Effect of exogenous melatonin on X-ray induced cellular toxicity in lymphatic tissue of Indian tropical male squirrel, *Funambulus pennanti*

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Effect of exogenous melatonin on X-ray induced cellular toxicity in lymphatic tissue of Indian tropical male squirrel, *Funambulus pennanti*

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Abstract

**Purpose:** The radioprotective ability of melatonin was investigated in the Indian tropical rodent, *Funambulus pennanti* during its reproductively inactive phase (RIP) when peripheral melatonin is high and the animal is under the influence of environmental stresses.

**Materials and methods:** Fifty male squirrels were divided into two sets of 25 each; with set 1 receiving 0.9% saline and set 2 receiving 25 μg melatonin/100 g body weight/day for four weeks during evening hours. Five saline-treated and five-melatonin treated squirrels were sacrificed at times of 4, 24, 48 and 72 hours following 2.06 Gy X-ray radiation. Remaining squirrels that were not exposed to X-ray were used as control and melatonin treated, respectively. Total leukocyte count (TLC) and lymphocyte count (LC) in peripheral blood and lipid peroxidation (LPO) status, superoxide dismutase (SOD) activity, total antioxidant status (TAS), apoptotic percentage on the basis of morphological changes and DNA fragmentation and caspase-3 activity were measured in the spleens of squirrels.

**Results:** Peripheral blood and spleens with higher intrinsic levels of environmental and X-radiation induced free radical generation had inhibited TLC, LC, SOD activity and TAS and increased LPO and apoptosis. Melatonin pre-treatment significantly reduced and altered the changes brought on by X-radiation.

**Conclusion:** Exogenous melatonin with its anti-apoptotic and antioxidant properties additively increased the immunity of the squirrels, by protecting their hematopoietic system and lymphoid organs against X-ray radiation induced cellular toxicity.

**Keywords:** Melatonin, X-radiation, oxidative damage, apoptosis, TUNEL, reproductive inactive phase

Introduction

X-rays are the most commonly used diagnostic tool by the clinicians though the ability of ionizing radiation to damage immune function and to induce cancer is well known. Disruption of the balance between production and detoxification of free radicals and reactive oxygen species (ROS) by ionizing radiation can lead to oxidative stress conditions causing damage to biological targets such as DNA, lipids and proteins and on defense systems of the cells. The defense systems composed of enzymes and antioxidants such as glutathione (Gul et al. 2000), can play a significant role in carcinogenesis (Martinez Cayuela 1995). Further, oxidative stress to biological membranes leads to the formation of damaged and altered lipid and protein molecules causing changes in membrane structures, permeability, fluidity, signaling pathways and enzyme activities (Sitasawad & Kale 1994).

Free radicals with a very short half-life are very hard to measure in the laboratory. A commonly used alternate approach measures the markers of free radicals rather than the actual radical such as thiobarbituric acid reactive substances (TBARS) assay which is a commonly measured parameter of lipid peroxidation (LPO) after oxidative stress (e.g., ionizing radiation exposure) (Noor et al. 2005). TBARS assay includes products like malondialdehyde (MDA), 4-hydroxyalkenals (HDA), 4-hydroxynonenal (HNE) which results from interactions of free radicals with polyunsaturated fatty acids (PUFA), this assay is easy to use to study the effects of treatments on lipid damage/lipid peroxidation (LPO).

Both endogenous and exogenous antioxidants prevent formation of metabolites damaging...
macromolecules, thereby protecting against free radical mediated damages. Thus, there is a need for a compound that can act both as an immunostimulator and free radical scavenger at the same time. Melatonin, an indole hormone synthesized mainly in the pineal gland of vertebrates and also in retina and gastrointestinal tract is known for its immune stimulation (Maestroni 1993, Bubenik 2002) and free radical scavenging activity (Hardeiland et al. 1993, Allegra et al. 2003). The discovery of melatonin as a direct free radical scavenger (Tan et al. 1993) and as an indirect antioxidant via its stimulatory actions on antioxidative enzymes (Reiter et al. 2000, Rodriguez et al. 2004) has greatly increased interest in the use of this agent in experimental and clinical settings.

