin occur by both direct free radical scavenging and indirect antioxidant mechanisms (Reiter et al. 1995). Presence of melatonin membrane and/or nuclear receptors on some immunocompetent cells (Guerrero et al. 1997, Gonzalez-Haba et al. 1995) has suggested the mediating actions of melatonin. The receptor-mediated effects of melatonin can act in at least two ways. (i) They can act through binding to specific receptors MT1, MT2 or MT3 present on plasma membranes which couple to guanosine triphosphate (GTP) binding proteins and regulates several second messengers including cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), diacylglycerol (DAG), inositol trisphosphate (IP_3), arachidonic acid, and intracellular calcium ion (Ca^{2+}) concentration (Vanecek 1998); (ii) They can act on nuclear orphan receptors

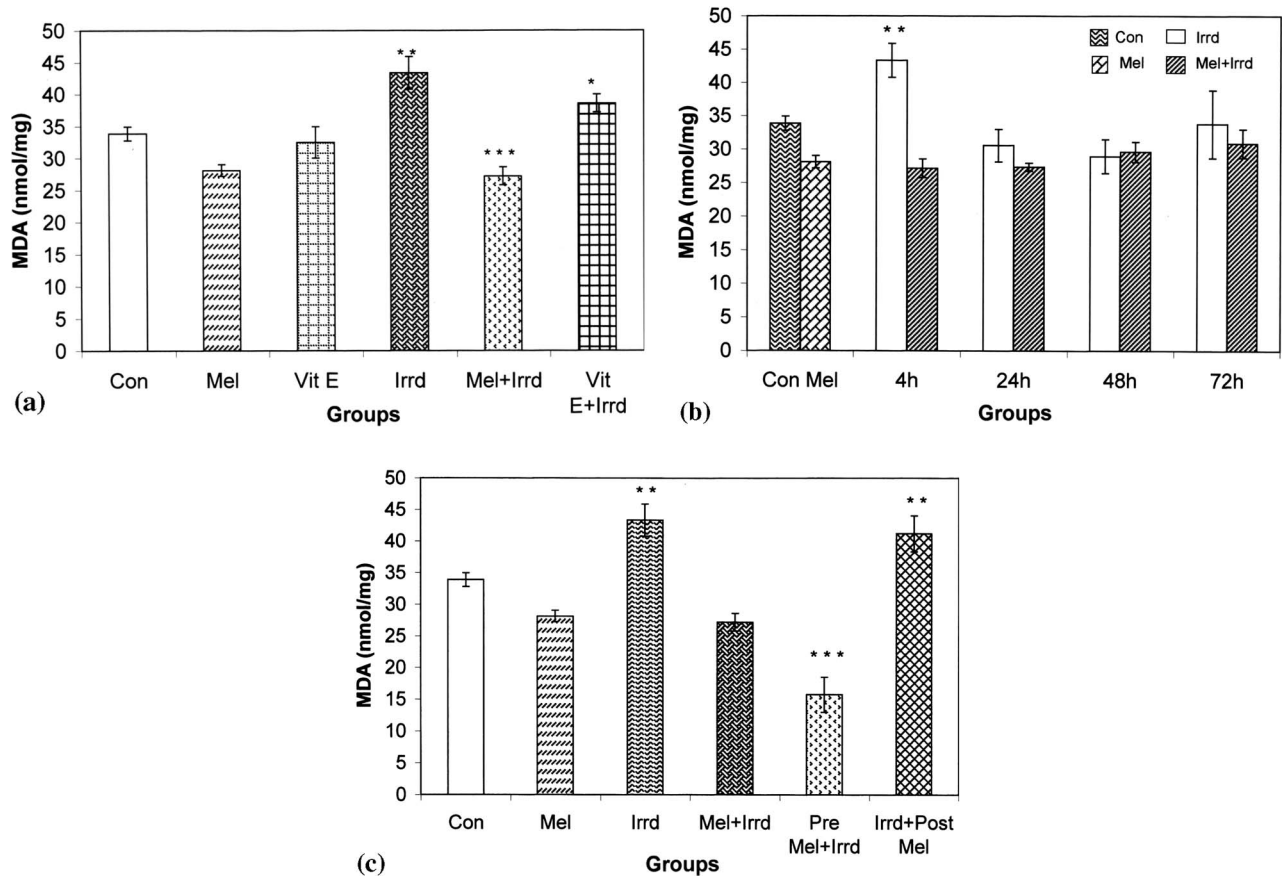

Figure 3. (a) Effect of pre-treatment with physiological doses of melatonin (25 μg/100 g bwt) and vitamin E (25 μg/100 g bwt) on malondialdehyde (MDA) levels of Indian palm squirrel, *Funambulus pennanti* during their reproductively active phase (March to June) after 2.06 Gy of X-ray irradiation. Histograms represent Mean $\pm$ SEM, $n = 5$ for each group. Con, Control; Mel, 25 μg/100 g bwt melatonin only; Vit E, 25 μg/100 g bwt vitamin E only; Irrd, Irradiation only; Mel + Irrd, 25 μg/100 g bwt melatonin treatment and irradiation; Vit E + Irrd, 25 μg/100 g bwt vitamin E treatment and irradiation; $^{**}p < 0.01$ when compared with control, $^{*}p < 0.05$ and $^{***}p < 0.01$ when compared with irradiated group. (b) Effect of pre treatment with physiological doses of melatonin (25 μg/100 g bwt) on malondialdehyde (MDA) levels of Indian palm squirrels, *F. pennanti* during reproductively active phase (March to June) after 2.06 Gy of X-ray irradiation. Mice were sacrificed at different time intervals of 4, 24, 48 and 72 h after irradiation. Histograms represent Mean $\pm$ SEM, $n = 5$ for each group. Con, Control; Mel, Melatonin only; Irrd, Irradiation only; Mel + Irrd, Melatonin treatment and irradiation; $^{**}p < 0.01$ when compared with control. (c) Effect