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UVB irradiation severely induces systemic tissue injury by augmenting oxidative load in a tropical rodent: Efficacy of melatonin as an antioxidant

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ABSTRACT

Tropical animals are regularly exposed to solar UV radiation. The generation and accumulation of free radicals as a result of UVB incidence causes tissue damage. In the present study we report that the irradiation of *Funambulus pennanti* by 1.5 J/cm^2 of UVB caused significant oxidative damage to the spleen. The systemic immunity suffered collateral damage as depicted by results of total leukocyte count (TLC) while an increase in the thiobarbituric acid reactive substances (TBARS) and decline in the activities of enzymes superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px) and Catalase (CAT) denoted oxidative tissue damage. Melatonin the indole-amine with known antioxidative properties when administered subcutaneously (s.c 100 μg/100 gm body weight), before the UVB irradiation recovered the damages caused by UVB radiation in the spleen. The action of melatonin was direct and might have involved its membrane receptor (MT1) as well as nuclear receptor (RORα) indicating the fact that the mode of action of melatonin in ameliorating UVB radiation induced free radical load may be receptor mediated. Our study hence reports for the first time that UVB radiation incurred oxidative damage to the spleen and suppressed the normal tissue functions. This UVB mitigated oxidative stress was recovered by the free radical scavenging and anti-apoptotic functions of melatonin when administered prior to UVB irradiation.

1. Introduction

The ultraviolet radiations are low wavelength non-ionizing waves that cause significant damage to cells and tissues. The UV rays are capable of inducing inflammation, immune suppression and extensive tissue damage [1]. The principal action of UVB radiation (UVBR) is triggering the generation of free radicals [2] which then cause oxidative damages like the peroxidation of membrane lipids and ultimately compels the apoptotic or necrotic death of the cells. Although much of the studies on UV radiation are dedicated to skin associated dysfunctions like erythema formation, local immune suppression of the skin, induction of non-melanoma skin cancers yet UV rays can also harm systemic immunity [3]. The spleen is an organ where melatonin is synthesized and also being a secondary lymphoid organ is an important component of the immune system and hence is a suitable organ for studying the damaging effects of UVB radiation.

Melatonin apart from having a wide array of physiological functions is one of the most important antioxidants in the living system. Apart from up-regulating the expression of the genes for antioxidative enzymes like superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px) and Catalase (CAT) [4], melatonin also directly scavenges free radicals due to its lipophilicity that enables it to enter the cellular milieu and detoxify the highly reactive free radicals [5]. Therefore we became interested to investigate whether melatonin can protect the spleen against free radical mediated damages incurred by UVB radiation and improve the suppressed immune repertoire of the body. Spleen is an organ where melatonin is locally synthesized and the local melatonergic system may also get affected due to the severe oxidative stress thereby further aggravating the conditions. This made us to investigate the local melatonin synthesis by measuring the activity of its rate limiting enzyme arylalkylamine N-acetyl transferase (AANAT).

We selected a diurnal tropical rodent, *Funambulus pennanti*, commonly known as the Indian palm squirrel as our animal model. This seasonal breeder is most frequently exposed to sunlight and other environmental biotic factors and is thus an appropriate model to study hazards of UVB radiation. The immune functions

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of *F. pennanti* are seasonally adjusted via photoperiod [6] as in the case of non tropical rodents where immune function varies with seasons [7]. Reports explain the effect of photoperiod and other seasonal stress on the immunity of the rodent only [6]. Therefore, we recorded the damaging effects of UVB radiation on the spleen, emphasizing on the induced oxidative load and studied the expression pattern of the melatonin receptors, which are present in the spleen (membrane as well as nuclear) for the evidences of the protective role of melatonin administration against UVB radiation. We also investigated the expression of the anti-apoptotic factor Bcl-2 to demonstrate whether the UVB mediated oxidative damage led to the induction of apoptosis and whether it can be prevented by a melatonin pre-treatment.

2. Material and methods

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

2.1. Ultraviolet radiation

The animals were irradiated in a specially designed chamber [8]. The UVB (Philips, India Pvt. Ltd.) tubes which emit light only in the UVB range (280–320 nm) were used to deliver a dose of 1.5 J/cm^2 for 30 minutes/day for 4 days with a skin to source distance of 30 cm as previously established from our lab [9]. The used doses were standardized in our lab. The dose in case of the study was selected such that it increases the free radical content of the skin to an extent that the generated ROS reaches systemic circulation through blood capillaries and affects the spleen.

2.2. Chemicals and treatments

All the chemicals, drugs and hormones such as Melatonin and Concanavalin A were purchased from Sigma-Aldrich Chemicals, St. Louis, Missouri, USA. Melatonin stock was freshly prepared every week by dissolving in trace amounts of absolute ethanol (10 μl) and diluting in 0.9% normal saline to get the desired concentration. It was stored at 4 °C in amber colored glass bottle to avoid photo-degradation. The injections were given subcutaneously (s.c.) during evening hours (17:00–17:30p.m).

