

Comparative Effect of Melatonin and Vitamin E on Phenylhydrazine-Induced Toxicity in the Spleen of *Funambulus pennanti*

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ABSTRACT: Phenylhydrazine (PHZ) oxidation resulting in free iron release followed by free radical generation has increased frequency of cancer. This study aims towards the dose-dependent response of PHZ and the role of melatonin in comparison with vitamin E following PHZ-induced toxicity within the lymphoid tissue (spleen) of Indian tropical seasonal breeder, *Funambulus pennanti*, during reproductively active phase. An increase in the damages in terms of lipid peroxidation (LPO), apoptosis percentage, and splenomegaly was observed following different doses of PHZ treatment, i.e., 0.025, 0.5, and 1 mg/100 g body weight (b.wt.), where dose of 1 mg/100 g b.wt. showed more significant damages. Both melatonin (0.5 mg/100 g b.wt.) and vitamin E (1 mg/100 g b.wt.) administration ameliorated oxidative damages of 1 mg/100 g b.wt. PHZ-treated group. Melatonin altered PHZ-induced responses significantly to a greater degree than vitamin E as evidenced by LPO status, SOD activity, and ABTS radical cation scavenging activity of antioxidants. Thus, melatonin might be able to restrict carcinogenic property of PHZ-induced oxidative stress by protecting macromolecules of the cell from harmful effects of PHZ and instead preserving cell viability. © 2008 Wiley Periodicals, Inc. *Environ Toxicol* 24: 1–9, 2009.

Keywords: melatonin; vitamin E; phenylhydrazine; oxidative stress; seasonal breeder

INTRODUCTION

Drug-induced hemolytic response has been associated with intracellular oxidative stress (Imanishi et al., 1985; Ferrali et al., 1992, 1997). Besides initiating hemolytic injury, reactive oxygen species (ROS) and free radicals have molecular targets of attacks on lipids and proteins (McMillan et al., 2005). Phenylhydrazine (PHZ), a known hematotoxic agent (Berger, 2007), is used as a model for studying hematinic effects (Biswas et al., 2005), as a model of reticulocyte research (Xie et al., 2002) and also used in industrial and

laboratory settings (Sharma et al., 1991; Golab et al., 2002, Xie, 2003). PHZ oxidation resulting in free iron release followed by free radical generation has increased likelihood of cancer and thus regarded as a potent carcinogen of hydrazine family (Parodi et al., 1981; Paiva et al., 1991; Toth, 2000; Ritz et al., 2006). Hydrazines are usually used in laboratory, industrial and therapeutic settings (Moloney et al., 1983; Kean et al., 2006). Relationships between iron overload, iron release, deoxyribonucleic acid (DNA) damage, and tumor initiation following subchronic intoxication of rats with PHZ already exist (Bonkovsky, 1991; Ferrali et al., 1997).

Melatonin because of its nature of scavenging free radicals and stimulating antioxidative enzymes activity (Tan et al., 1993; Reiter et al., 2000; Rodriguez et al., 2004) has been frequently used in experimental and clinical settings. Physiologic and pharmacologic concentrations of melatonin have shown chemopreventive, oncostatic, and tumor

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inhibitory effects in a variety of *in vitro* and *in vivo* experimental models of neoplasia (Jung and Ahmad, 2006). Karbownik et al. (2000) has reported that melatonin administration reduced oxidative stress induced by PHZ and iron overload in liver and spleen of rats according to the suppression of lipid peroxidation (LPO).

Our animal model *Funambulus pennanti*, a semidomestic tropical seasonal breeder, commonly known as Indian palm squirrel, is usually found in Northern India. These seasonal breeders are always under the influence of seasonal changes and hence sensitive to environmental stress. They show annual competence of reproductive functions and seasonal adjustments of immune functions with photoperiod (Haldar et al., 2001). *F. pennanti* habitats near the human population; thus, it is usual to get exposed to industrial toxins and hemolytic drug, PHZ, during laboratory purposes.