Injury due to ionizing radiation and the potential of antioxidants to reduce these deleterious effects have been widely studied in laboratory animal models but not in the case of tropical seasonal breeders, which show annual competence of reproductive functions, seasonal adjustments of immune functions with photoperiod and are continuously under stress of environmental influences and natural radiations. The Indian tropical squirrel, Funambulus pennantii, one such seasonal breeder possesses a peculiar feature of two extremely varied phases in its annual reproductive cycle, namely a reproducitively active phase (RAP) and reproductively inactive phase (RIP) with the presence of a high melatonin level in the internal milieu during RIP (Haldar et al. 2001). These seasonal breeders augment their free radical load due to winter bound stress e.g. low ambient temperature, need of shelter and food shortage. In this study, we have attempted to develop an improved antioxidant based strategy for lowering the risk of radiation induced free radical damages in the Indian palm male squirrel, F. pennantii. We have done this by examining the role of exogenous melatonin along with endogenous melatonin on the response to X-ray radiation-induced cellular toxicity by measuring total leukocyte count (TLC) and lymphocyte count (LC) in peripheral blood and LPO, superoxide dismutase (SOD) activity, total antioxidant status (TAS), percent apoptosis based on morphological changes by Acridine Orange staining (confirmation by DNA fragmentation measurements) and caspase-3 activity in splenocytes.

**Methods**

All the experiments on animals were conducted in accordance with Institutional Practice and within the framework of Revised Animals (Scientific Procedures) Act of 2002 of Government of India on Animal Welfare.

**Animal care and maintenance**

Fifty young adult male squirrels (*Funambulus pennantii*), weighing 100–120 g were obtained from the local animal suppliers of Varanasi (Lat. 25°18’ N; Long. 83°01’ E), as they are easily available in this vicinity. All squirrels were kept in a well-aerated animal room. The room temperature was maintained at 25 ± 2°C with a photoperiod of 10 h light and 14 h dark with lights on from 07:00 hours to 17:00 h equivalent to ambient conditions. The squirrels were fed soaked gram seed (*Cicer arietinum*), 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. Sterile and pyrogen-free glassware, syringes, needles and pipettes were used during the experiment. After acclimatization for two weeks to laboratory conditions, fifty male squirrels were divided into two sets of 25 each. Set 1 received 0.9% normal saline (NaCl) and set 2 received 25 µg melatonin/100 g body weight daily for four weeks. Melatonin was prepared fresh every 7 days by dissolving it in trace amounts of absolute ethanol, diluting in 0.9% normal saline and storage at 4°C in an amber glass bottle for its stability. The injections were given subcutaneously (s.c.) during evening hours at 17:00 h in red light or the dark. Twenty saline treated squirrels from set 1 and twenty melatonin treated squirrels from set 2 were anesthetized with 1.75 mg/kg of thiopental sodium (s.c.) and irradiated abdominally (near the splenic region) for 60 s with 2.06 Gy of X-ray radiation in a single fraction during morning hours around 11:00 a.m. The X-ray machine (1070D, Rocks, Mumbai, India) was operated at 70 kV potential, 10 mA current with 1.5 mm aluminium filter and source to skin distance of 10 cm. A minimum of ten squirrels, i.e., five saline treated and five melatonin treated were sacrificed for spleen and peripheral blood at 4, 24, 48 and 72 hours following X-ray irradiation. The remaining ten animals were not exposed to X-rays and used as controls and melatonin treated only, respectively.

**Total leukocyte count (TLC).** Blood was collected from heart puncture under ether anesthesia in a heparinized tube and processed for TLC. The number of white blood cells was counted (no./mm³) in Neubauer counting chamber (Paul Marienfeld GmBH & Co. KG, Lauda-Königshofen, Germany) using a Nikon microscope (Nikon, Kawasaki, Japan).

**Lymphocyte count (LC).** A thin film of blood was prepared on a glass slide, stained with Leishman’s
Lipid peroxidation (LPO) assay by TBARS level estimation. All spleens were excised and weighed. The tissues were homogenized in a ten fold excess of 20 mM Tris Hydrochloride (HCl) buffer (pH 7.4) and the 10% homogenates were centrifuged for 15 min at 3000 g at 4°C. The supernatant was subjected to thiobarbituric acid (TBA) assay by mixing with 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid, 0.8% TBA and boiling for 1 h at 95°C. The reaction mixture was immediately cooled in running water, vigorously shaken with n-butanol and pyridine reagent (15:1) and centrifuged for 10 min at 1500 g (Ohkawa et al. 1978). The absorbance of the upper phase was measured at 534 nm. LPO was expressed as TBARS in nmol/g tissue wt., by taking 1,1,3,3 tetraethoxy propane (TEP) as a standard. The standard curve was calibrated using a 10 nM concentration of TEP.