of pre-treatment with physiological doses (25 μg/100 g bwt) and pre- and post-treatment with a high dose (0.5 mg/100 g bwt) of melatonin on malondialdehyde (MDA) levels of Indian palm squirrel, *F. pennanti* during their reproductively active phase (March to June) following 2.06 Gy of X-ray irradiation. Histograms represent Mean $\pm$ SEM, $n = 5$ for each group. Con, Control; Mel, 25 μg/100 g bwt melatonin only; Irrd, Irradiation only; Mel + Irrd, 25 μg/100 g bwt melatonin treatment and irradiation; Pre-Mel + Irrd, 0.5 mg/100 g bwt melatonin prior to irradiation; Irrd + Post Mel, 0.5 mg/100 g bwt melatonin after irradiation; $^{**}p < 0.01$ when compared with control, and $^{***}p < 0.01$ when compared with irradiated group.

RZR/RORα which get activated and bind to DNA turning genes on/off, regulating specific RNA transcription and activating antioxidative enzymes (Becker-Andre et al. 1994, Wiesenberg et al. 1995), thus supporting the role of melatonin as an indirect antioxidant. Melatonin inhibits the activation of transcription factor nuclear factor-kappa B (NF-κB) by preventing its translocation to the nucleus and, in turn, preventing DNA binding (Mohan et al. 1995, Chuang et al. 1996). Thus, melatonin reduces tissue damage during inflammatory responses due to free radicals via direct free radical scavenging and indirectly by lowering cytokines production that contributes to cellular destruction.

There have been many reports that ionizing radiation induces the formation of MDA, the main product of lipid peroxidation in the liver, lung, brain, colonic and intestinal tissues (Bouzouf et al. 2005, Varshney & Kale 1990, Taysi et al. 2003, Sener et al. 2003). In the present study, when squirrels were exposed to a single dose of radiation MDA levels were found to be significantly increased in the spleen cells of X-ray-exposed squirrels compared with control squirrels, whereas pre-treatment of melatonin prevented the increase in MDA levels. We compared this efficiency of melatonin with that of another known antioxidant vitamin E (25 μg/100 g bwt) and found melatonin to be more powerful