Till date, erythrocyte has been one of the most promising cellular models for studying the effect of PHZ oxidation (Snyder et al., 1985; Berger, 2007). Since, PHZ-induced free iron release followed by free radical load within the spleen will further affect the splenic cells, which are mostly lymphocytes, in turn affecting the immunity of the animal, thus present study was designed to study two major objectives. The first being the effect of different doses of PHZ on spleen, which has not been reported so far. *In vivo* melatonin treatment enhances various aspects of immune function in nontropical rodents (Nelson and Drazen, 2000) and tropical rodents (Haldar et al., 2001; Rai and Haldar, 2003). Melatonin and vitamin E are free radical scavengers. After investigating the dose-dependent effect of PHZ, our second objective was to note the protective efficacy of melatonin in comparison with vitamin E against PHZ-induced oxidative damage in the lymphatic tissue of *F. pennanti* during reproductively active phase, when the level of immunity is low due to the presence of high gonadal steroids.

MATERIALS AND METHODS

All the experiments 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.

Animals' Care and Maintenance

Forty male squirrels, weighing 100–130 g were collected in the vicinity of Varanasi (Latitude 25°18' N; Longitude 83°01' E) and kept in a well-aerated animal room (temperature maintained at 25 ± 2°C and at photoperiod of 14-h light and 10-h dark cycle with lights on from 06:00 h to 20:00 h) and fed gram seeds (*Cicer arietinum*), seasonal nuts, grains along with water *ad libitum*.

Experimental Design

All the chemicals used were purchased from Sigma-Aldrich Chemicals, St Louis, MO. After acclimatization for 2 weeks to laboratory conditions, the squirrels were divided into eight groups, namely A as Control group, E as melatonin-treated group, G as Vitamin E-treated group, D as PHZ-treated group, F as Melatonin plus 1 mg/100 g body weight (b.wt.) PHZ cotreated group, H as vitamin E plus 1 mg/100 g b.wt. PHZ cotreated group. All the injections were given daily subcutaneously (s.c.) for 7 days with 0.025 mg, 0.5 mg, 1 mg/100 g b.wt. PHZ at 09:00 h in morning, 0.5 mg/100 g b.wt. melatonin at 18:00 h in dim light, 1 mg/100 g b.wt. vitamin E and 0.9% saline to control at the same time of melatonin, vitamin E, and PHZ injections. Volume of the each treatment administered daily was 0.1 mL PHZ cotreated group. PHZ was freshly prepared in 0.9% normal saline (NaCl). Melatonin was freshly prepared every 7 days in trace amounts of absolute ethanol, diluted in 0.9% NaCl, and was stored in an amber glass bottle at 4°C for its stability. Vitamin E was prepared in 0.9% NaCl and 20% ethanol. After 24 h of the last injection, all the squirrels were anesthetized with 1.75 mg/kg of thiopental sodium (s.c.) and sacrificed for spleen and peripheral blood. Spleen was stored at –80°C. Blood was collected for total leukocyte count (TLC) and spleen for apoptosis percent of cells, LPO status, superoxide dismutase (SOD) activity, and total antioxidant activity (TAA) of the tissue homogenate and mass index of the splenic tissue.

Total Leukocyte Count

Blood was collected directly from heart under ether anesthesia in heparinized tube and processed for TLC in Neubauer's counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) under the microscope (Nikon, Kawasaki, Japan).

Lipid Peroxidation Assay by Thiobarbituric Acid Reactive Substances Level Estimation

All the spleens were excised and weighed. The tissues were homogenized in 20 mM Tris HCl buffer (pH 7.4) and 10% homogenates were centrifuged for 15 min at 3000 × g, at 4°C. Supernatant was subjected to thiobarbituric acid (TBA) assay by mixing with 8.1% sodium dodecyl sulfate, 20% acetic acid, 0.8% TBA, and boiling for 1 h at 95°C (Ohkawa et al., 1978). The reaction mixture was immediately cooled in running water and vigorously shaken with *n*-butanol and pyridine reagent (15:1) and centrifuged for 10 min at 1500 × g. The absorbance of the upper phase was taken at 534 nm. Total TBARS were expressed as nmol/g tissue wt., taking 1,1,3,3-tetraethoxy propane (TEP) as standard. The standard curve was calibrated using 10 nM concentration of TEP.