2.3. Study design

A total of twenty four male squirrels (body weight ~100 g) and age ~10 months, as judged by the cranium diameter and incisor length [10] were maintained in an animal house with controlled photoperiodic conditions of 12 L:12 D (lights on from 06:00 to 18:00 h) equivalent to ambient conditions (temperature and humidity) of the months of August–October (Autumn) and fed with food (soaked gram seeds and seasonal fruits) and water *ad libitum*.

The squirrels were divided into four groups comprising six animals in each. The groups were designated as: Group A- saline treated controls, Group B- irradiated with 1.5 J/cm^2 of UVB, Group C- treated with 100 μg/100 g b. wt. melatonin and Group D- 100 μg/100 g body weight melatonin pre-treatment followed by 1.5 J/cm^2 of UVB irradiation. The ventral abdominal skin of the squirrels was shaved (12 cm^2 area) prior to UVB irradiation. Group A and B squirrels were shaved but not irradiated and served as sham-controls but all the animals of groups C and D were exposed to UVB radiation group wise at a time. The pre-treatment of melatonin to groups B and D was also for 4 days. After the stipulated time of treatment and UVB radiation, all the squirrels were sacrificed under deep ether anaesthesia during late afternoon hours. The blood was collected directly from heart into heparinised syringes for total leukocyte count (TLC). Spleens were dissected out on ice, cleaned and then immediately processed for splenocyte culture. The remaining half of the spleens was stored at -80^{0}c for preparation of homogenates required for biochemical estimations and western blot analysis.

2.4. Total leukocyte count

Blood collected in heparinised tubes was evaluated for TLC by mixing with Turk's solution (1:20). The counting was done in a Neubauer's counting chamber (Paul Marienfeld GmBH & Co. KG, Lauda-Königshofen, Germany) and expressed as TLC/mm^3.

2.5. Lymphocyte proliferation assay

2.5.1. Cell suspension preparation

The spleens were minced and erythrocytes were lysed by hypotonic shock using equal volume of cold ammonium chloride tris buffer (tris hydroxymethylene aminomethane, BDH, UK); 0.5% tris buffer and 0.84% NH_4Cl mixed in 1:10 ratio; pH 7.2. Viability and cell count were determined by trypan blue dye exclusion method and Turk solution respectively. Splenocyte suspensions were seeded in 35 mm sterile culture petri plates at a density of 2×10^6 cells per ml in complete RPMI 1640 medium containing penicillin 100 U/ml, streptomycin 100 mg/ml and 10% fetal calf serum (Sigma, USA). The cell suspension was divided such that one ml of suspension contained 2×10^6 cells per plate with and without the T-cell mitogen concanavalin A (Con A) at 10 μg/ml. The plates were kept in 37 °C incubator with 5% CO_2 atmosphere for 48 hrs.

2.5.2. Cell harvesting

100 μl of Dimethyl thiazolyl diphenyl tetrazolium salt MTT (5 mg/ml) was added to each culture 3 hrs before the scheduled harvesting at 48 h and the OD was taken at 590 nm. The blastogenic response of the splenocytes (%SR) was calculated as follows:

$$\%SR = \frac{\text{OD of Mitogen stimulated cells}}{\text{OD of Basal cells}} \times 100$$

2.6. Lipid peroxidation assay by TBARS level estimation

Spleens were excised and weighed for the preparation of 10% tissue homogenates in 20 mM Tris Hydrochloride (Tris-HCl) buffer (pH 7.4). The homogenates were centrifuged at 3000*g* for 15 min at 4 °C and supernatant was subjected to thiobarbituric acid (TBA) assay as described by Ohkawa et al., 1978 [11]. The supernatant was mixed with 2.8 mM Butylated hydroxyl Toluene (BHT), 8.1% SDS, 20% Glacial Acetic acid and 0.8% Thiobarbituric acid (TBA) and boiled for 1 h at 100 °C. The reaction was immediately cooled in running water and vigorously shaken with n-butanol: pyridine (15:1). The mixture was centrifuged at 1500*g* for 10 mins and the absorbance of the upper phase was checked at 534 nm.

2.7. Superoxide dismutase assay

10% homogenates of spleen tissues were processed for SOD activity assay as per the mentioned protocol [12]. 100 μl of the spleen homogenate was mixed in a 1.4 ml reaction mixture containing 20 mM L-Methionine, 1% (v/v) Triton X, 10 mM Hydroxylamine hydrochloride and 50 mM EDTA. 80 μl of 50 μM Riboflavin was then added and the whole mixture was incubated under 20 W white light for 10 mins. The reaction was stopped by adding freshly prepared Greiss reagent and the OD was taken at 543 nm.