Superoxide dismutase (SOD) assay. 10% homogenates of all spleen tissues were prepared in 150 mM phosphate buffered saline (PBS, pH 7.4) and centrifuged for 30 min at 12,000 g at 4°C. The supernatant was again centrifuged for 60 min at 12,000 g at 4°C and then processed for enzymic activity based on a modified spectrophotometric method using nitrite formation by superoxide radicals (Das et al. 2000). 0.5 ml of homogenate was added to 1.4 ml of reaction mixture comprised of 50 mM phosphate buffer (pH 7.4), 20 mM L-Methionine, 1% (v/v) Triton X-100, 10 mM Hydroxyamine hydrochloride, 50 mM ethylene diamine tetraacetic acid (EDTA) followed by a brief pre-incubation at 37°C for 5 min. Next, 0.8 ml of riboflavin was added to all samples along with a control containing buffer instead of sample and then exposed to two 20 W fluorescent lamps fitted parallel to each other in an aluminium foil coated wooden box. After 10 min of exposure, 1 ml of Greiss reagent was added and absorbance of the color formed was measured at 543 nm. One unit of enzyme activity is defined as the amount of SOD inhibiting 50% of nitrite formation under assay conditions.

Total antioxidant status (TAS). ABTS, 2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation decolourisation test is a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. The radical scavenging activity of antioxidants for ABTS radical cations was measured according to the method of Re et al. (1999). A stock solution of ABTS radical cations was prepared one day before the assay by mixing 5 ml of 7 mM ABTS with 1 ml of 14.7 mM potassium persulfate, followed by storage in the dark at room temperature. The stock solution of ABTS radical cations was diluted with water or ethanol. ABTS radical cation was generated by oxidation of ABTS with potassium persulfate. 2.95 ml of ABTS cation solution were mixed with 50 µl of 10% homogenate and the decrease in absorbance was monitored for 60 min at 10 min intervals at 734 nm.

Morphological analysis of apoptotic cells. Apoptosis of splenocytes was performed following acridine orange (AO) staining. 0.4 ml of acid-detergent solution (0.1% Triton X-100, 0.08 N HCl, 0.15 M NaCl, pH = 1.2) was admixed gently with 0.2 ml of the diluted sample (1 x 10^6 cells/ml in PBS). After 15 s, the samples were stained by adding 1.2 ml of AO in 0.001 M EDTA on ice for 15 min. A drop of this mixture was taken on a clean slide and mounted with a cover glass and observed immediately under a fluorescence microscope (Leitz MPV3, Wetzlar, Hesse, Germany) at 440 nm to 520 nm.

DNA fragmentation detection by TUNEL assay. DNA fragmentation was detected using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) kit according to the manufacturer’s instructions (R&D Systems, Inc. MN, USA). In short, the cells of control, irradiated and melatonin pre-treated irradiated groups were fixed in 3.7% formaldehyde in PBS for 15 min at 25°C and then dropped were placed separately on clean glass slides and air-dried. The slides were treated with 0.05 ml of proteinase K solution for 30 min and incubated with quenching solution for 3–4 min. Then the slides were immersed in 1 x TdT labeling buffer for 5 min and incubated with 0.05 ml of labeling reaction at 37°C for 60 min. The reaction was terminated by immersing slides in 1 x TdT stop buffer. Then the slides were incubated with 0.05 ml of diluted (1:500) anti-BrdU at 37°C for 60 min. Slides were washed with PBS containing 0.05% Tween-20 and then treated with 0.05 ml of Streptavidin-horse radish peroxidase (HRP) solution for 10 min. Washed slides were immersed in 3,3' dianobenzidine (DAB) solution for 5 min and then in methyl green solution for 2 min after washing. The slides were again washed sequentially by dipping at least 10 times in distilled water, 50%, 70%, 95%, 100% ethanol and finally in xylene for two changes each. Slides were mounted in Depex Polystyrene (DPX) and then analyzed for TUNEL positive staining under phase contrast microscope at 400 x magnification (Chauke et al. 2005). More than 300 cells per slide were counted on randomly selected areas. Cells stained with brown fluorescence
were considered as TUNEL positive cells and data were expressed as the percentage of the total sample.