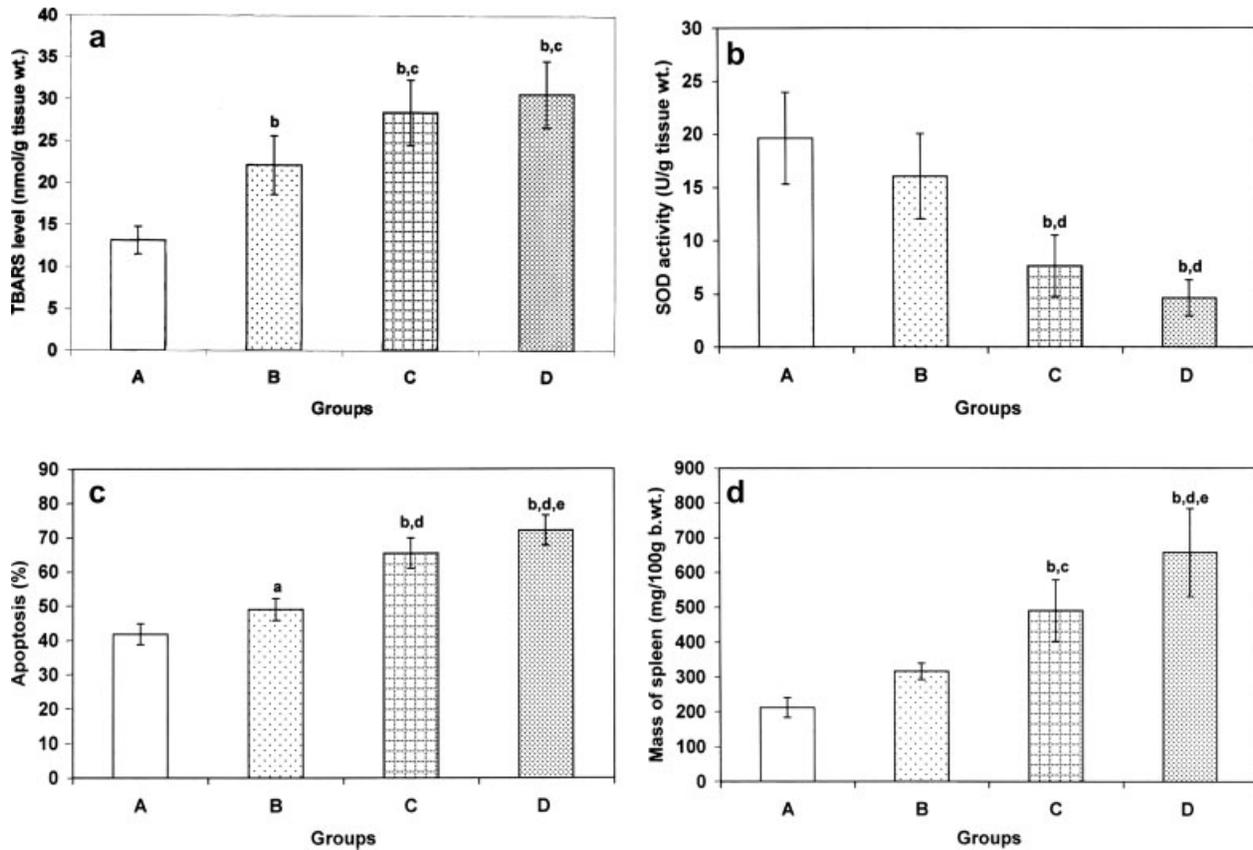


Fig. 1. (a) Effect of increasing doses of phenylhydrazine on spleen TBARS level of *F. pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = control, B = 0.025 mg/100 g b.wt. PHZ-treated group, C = 0.5 mg/100 g b.wt. PHZ-treated group, D = 1 mg/100 g b.wt. PHZ-treated group; b = $P < 0.01$ when compared with A, c = $P < 0.05$ when compared with B. (b) Effect of increasing doses of phenylhydrazine on spleen SOD activity of *F. pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = control, B = 0.025 mg/100 g b.wt. phenylhydrazine-treated group, C = 0.5 mg/100 g b.wt. phenylhydrazine-treated group, D = 1 mg/100 g b.wt. phenylhydrazine-treated group; b = $P < 0.01$ when compared with A, d = $P < 0.01$ when compared with B. (c) Effect of increasing doses of phenylhydrazine on percent apoptosis of spleen of *F. pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = control, B = 0.025 mg/100 g b.wt. phenylhydrazine-treated group, C = 0.5 mg/100 g b.wt. phenylhydrazine-treated group, D = 1 mg/100 g b.wt. phenylhydrazine-treated group; a = $P < 0.05$ and b = $P < 0.01$ when compared with A, d = $P < 0.01$ when compared with B, e = $P < 0.05$ when compared with C. (d) Effect of increasing doses of phenylhydrazine on spleen mass of *Funambulus pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = control, B = 0.025 mg/100 g b.wt. phenylhydrazine-treated group, C = 0.5 mg/100 g