antioxidant, on a w/w basis, in this animal model during the reproductively active phase. This might be due to higher potency of melatonin in scavenging various free radicals generated after irradiation and in stimulating other antioxidative enzymes such as reduced glutathione (GSH) while vitamin E does not contribute to GSH synthesis as directly as melatonin (Urata et al. 1999). Our data is consistent with the scavenging action of melatonin where one molecule of melatonin can scavenge four oxygen-based free radicals compared to one molecule of vitamin E which can scavenge only two radicals, indicating melatonin to be a more potent antioxidant molecule per molecule than vitamin E (Pieri et al. 1994). Though vitamin E treatment showed positive effects against X-ray irradiation by reducing MDA levels in spleens of squirrels we noticed a significant difference in mean MDA levels of both melatonin treated plus irradiated and vitamin E plus irradiated groups. Siu et al. (1998) compared both vitamin E and melatonin antioxidative capacity and found a dose-dependent response to both antioxidants. Melatonin was 7.2 times more potent than vitamin E. This result was further supported by Gitto et al. (2001) for rat liver homogenates and by Erol et al. (2004) for rat brain exposed to gamma irradiation. Both melatonin and vitamin E prevented peroxidation of polyunsaturated membrane fatty acids by scavenging peroxyl radicals. Vitamin E protects cell membrane and other lipid-containing substances in the body from free radical damage by donating electrons to free radicals in order to neutralize them. Once vitamin E donates an electron, it becomes a radical and loses its antioxidant activity but needs vitamin C to restore the properties whereas the antioxidant action of melatonin involves donation of two electrons thereby ensuring that it does not become a free radical. Unlike other antioxidants, the reaction products of melatonin with free radicals are themselves antioxidants. The high-dose 0.5 mg melatonin/100 g bwt prevented the rise in MDA level to a greater degree than a physiological dose of 25 μg melatonin/100 g bwt. This result supports the role of melatonin in reducing MDA formed after irradiation. Post treatment with melatonin showed no reduction in MDA indicating the inability of high dose (0.5 mg/100 g bwt) melatonin to ameliorate lipid damage once it is formed. This is explained by a high level of lipid degradation products immediately after 2.06 Gy X-ray exposure. The presence of melatonin in immune cells prior to the X-ray exposure prevented damage to cellular membranes but post treatment of 0.5 mg melatonin/100 g bwt could not prevent the damage once formed. However, lipid damage was reduced at 24 h in the irradiated group suggesting the activation of internal antioxidant defence systems at later times.

The role of melatonin in scavenging free radicals and stimulating cellular antioxidant defences makes this molecule of great interest in terms of altering the responses of cells to apoptotic stimuli. A marked reduction was observed in the radiation-induced apoptosis in splenocytes of melatonin-treated squirrels compared to the irradiation only group. Like lipid peroxidation, high dose (0.5 mg/100 g bwt) post treatment with melatonin was unable to reduce the percentage of apoptosis whereas pre treatment with the same dose reduced the apoptosis percentage in the spleen. These results clearly suggest that cellular damage due to X-rays could not be modulated by post treatment with melatonin. In contrast, pre-melatonin treatment with either physiological or high doses is effective. Numerous reports have already shown that melatonin protects lipid, proteins and DNA from the harmful effects of free radicals and are typically associated with preservation of cell viability (Reiter et al. 2000, Sainz et al. 2003). The radioprotective effect of melatonin in human cells was confirmed by Vijayalaxmi et al. (1995, 1995a) as melatonin-pretreated human blood lymphocytes exhibited a significant and dose-dependent reduction in the observed incidence of chromosome abberations and micronuclei.

The present study was performed during a reproductively active phase of squirrels when the pineal hormone melatonin is peripherally low (Haldar 1996) so that the protective role of exogenous melatonin following X-ray irradiation becomes more evident. The data obtained suggest for the first time that in a seasonal breeder, while endogenous level of melatonin are low, X-ray exposure caused oxidative damage but exogenous pre-treatment with melatonin prevented the oxidative as well as hematopoietic damage while post treatment failed to do so.

Conclusion

Pre-treatment with melatonin has clear antioxidant properties against X-ray-induced oxidative damage and could be of high clinical value.