Detection of caspase-3 activity of splenocytes. The spleens of control, melatonin treated, irradiated and melatonin pre-treated plus irradiated groups were minced in a watch glass using sterile blades and passed through sterile, stainless steel wire mesh. The splenic cells were suspended in ice-cold 1× PBS and erythrocytes were lysed by incubating in 0.84% Tris NH₄Cl for 10 min at cold temperature. The cell suspension was washed thrice before determining cell viability and cell count. The cells were collected by centrifugation at 500 g for 10 min at 4°C. The supernate is gently removed while the pellet was lysed by the addition of 50 μl of cold lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton-X100, pH 6.0) per 2×10⁶ cells and incubated in ice for 10 min. After 10 min, the lysates were centrifuged at 10,000 g for 1 min at 4°C. The supernate was transferred to a fresh tube, kept on ice and processed for caspase-3 activity using caspase-3 colorimetric assay kit according to the manufacturer’s instructions (R&D Systems, Inc. MN, USA), which was carried out in 96-well flat bottom microplate. Each enzymatic reaction required 50 μl of cell lysate, 50 μl of reaction buffer, 5 μl of caspase-3 colorimetric substrate (DEVD-pNA) and the microplate was incubated at 37°C for 2 h with a substrate blank and sample blank. At the end of incubation period, absorbance of enzymatically released chromophore p-nitroanilide (pNA) was measured at 405 nm in the microplate reader (Tecan, Spectra II-microelisa plate reader, Austria). The caspase-3 activity was determined by comparing the absorbance of pNA from apoptotic sample with untreated control. The activity is presented as fold increase of optical density (OD)/10⁶ cells per ml (Chaube et al. 2005).

Statistical analysis
All the data are expressed as mean ± SEM of at least five animals per group. The statistical analysis was performed with ANOVA followed by Student Newman-Keuls’ multiple range tests. The differences were considered statistically significant when \( p < 0.05 \).

Results
Effect of X-ray radiation and melatonin pre-treatment on total leukocyte count
The melatonin treated only group had a significantly higher total leukocyte count compared to the control group. X-radiation of 2.06 Gy caused a significant reduction in total leukocyte count \( (p < 0.05) \) when compared to control group. The count was highly increased in the melatonin pre-treated plus irradiation group \( (p < 0.01) \) when compared to the irradiated only group at 4 h. The count in the irradiated group was similar to the control group at 24 and 48 hours and a little less (non-significant difference) at 72 h. The count in the melatonin plus irradiation group was similar at 4, 24 and 48 h and reached a level similar to the control group at 72 h. There was a highly significant difference at 4 and 24 h \( (p < 0.01) \) and significant difference at 72 h \( (p < 0.05) \) between the irradiation alone and melatonin treated plus irradiated groups (Figure 1).

Effect of X-ray radiation and melatonin pre-treatment on lymphocyte count
The melatonin treated only group had a significantly higher LC compared to the control group. Like TLC, radiation also suppressed LC \( (p < 0.01) \) when compared to the control group. Exogenous melatonin administration significantly increased the count when the irradiation alone group is compared to the melatonin treated plus irradiation group \( (p < 0.01) \) at 4 h. Subsequently, the LC difference was reduced but remained significant in the irradiation alone groups at 24 h \( (p < 0.01) \), at 48 h \( (p < 0.01) \) and 72 h \( (p < 0.05) \) when compared to controls. LC counts of the irradiation only groups and melatonin plus irradiation groups were gradually restored with time and the differences between the two groups diminished with time (Figure 2).
Melatonin pre-treatment reduces X-ray radiation induced lipid peroxidation

The TBARS level in the splenic tissue of the control group was higher than in the melatonin treated only group. The TBARS level in the splenic tissue of X-ray irradiated only group increased significantly \((p < 0.05)\), i.e., 1.37-fold at 4 h when compared to the non-irradiated control. The TBARS levels of the melatonin treated irradiated groups was lower than that of the irradiation only groups under all observed times after irradiation though the difference was not significant at 4 and 24 h. The melatonin pre-treated groups exposed to radiation showed a highly significant reduction in TBARS level at 48 h and 72 h \((p < 0.01)\) when compared to the respective irradiation only groups (Figure 3).