b.wt. phenylhydrazine-treated group, D = 1 mg/100 g b.wt. phenylhydrazine-treated group; b = $P < 0.01$ when compared with A, c = $P < 0.05$ and d = $P < 0.01$ when compared with B, e = $P < 0.05$ when compared with C.

Superoxide Dismutase Activity Estimation

Ten percent homogenates of all spleen tissues were prepared in 150 mM phosphate-buffered saline (PBS) (pH 7.4) and centrifuged for 30 min at $12\,000 \times g$ at 4°C . The super-

natant was again centrifuged for 60 min at $12\,000 \times g$, at 4°C , and then processed for enzymic activity estimation using nitrite formation by superoxide radicals (Das et al., 2000). Half milliliter (0.5 mL) of homogenate was added to 1.4 mL of reaction mixture comprising of 50 mM

phosphate buffer (pH 7.4), 20 mM L-methionine, 1% (v/v) Triton X-100, 10 mM hydroxylamine hydrochloride, 50 mM ethylene diamine tetraacetic acid (EDTA) followed by a brief preincubation at 37°C for 5 min. Next, 0.8 mL of riboflavin was added to all samples along with control containing buffer instead of sample and then exposed to two 20-W fluorescent lamps fitted parallel to each other in an aluminum 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 condition.

Total Antioxidant Activity

ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt radical cation decolorisation test is widely used for the assessment of TAA of various substances. The scavenging activity or capacity of antioxidants for