Acknowledgements

The help of Dr K. Dixit for providing the X-ray irradiation facility and financial assistance from Nuclear Science Centre, University Grant Commission, New Delhi, India, are gratefully acknowledged.

References

Allegra M, Reiter RJ, Tan DX, Gentile C, Tesoriere L, Livrea MA. 2003. The chemistry of melatonin's interaction with reactive species. Journal of Pineal Research 34: 1–10.

Badr FM, El Habit OHM, Harraz M. 1999. Radioprotective effect of melatonin assessed by measuring chromosomal damage in mitotic and meiotic cells. Mutation Research 444:367–372.

Bakan E, Taysi S, Polat MF, Dalga S, Umudum Z, Bakan N, Gumus M. 2002. Nitric oxide levels and lipid peroxidation in plasma of patients with gastric cancer. Japanese Journal of Clinical Oncology 32:162–166.

Becker-Andre M, Wiesenberg I, Schaeren-Wiemers N, Andre E, Missbach M, Saurat J-H, Carlberg C. 1994. Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. Journal of Biological Chemistry 269:28531–28534.

Bouzouf M, Martinez-Cruz F, Molinero P, Guerrero JM, Osuna C. 2005. Melatonin prevents hyperhomocysteinemia and neural lipid peroxidation induced by methionine intake. Current Neurovascular Research 2:175–178.

Chuang JI, Mohan N, Meltz ML, Reiter RJ. 1996. Effect of melatonin of NF-κB DNA binding activity in rat spleen. Cell Biology International 20:687–692.

Costa EJX, Shida CS, Biaggi MH, Ito AS, Lamy-Freund MT. 1997. How melatonin interacts with lipid bilayers: A study by fluorescence and ESR spectroscopies. Federation of European Biochemical Societies Letters 416:103–106.

Daly JM, Bertagnolli M, De Cosse JJ, Morton DM. 1999. Oncology. In: Schwartz SI, Spencer FC, Galloway AC, Shires GT, Daly JM, Fischer JE, editors. Principles of surgery. 7th ed. New York: McGraw Hill Co. pp 1: 335.

Erol FS, Topsakal C, Ozveren MF, Kaplan M, Ilhan N, Ozercan IH, Yildiz OG. 2004. Protective effects of melatonin and vitamin E in brain damage due to gamma radiation. Neurosurgical Review 27:65–69.

Ganasoundari A, Uma Devi P, Rao MNA. 1997. Protection against radiation-induced chromosome damage in mouse bone marrow by *Ocimum sanctum*. Mutation Research 373: 271–276.

Gitto E, Tan DX, Reiter RJ, Karbownik M, Manchester LC, Cuzzocrea S, Fulia F, Barberi I. 2001. Individual and synergistic antioxidative actions of melatonin: Studies with vitamin E, vitamin C, glutathione and desferrioxamine (desferoxamine) in rat liver homogenates. Journal of Pharmacy and Pharmacology 53:1393–1401.

Gonzalez-Haba MG, Garcia-Maurino S, Calvo JR, Goberna R, Guerrero JM. 1995. High-affinity binding of melatonin by human circulating T lymphocytes ($CD4^+$). The Federation of American Societies for Experimental Biology Journal 9:1331–1335.

Guerrero JM, Rafii-El-Idrissi M, Garcia-Perganeda A, Garcia-Maurino S, Gil-Haba M, Pozo D, Calvo JR. 1997. Mechanism of action of melatonin on the human immune system. Membrane versus nuclear receptors. In: Maestroni GJM, Conti A, Reiter RJ, editors. Therapeutic potential of melatonin. Basel: Karger. pp 43–51.

Haldar C. 1996. Patterns of plasma melatonin in Indian tropical palm squirrel, *F. pennanti*. In: Haldar C, editor. Role of pineal gland: Its molecular signals. New Delhi: HPCL. pp 95–117.

Haldar C, Singh R. 2001. Pineal modulation of thymus and immune function in a seasonally breeding rodent *F. pennanti*. Journal of Experimental Zoology 287(2):90–98.