Effect of X-ray radiation and melatonin on SOD activity

Figure 4 shows that melatonin treatment alone significantly increased the SOD levels in splenic tissue compared to control tissue. SOD activity of the irradiation only group was reduced 2.25-fold at 4 h compared to non-irradiated controls \((p < 0.01)\) and gradually recovered to near control levels by 72 h. The melatonin treated group exposed to radiation showed an increase of 1.55-fold in SOD activity compared to the radiation only group \((p < 0.05)\) at 4 h and SOD levels gradually increased to near to the melatonin only group level by 48 h.

Total antioxidant status of the tissue against ABTS radical cation

This method described in detail in the materials and methods section gives a measure of the antioxidant activity determined by decolorization assay of the ABTS radical cation, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm. A significant decrease in percentage inhibition or elimination of ABTS radical...
cation was noted in the splenic tissue of the irradiation alone group at 5 min ($p < 0.01$), at 10 min and at 15 min ($p < 0.05$) when compared to the control group. A similar pattern was seen for the melatonin treated irradiated group (Figure 5A). Total antioxidant activity started increasing from 24 h onwards in the irradiation only group when compared to the control group but was still significantly lower ($p < 0.05$) when observed at 48 and 72 h (Figure 5B). The melatonin pretreated treated group showed a significant increase in percentage cation radical scavenging activity when compared to control ($p < 0.05$, Figure 5A). The melatonin pretreated group when irradiated had significantly increased percentage ABTS radical cation scavenging activity at 48 h when compared to the irradiation alone group ($p < 0.01$ at 5 min and $p < 0.05$ at 15 min, Figure 5C).

Figure 5. (A) Time course of ABTS radical cation scavenging activity (indicated as %) in the spleen of control, melatonin (25 μg/100 g b. wt.), 2.06 Gy X-ray irradiated and melatonin pre-treated plus irradiated Funambulus pennanti during reproductively inactive phase (October–December). Vertical bars represent mean ± SEM, $n = 5$ for each group. Con, Control; Mel, Melatonin only; Irrd, Irradiated group; Mel + Irrd, Melatonin treatment and irradiation; 5' = 5 min, 10' = 10 min, 15' = 15 min. a = $p < 0.01$ at 5 min; b = $p < 0.05$ and c = $p < 0.01$ at 15 min when compared with their respective controls. (B). Time course of ABTS radical cation scavenging activity (indicated as %) in the spleen of control and 2.06 Gy X-ray irradiated Funambulus pennanti during reproductively inactive phase (October–December) and sacrificed at 4, 24, 48 and 72 hours after irradiation. Vertical bars represent mean ± SEM, $n = 5$ for each group. Con, Control; Irrd, Irradiation only; 5' = 5 min, 10' = 10 min, 15' = 15 min. a = $p < 0.01$ at 5 min; b = $p < 0.01$ at 10 min; c = $p < 0.01$ at 15 min when compared with their respective controls. (C) Time course of ABTS radical cation scavenging activity (indicated as %) in the spleen of control, melatonin (25 μg/100 g b. wt.), 2.06 Gy X-ray irradiated and melatonin pre-treated and irradiated Funambulus pennanti during the reproductively inactive phase (October–December) and sacrificed at 48 hour. Vertical bars represent mean ± SEM, $n = 5$ for each group. Con, Control; Mel, Melatonin only; Irrd 48 h, Irradiated group sacrificed after 48 hour; Mel + Irrd 48 h, Melatonin treated and Irradiated group sacrificed after 48 hour; 5' = 5 min, 10' = 10 min, 15' = 15 min. a = $p < 0.01$ at 5 min and c1 = $p < 0.05$ at 15 min when compared with their respective controls.
Melatonin pre-treatment restores X-ray radiation induced apoptotic index

Melatonin treatment alone caused a significant decrease in the percent apoptosis compared to control splenocytes, Figure 6. A significant increase was seen in the percent apoptosis of splenocytes in the X-irradiated only group when compared to the control group \((p < 0.05)\) and melatonin pre-treatment resulted in decrease in the melatonin plus irradiation group when compared to irradiation only group at 4 h \((p < 0.01)\). X-irradiation caused a highly significant increase in percent apoptosis, which was reduced in melatonin treated plus irradiation groups at all observed time points with statistically significant reductions at 4, 24 and 48 h as compared with the irradiation alone groups \((p < 0.01)\).