Fig. 2. (a) Effect of melatonin (0.5 mg/100 g b.wt.) and vitamin E (1 mg/100 g b.wt.) on total leukocyte count of *F. pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = Control, E = 0.5 mg/100 g b.wt. melatonin-treated group, G = 1 mg/100 g b.wt. vitamin E-treated group, D = 1 mg/100 g b.wt. phenylhydrazine-treated group, F = 0.5 mg/100 g b.wt. melatonin plus 1 mg/100 g b.wt. phenylhydrazine cotreated group, H = 1 mg/100 g b.wt. vitamin E plus 1 mg/100 g b.wt. phenylhydrazine cotreated group; b = $P < 0.01$ when compared with A, c = $P < 0.01$ when compared with D. (b) Effect of melatonin (0.5 mg/100 g b.wt.) and vitamin E (1 mg/100 g b.wt.) on spleen TBARS level of *F. pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = Control, E = 0.5 mg/100 g b.wt. melatonin-treated group, G = 1 mg/100 g b.wt. vitamin E-treated group, D = 1 mg/100 g b.wt. phenylhydrazine-treated group, F = 0.5 mg/100 g b.wt. melatonin plus 1 mg/100 g b.wt. phenylhydrazine cotreated group, H = 1 mg/100 g b.wt. vitamin E plus 1 mg/100 g b.wt. phenylhydrazine cotreated group; a = $P < 0.05$ and b = $P < 0.01$ when compared with A, c = $P < 0.01$ when compared with D, d = $P < 0.05$ when compared with H. (c) Effect of melatonin (0.5 mg/100 g b.wt.) and vitamin E (1 mg/100 g b.wt.) on spleen SOD activity of *F. pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = Control, E = 0.5 mg/100 g b.wt. melatonin-treated group, G = 1 mg/100 g b.wt. vitamin E-treated group, D = 1 mg/100 g b.wt. phenylhydrazine-treated group, F = 0.5 mg/100 g b.wt. melatonin plus 1 mg/100 g b.wt. phenylhydrazine cotreated group, H = 1 mg/100 g b.wt. vitamin E plus 1 mg/100 g b.wt. phenylhydrazine cotreated group; a = $P < 0.05$ and b = $P < 0.01$ when compared with A, c = $P < 0.01$ when compared with D, d = $P < 0.05$ when compared with H. (d) Effect of melatonin (0.5 mg/100 g b.wt.) and vitamin E (1 mg/100 g b.wt.) on percent apoptosis of splenocytes of *F. pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = Control, E = 0.5 mg/100 g b.wt. melatonin-treated group, G = 1 mg/100 g b.wt. vitamin E-treated group, D = 1 mg/100 g b.wt. phenylhydrazine-treated group, F = 0.5 mg/100 g b.wt. melatonin plus 1 mg/100 g b.wt. phenylhydrazine cotreated group, H = 1 mg/100 g b.wt. vitamin E plus 1 mg/100 g b.wt. phenylhydrazine cotreated group; a = $P < 0.05$ and b = $P < 0.01$ when compared with A, c = $P < 0.01$ when compared with D. (e) Effect of melatonin (0.5 mg/100 g b.wt.) and vitamin E (1 mg/100 g b.wt.) on spleen mass of *F. pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = Control, E = 0.5 mg/100 g b.wt. melatonin-treated group, G = 1 mg/100 g b.wt. vitamin E-treated group, D = 1 mg/100 g b.wt. phenylhydrazine-treated group, F = 0.5 mg/100 g b.wt. melatonin plus 1 mg/100 g b.wt. phenylhydrazine cotreated group, H = 1 mg/100 g b.wt. vitamin E plus 1 mg/100 g b.wt. phenylhydrazine cotreated group; b = $P < 0.01$ when compared with A, c = $P < 0.01$ when compared with D. (f) Time dependent ABTS radical cation scavenging rate by antioxidants (indicated as %) in the spleen of control, melatonin-, vitamin E-, and phenylhydrazine-treated *F. pennanti* during reproductively active phase (March–July); vertical bars represent mean \pm SEM, $n = 5$ for each group; A = Control, E = 0.5 mg/100 g b.wt. melatonin-treated group, G = 1 mg/100 g b.wt. vitamin E-treated group, D = 1 mg/100 g b.wt. phenylhydrazine-treated group, F = 0.5 mg/100 g b.wt. melatonin plus 1 mg/100 g b.wt. phenylhydrazine cotreated group, H = 1 mg/100 g b.wt. vitamin E plus 1 mg/100 g b.wt. phenylhydrazine cotreated group; 0.5' = 0.5 min, 5' = 5 min, 10' = 10 min.