Hardeland R, Reiter RJ, Poeggeler B, Tan DX. 1993. The significance of the metabolism of the neurohormone melatonin: Antioxidative protection and formation of bioactive substances. Neuroscience Biobehavioral Reviews 17:347–357.

Hussein Mahmoud R, Abu-Dief Eman E, Abd el-Reheem, Mohammad H, Abd-Elrahman Ali. 2005. Ultra structural evaluation of the radioprotective effects of melatonin against X-ray-induced skin damage in albino rats. International Journal of Experimental Pathology 86(1):45–55.

Karbownik M, Reiter RJ. 2000. Antioxidative effects of melatonin in protection against cellular damage caused by ionizing radiation, Proceedings of the Society for Experimental Biology and Medicine 225:9–22.

Koc M, Buyukokuroglu ME, Taysi S. 2002. The effect of melatonin on peripheral blood cells during total body irradiation in rats. Biological & Pharmaceutical Bulletin 25:656–657.

Konapacka M, Widel M, Rzeszowska WR. 1998. Modifying effect of vitamins C, E and beta-carotene against gamma ray induced DNA damage in mouse cells. Mutation Research 417:85–94.

Maestroni GJ. 1993. The immuno neuroendocrine role of melatonin. Journal of Pineal Research 14(1):1–10.

Mates JM, Perez-Gomez C, De Castro IN. 1999. Antioxidant enzymes and human diseases. Clinical Biochemistry 32: 595–603.

Mayo JC, Sainz RM, Antolin I, Herrera F, Martin V, Rodriquez C. 2002. Melatonin regulation of antioxidant enzyme gene expression. Cellular and Molecular Life Sciences 59:1706–1713.

Mayo JC, Tan DX, Sainz RM, Lopez-Burillo S, Reiter RJ. 2003. Oxidative damage to catalase induced by peroxyl radicals: Functional protection by melatonin and other antioxidants. Free Radical Research 37:543–553.

Mohan N, Sadeghi K, Reiter RJ, Meltz ML. 1995. The neurohormone melatonin inhibits cytosine, mitogen and ionizing radiation induced NF-κB. Biochemistry and Molecular Biology International 37:1063–1070.

Ohkawa H, Ohishi N, Yagi K. 1978. Reaction of linoleic acid hydroperoxide with thiobarbituric acid. Journal of Lipid Research 19:1053–1057.

Parthasarathy S, Santanam N, Ramachandran S, Meilhac O. 1999. Oxidants and antioxidants in atherogenesis; an appraisal. Journal of Lipid Research 40:2143–2157.

Pieri C, Marra M, Moroni F, Decchioni R, Marcheselli F. 1994. Melatonin: A peroxyl radical scavenger more effective than vitamin E. Life Sciences 55:271–276.

Rai S, Haldar C. 2003. Pineal control of immune status and hematological changes in blood and bone marrow of male squirrels (*Funambulus pennanti*) during their reproductively active phase. Comparative Biochemistry and Physiology C 136:319–328.

Reiter RJ, Melchiorri D, Sewerynek E, Poeggeler B, Barlow-Walden LR, Chuang JI, Ortiz GG, Acuna-Castroviejo D. 1995. A review of the evidence supporting melatonin's role as an antioxidant. Journal of Pineal Research 18:1–11.

Reiter RJ, Tan DX, Osuna C, Gitto E. 2000. Actions of melatonin in the reduction of oxidative stress: A review. Journal of Biomedical Science 7:444–458.

Reiter RJ, Tan DX. 2003. Melatonin: A novel protective agent against oxidative injury of the ischemic/reperfused heart. Cardiovascular Research 58:10–19.

Rodriguez AB, Marchena JM, Nogales G, Duran J, Barriga C. 1999. Correlation between the circadian rhythm of melatonin, phagocytosis and superoxide anion levels in ring dove heterophils. Journal of Pineal Research 26:35–42.

Rodriguez C, Mayo JC, Sainz RM, Antolin I, Herrera F, Martin V, Reiter RJ. 2004. Regulation of antioxidant enzymes: A significant role for melatonin. Journal of Pineal Research 36(1):1–9.