Melatonin attenuates radiation induced DNA damage

Figure 7 shows the results of the TUNEL assay performed on formaldehyde fixed normal and apoptotic splenocytes. The cells of the melatonin pre-treated group were resistant to radiation as indicated by low the incidence of TUNEL positivity (Figure 7C). TUNEL positivity was observed in nuclear regions by chromatin condensation and DNA fragmentation. Two distinct populations of cells were observed, one resembling control cells indicated with orange arrowhead (Figures 7A, 7C) and another population resembling apoptotic cells indicated with blue arrowhead (Figures 7B, 7C). The nuclear region of apoptotic cells stained brown with cell shrinkage and membrane blebbing feature whereas normal cells stained green with intact membranes and no chromatin condensation. We observed a significant increase in TUNEL positive cells of X-irradiation only group when compared to control \((p < 0.01)\) which was significantly reduced in the melatonin pre-treated plus irradiation group when compared to the irradiation alone group at 4 h \((p < 0.01, \text{ Figure 7D})\).

Effect of radiation on caspase-3 activity of splenocytes

The melatonin alone group showed a highly significant reduction in caspase-3 activity of splenocytes compared to the control group as measured by the optical density of released pNA at 405 nm, Figure 8. A significant increase, 2.2-fold, in caspase-3 activity of splenocytes from the irradiation alone group was observed compared to the control group at for hours after irradiation with 2.06 Gy \((p < 0.01)\). There was 1.6-fold decrease in caspase-3 activity of the melatonin pretreated plus irradiation group when compared with the results for the irradiation alone group \((p < 0.01)\).

Discussion

Irradiation triggers multiple cellular events that lead to cell death, in particular to apoptosis involving ROS. The apoptotic death of lymphocytes could induce impairment of specific immune responses. Patients exposed to doses of radiation can have hematopoietic failure and/or secondary immunodeficiency. Hence, agents which protect the hematopoietic system and lymphoid cells from radiation induced damage need to be identified. Previous studies have described the use of synthetic radioprotectors such as amifostine and other sulfhydryl compounds caused undesirable side effects (Links & Lewis 1999) compared to melatonin. Melatonin directly/indirectly detoxifies free radicals and ROS such as peroxynitrite anion, superoxide anion radical, singlet oxygen, hydrogen peroxide and nitric oxide (Reiter et al. 1999). Hematopoietic tissue and spleen, being proliferating tissues are radiosensitive and susceptible to radiation induced oxidative damage. Koc et al. (2002) have confirmed the marked effects of radiation on stem cells, leukocytes, lymphocytes and platelets.

Our results demonstrated 2.06 Gy of X-ray irradiation induced damage to peripheral blood with decreases in immune parameters like TLC and LC and to the spleen by an increased apoptotic index (percentage of apoptotic cells), increased LPO (TBARS), decreased SOD activity and increases in TAS. We studied the changes in TLC and LC in squirrels, at various times following radiation, since...
these are the important components of the immune system and affect the immune status of animals (Ohkoshi et al. 1991). The circulating TLC and LC were found to be significantly suppressed following radiation. Suppression of the leukocyte population was found to be mainly due to the decreased population of lymphocytes in the blood circulation. The LC in the irradiation only groups was significantly below control levels at all times (4–72 hours) after 2.06 Gy irradiation. However, exogenous melatonin counteracted radiation-induced suppression of leukocyte and lymphocyte population.

It has been shown that peak time for immune system variables (e.g., circulating lymphocytes, NK cell activity) directly corresponds with melatonin levels (Levi et al. 1989). The correlation of immune system variables with melatonin levels seen over the circadian cycle increased after melatonin treatment (Litvinenko et al. 2006). Our results are in agreement with Koc et al. (2002) and Vijayalaxmi et al. (1998) where melatonin administration prior to irradiation prevented damage to peripheral blood cells in rats and reduced the extent of primary DNA damage and chromosomal aberrations in human blood lymphocytes. In the present work irradiation of melatonin treated groups caused reduced damage to hematopoietic and lymphatic tissues, as compared with the irradiation alone groups. This is the first report of its kind, for a wild species, where exogenous melatonin has shown prominent radioprotection during the inactive phase of the animal when melatonin is peripherally high. The improved protection is likely due to the additive effect of exogenous melatonin with the high peripheral...
Melatonin protects Indian squirrel against X-radiation

leads to a reduction in the antioxidant activities of the enzymes such as SOD. Exogenous melatonin significantly reduced the X-radiation induced reduction in SOD activity at all observed time points. Antolin et al. (1996) and Kotler et al. (1998) have shown that exogenous melatonin can elevate antioxidant enzyme activity and expression. Further, Mayo et al. (2002) has reported that melatonin, at physiological serum concentrations, increases mRNA levels of both SOD and glutathione peroxidase (GSH-Px). Thus melatonin shows a two-fold radioprotective action as a free radical scavenger and as an enhancer of mRNA synthesis for antioxidant enzymes. The night-time increase in GSH-Px and SOD activities initially reported in several tissues of chicks (Albarran et al. 2001, Martin et al. 2003) were subsequently confirmed in rodents (Tomas-Zapico et al. 2003).