ABTS radical cations was measured based on the method of Re et al. (1999). A stock solution of ABTS radical cations was prepared 1 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 dark at room temperature. The stock solution of ABTS radical cations was diluted with water. ABTS radical cation was generated by oxidation of ABTS with potassium persulfate. ABTS cation solution of volume 2.95 mL was mixed with 50 μ L of 10% homogenate and the decrease in absorbance was monitored for 10 min at 734 nm.

Morphological Analysis of Apoptotic Cells

Cell death was microscopically analyzed following Acridine Orange–Ethidium Bromide (AO–EB) double staining. Acridine orange (AO) stained both apoptotic and viable cells emitting green fluorescence when bound to double-stranded DNA and red fluorescence when bound to single-stranded RNA. Viable cells fluoresced uniform bright green nuclei with organized structure while early apoptotic cells showed intact membranes and chromatin condensation as bright green patches or fragments and late apoptotic cells

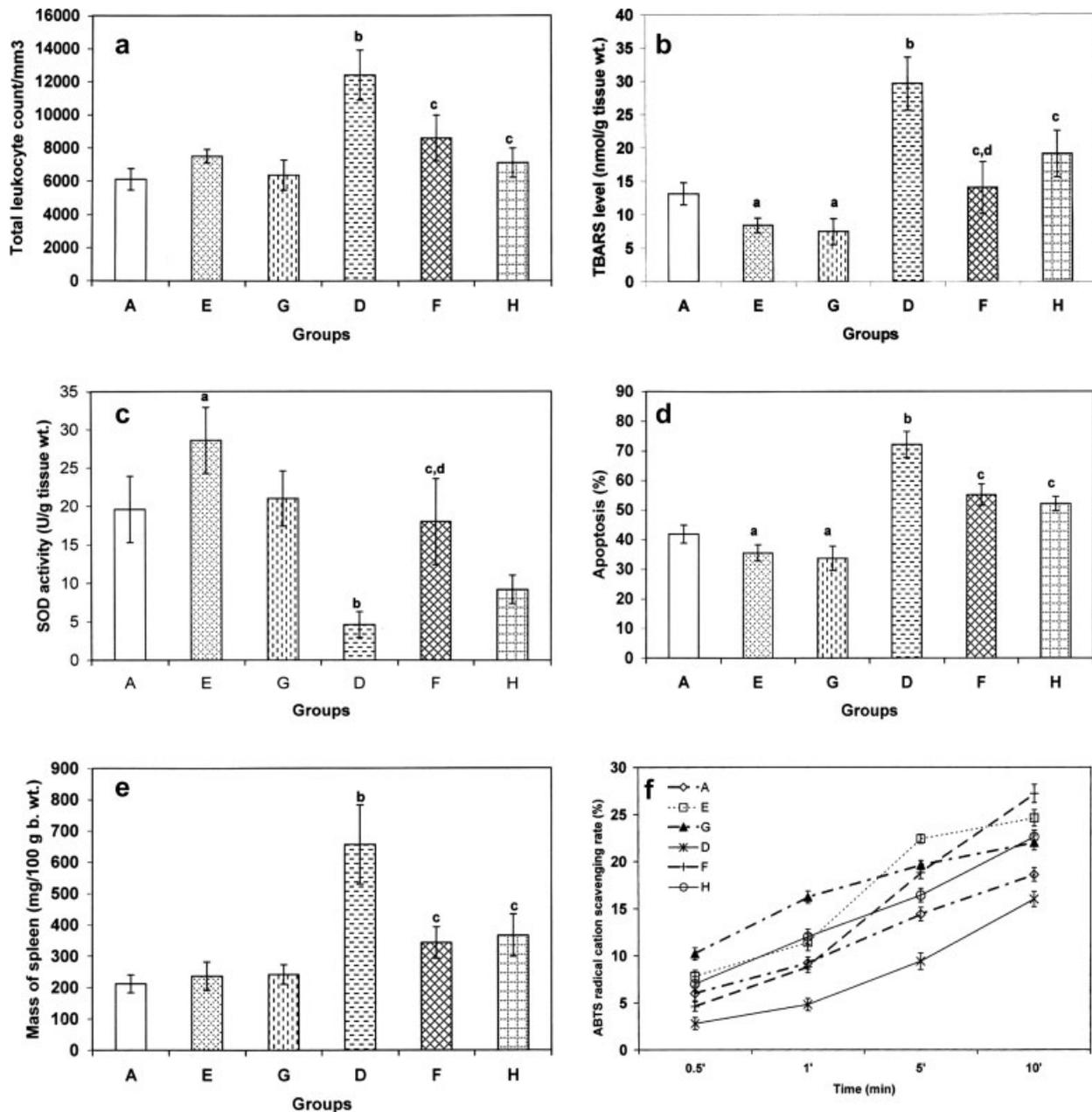


Fig. 2.

showed orange to red nuclei with condensed or fragmented chromatin. Ethidium bromide (EB) is taken up only by dead cells and emitted red fluorescence when intercalated with DNA. Necrotic cells showed a uniformly orange to red nuclei with organized structure. AO-EB dye of volume 0.01 mL (1×) was admixed gently with 0.2 mL of the diluted sample (1×10^6 cells/mL in PBS). A drop of this mixture was placed underneath coverslip on a clean slide and cells were observed immediately under fluorescence microscope (Leitz MPV3, Wetzlar, Hesse, Germany) at 440–520 nm at 920× magnification. A minimum of 200 cells was counted in every sample.

Statistical Analysis

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. Values were considered as significant if $P < 0.05$.

RESULTS

In Vivo Dose-Dependent Response of Splenocytes to Phenylhydrazine