Sainz RM, Mayo JC, Rodriguez C, Tan DX, Lopez-Burillo S, Reiter RJ. 2003. Melatonin and cell death: Differential actions on apoptosis in normal and cancer cells. Cellular and Molecular Life Sciences 60:1407–1426.

Sarma I, Kesavan PC. 1993. Protective effects of vitamins C and E against gamma ray induced chromosomal damage in mouse. International Journal of Radiation Biology 63:759–764.

Satyamitra M, Uma Devi P, Murase H, Kagiya VT. 2001. *In vivo* radioprotection by α-TMG: preliminary studies. Mutation Research 479:53–61.

Sener G, Jahovic N, Tosun O, Atasoy BM, Yegen BC. 2003. Melatonin ameliorates ionizing radiation-induced oxidative organ damage in rats. Life Sciences 74:563–572.

Shida CS, Castrucci AML, Lamy-Freund MT. 1994. High melatonin solubility in aqueous media. Journal of Pineal Research 16:198–201.

Sitasawad SL, Kale RK. 1994. Divalent cations and radiation induced lipid peroxidation. Indian Journal of Experimental Biology 32:55–59.

Siu AW, Reiter RJ, To CH. 1998. The efficacy of vitamin E and melatonin as antioxidants against lipid peroxidation in rat retinal homogenates. Journal of Pineal Research 24:239–244.

Sudheer Kumar M, Unnikrishnan MK, Uma Devi P. 2003. Effect of 5-aminosalicylic acid on radiation induced micronuclei in mouse bone marrow. Mutation Research 527:7–14.

Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ. 1993. Melatonin: A potent endogenous hydroxyl radical scavenger. Endocrine Journal 1:57–60.

Tan DX, Reiter RJ, Manchester LC, Yan MT, El-Sawi M, Sainz RM, Mayo JC, Kohen R, Allegra M, Hardeland R. 2002. Chemical and physical properties and potential mechanisms: Melatonin as a broad-spectrum antioxidant and free radical scavenger. Current Topics in Medicinal Chemistry 2:181–198.

Taysi S, Koc M, Buyukokuroglu ME, Altinkaynak K, Sahin YN. 2003. Melatonin reduces lipid peroxidation and nitric oxide during irradiation-induced oxidative injury in the rat liver. Journal of Pineal Research 34:173–177.

Toule R. 1987. Radiation-induced DNA damage and its repair. International Journal of Radiation Biology 51:573–589.

Umegaki K, Ishikava T. 1994. Decrease in vitamin E levels in the bone marrow of mice receiving whole body X-ray irradiation. Free Radical Biology & Medicine 17:439–444.

Urata Y, Honma S, Goto S, Todoroki S, Iida T, Cho S, Honma K, Kondo T. 1999. Melatonin induces gamma-glutamyl-cysteine synthase mediated by activator protein 1 in human vascular endothelial cells. Free Radical Biology and Medicine 27:838–847.

Vanecek J. 1998. Cellular mechanisms of melatonin action. Physiological Reviews 78:687–721.

Varshney R, Kale RK. 1990. The effects of calmodulin antagonists on radiation induced lipid peroxidation in microsomes. International Journal of Radiation Biology 58:733–743.

Vijayalaxmi, Reiter RJ, Meltz ML. 1995. Melatonin protects human blood lymphocytes from radiation-induced chromosome damage. Mutation Research 346:23–31.

Vijayalaxmi, Reiter RJ, Sewerynek E, Poeggeler B, Leal BZ, Meltz ML. 1995a. Marked reduction of radiation-induced micronuclei in human blood lymphocytes pretreated with melatonin. Radiation Research 143:102–106.

Vijayalaxmi, Reiter RJ, Herman TS, Meltz ML. 1998. Melatonin reduces gamma radiation-induced primary DNA damage in human blood lymphocytes. Mutation Research 397:203–208.

Wiesenberg I, Missbach M, Kahlen J-P, Schräder M, Carlberg C. 1995. Transcriptional activation of the nuclear receptor RZRα by the pineal gland hormone melatonin and identification of CGP 52608 as a synthetic ligand. Nucleic Acids Research 23:327–333.