Melatonin regulates antioxidant enzyme gene expression and activity with activation of MT1/2 receptors, via G inhibitory (Gi) protein (Mayo et al. 2002, Rodriguez et al. 2004). The decrease of free radicals by melatonin would allow repression of redox-sensitive transcription factors, i.e., nuclear factor-kappa B (NFkB), activating protein-1 (AP-1) (Chuang et al. 1996). Although the free radical scavenging action of melatonin is not receptor-mediated, its involvement in the regulation of antioxidant enzymes can not be ruled out. Melatonin administration can change the cellular redox state towards a more reducing environment and reducing proteins, which may lead to activation and increased concentrations of antioxidant enzymes (Becker-Andre et al. 1994, Wiesenberg et al. 1995). The oxidative status of the cell is the primary factor regulating gene expression and activity of these enzymes (Rodriguez et al. 2004). The role of melatonin in stimulating the protective activity of antioxidant enzymes was measured by ABTS radical cation reduction. We observed that presence of antioxidants in the system (splenic homogenate) resulted in a reduction of preformed ABTS radical cation radical at 8 and 24 h. Antioxidants cause a reduction of ABTS radical cation and not prevention of its formation (Bartosz & Bartosz 1999). Herraiz and Galisteo (2004) reported that many indoleamines including melatonin acted as free radical scavengers and antioxidants in an ABTS assay measuring total antioxidant activity. The total antioxidant activity against ABTS radical cation was progressively lost in both irradiated group and melatonin-treated plus irradiated group when compared to controls. But a significant increase in the percentage inhibition of the ABTS radical cation at 48 hours in melatonin treated plus irradiation group as compared with irradiation alone group might be because of the better TAS during that period, which was not observed at 4 and 24 h. At 72 h, TAS of

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Figure 8. Effect of melatonin on 2.06 Gy X-radiation induced caspase-3 activity in the splenocytes of Funambulus pennanti sacrificed at 4 hours after irradiation. Vertical bars represent mean ± SEM, n = 5 for each group. Con, Control; Mel, Melatonin only; Irrd, Irradiated group; Mel + Irrd, Melatonin treatment and irradiation. **p < 0.01 when compared with control; +p < 0.01 when compared with irradiated group.

melatonin present during this inactive phase (Haldar et al. 2001). Nelson and Drazen (2000) reported that enhanced immune function during the inactive phase of animals is due to an increase in the duration of melatonin secretion. During the inactive phase, the presence of high melatonin levels likely contribute to the ability of F. pennanti to deal with radicals generated by radiation as well as the stressful conditions of winter.

LPO in cell membranes is devastating to the functional integrity of these structures and severe damage leads to cell death. Melatonin, due to its small molecular structure, high lipophilicity and modest hydrophilic nature, efficiently penetrates cell membranes and acts as an extracellular and intracellular free radical scavenger against X-ray induced damage (Kim et al. 2001). The ability of melatonin to suppress LPO has already been studied in liver, lung, brain, intestine and colon of rats and mice (Sener et al. 2003, Tayisi et al. 2003, Bouzouf et al. 2005). TBARS, such as MDA are the lipid breakdown products which form adducts with proteins and introduces cross-links causing alterations in their biochemical properties (Slatter et al. 2000). Our data showed a significant inhibitory effect of melatonin against radiation induced LPO in splenic tissue which might be due to the ability of melatonin to stop the initiation of LPO, breaking the LPO propagation chain reaction and its interaction with MDA, thus neutralizing further damaging consequences on the cell (Li et al. 2005).