PHZ treatment significantly increased ($P < 0.01$) LPO levels in 0.025, 0.5 and 1.0 mg treated groups when compared to sham-injected controls. The comparison between the different doses of PHZ revealed that the dose of 1.0 mg induced more damage to cell membrane (1.0 mg $>$ 0.5 mg $>$ 0.025 mg) [Fig. 1(a)].

A significant reduction ($P < 0.01$) in the mean SOD activity of PHZ-treated groups was observed in case of 0.5 mg and in 1 mg of PHZ groups, but no significant change in SOD activity was observed when treated with 0.025 mg PHZ group [Fig. 1(b)].

There was a significant PHZ dose-dependent increase ($P < 0.01$) in apoptosis percent of splenocytes (0.025 mg $<$ 0.5 mg $<$ 1 mg) when compared with controls. Both 0.5 and 1 mg PHZ groups showed significant increase ($P < 0.05$) in apoptosis percent *versus* 0.025 mg, whereas no significant change was observed between 0.5 and 1 mg [Fig. 1(c)]. Different morphological changes in splenocytes was noted after staining with AO-EB dye (Fig. 3).

There was an increase in the splenic mass with the increased doses of PHZ in 0.025, 0.5 and 1.0 mg treated groups, respectively, when compared with sham-injected controls. There was a significant induction of splenomegaly with 0.5 mg of PHZ ($P < 0.05$) and also with 1 mg of PHZ ($P < 0.01$) when compared with lower dose of PHZ, i.e., 0.025 mg/100 g b.wt. [Fig. 1(d)].

Comparative Effect of Melatonin and Vitamin E Injections Following PHZ Administration on Splenocytes and Peripheral Blood of Squirrels

TLC of PHZ-treated group was higher than sham-injected controls ($P < 0.01$). Melatonin supplementation reduced the PHZ-enhanced TLC, while vitamin E reduced the count more significantly [$P < 0.05$, Fig. 2(a)].

The mean TBARS level in PHZ-treated group was higher than sham-injected controls ($P < 0.01$). Seven days of melatonin supplementation reduced TBARS level significantly ($P < 0.01$), while the reduction by vitamin E was less ($P < 0.05$) when compared to sham-injected controls [Fig. 2(b)].