The antioxidative enzymes present in the spleen suppress the effect of ROS and free radicals, thus protecting cells from oxidative damage. The increased rate of ROS production due to radiation
both the irradiation alone group and melatonin-treated plus irradiation group was nearly the same indicating the protective effect of endogenous melatonin. The presence of high levels of free radicals in the melatonin treated tissue showed a lag in percentage inhibition of ABTS\(^+\) radical cation while the presence of less free radicals showed comparative increases in ABTS percentage inhibition. This might be due to the engagement of melatonin in combating free radicals and ABTS\(^++\). Therefore, the more free radicals, the less ABTS percentage inhibition occurs and vice-versa.

Melatonin as free radical scavenger and cellular antioxidant defense stimulator alters the response of cells to apoptotic stimuli. Significant decreases in the percentage apoptotic cells was seen in splenocytes from animals pretreated with melatonin and irradiated compared with irradiation alone groups. Numerous reports have shown that melatonin protects lipids, proteins and DNA from harmful effects of free radicals and is associated with preservation of cell viability (Sainz et al. 2003). Nuclear DNA damage involves two distinct mechanisms: (i) Oxidative injury, which involves a direct attack of free radicals on DNA, and (ii) endonuclease-mediated DNA fragmentation which is accompanied by morphological features of apoptosis and caspase cascade activation (Sun et al. 2002). TdT-mediated dUTP nick end labeling (TUNEL), a widely used technique to detect primarily DNA double strand breaks (DSB), was used in the present study to confirm apoptosis in shrunken splenocytes. Nuclear DNA fragmentation is a late stage apoptosis hallmark and makes cell death irreversible (Walker & Sikorska 1997). Free 3′-OH DNA ends accompanying chromatin fragmentation can be detected by the TUNEL assay (Gavrieli et al. 1992). As shown in Figure 7B, free 3′-OH ends were available for TdT labeling in the apoptotic cells where TUNEL positivity was detected whereas melatonin pre-treatment has markedly reduced TUNEL positivity and increased the number of viable cells as compared to the irradiation alone group (Figure 7C, 7D). This suggests a role of melatonin in DNA repair besides its anti-apoptotic and antioxidant effects.

Apoptosis is actively executed by a set of dormant cysteine proteases, the caspases. Caspases acting via receptor-mediated (Fas ligand or tumor necrosis factor z-mediated) pathway and the mitochondrial pathway, on activation, initiate the death program by destroying key components of the cellular infrastructure and activating factors that mediate damage to the cells (Takahashi & Earnshaw 1996). Caspase-3, on activation, is translocated from cytoplasm to nucleus where it cleaves genomic DNA at internucleosomal regions resulting in 180–200 base pair DNA oligonucleosomal fragments (Liu et al. 1998, Kamada et al. 2005). DNA fragmentation as analysed by TUNEL staining (Figure 7B) was mostly caspase-3 dependent (Figure 8), which is also supported by the work of Inagaki-Ohara et al. (2002) and Chaube et al. (2005). As observed in Figure 8, caspase activity of the melatonin pre-treated group and irradiated group was decreased in comparison to the irradiation alone group. This result suggests that melatonin might have a role in reducing apoptosis incidence through the caspase-3 mediated pathway by blocking the activity of caspase-3.

The above findings demonstrate that peripheral blood and spleen were sensitive to higher intrinsic levels of environmental and X-ray radiation induced free radicals generated by 2.06 Gy irradiation. Melatonin pre-treatment significantly protected against radiation effects on TLC, SOD activity, TAS, LPO and apoptosis in irradiated groups decreasing intrinsic oxidative stress and acting as a beneficial factor for the organism’s survival. Recently, Sankaran and Subramanian (2006) reported that long-term administration of exogenous melatonin enhanced the circulating level of melatonin in rats. The long-term administration of exogenous melatonin might have elevated its endogenous level contributing to the protection of irradiated squirrels seen in the present study.

**Conclusion**

The potency of melatonin as free radical scavenger, antioxidant activity stimulator and immune enhancer has protected the seasonally breeding rodent, *F. pennanti* against X-radiation during its reproductively inactive phase. The possibility that melatonin is modulating endogenous DNA repair activity, as evidenced in TUNEL assay results, needs further investigation. Alternatively, long term melatonin treatment may have protected the splenocytes of *F. pennanti* from X-ray radiation induced apoptosis by inhibiting caspase-3 activity, which is mediated through the caspase-3 pathway. Our result suggests that radio-protective property of melatonin can be of therapeutic use in areas of medical/accidental exposure to radiations.

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Melatonin protects Indian squirrel against X-radiation


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