Mean SOD activity was lower in PHZ group than in sham-injected controls ($P < 0.01$). Melatonin restored SOD activity of squirrel's spleen when compared with PHZ group ($P < 0.05$). However, no significant change in SOD activity was observed after vitamin E supplementation [Fig. 2(c)].

Significant increase ($P < 0.01$) in apoptosis of PHZ group was noted when compared with sham-injected control. This was significantly reduced by melatonin and vitamin E supplementation ($P < 0.01$) alone when compared with PHZ group [Fig. 2(d)]. Figure 3 presented morphologies of splenocytes stained with AO-EB.

Splenomegaly induced by PHZ treatment was significantly inhibited when melatonin was given along with PHZ treatment ($P < 0.01$). Similarly, vitamin E also reduced the effect of PHZ-induced splenomegaly [$P < 0.01$, Fig. 2(e)].

Significant decrease in ABTS radical cation scavenging capacity of antioxidants was noted in PHZ group D (1 mg/100 g b.wt.) at all time points when compared to rest of the groups [Fig. 2(f)]. The scavenging capacities of the antioxidants were time-dependent. The majority of interaction of antioxidants in melatonin and PHZ cotreated group F occurred from 1 to 10 min surpassing the scavenging rate of antioxidants in vitamin E and PHZ cotreated group H after 1 min.

DISCUSSION

PHZ-induced ROS and free radicals play a vital role in carcinogenesis, tumor promotion, and progression. Organisms possess natural defenses against free radical overload in the form of antioxidants and antioxidant enzymes, which are particularly effective for neutralizing specific ROS/reactive nitrogen species (RNS) such as SOD-neutralizing superoxide radical and catalase-neutralizing H_2O_2 .

The reason for observed splenomegaly in *F. pennanti* following PHZ treatment might be because of the increase in erythrophagocytosis, primarily in red pulp resulting in increased numbers of macrophages, increased size of macrophages, and retention of erythrocytes, which

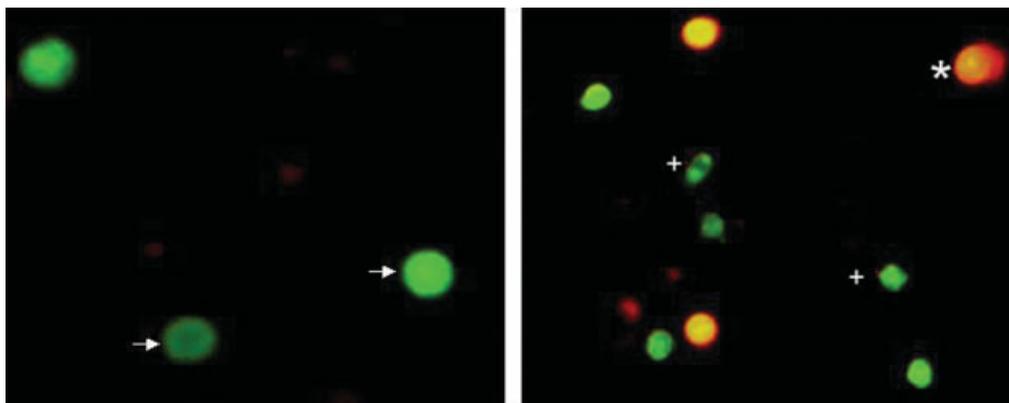


Fig. 3. Morphological changes observed in splenocytes stained with Acridine Orange–Ethidium Bromide (AO–EB) dye observed under fluorescence microscope at 920 \times magnification. Arrows (\rightarrow) indicate control, Plus (+) indicate early apoptotic cells, and asterisk (*) indicate late apoptotic cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

altogether combined to enlarge the spleen (Sorrell and Weiss, 1982). Gudima and Taylor (2001) have reported that PHZ injections into the peritoneum of the mouse destroyed erythrocytes and induced anemia that in turn produced an increase in β -globin mRNA in the spleen. In the present study, spleen underwent major increase in mass by day 7 as a consequence of three increasing doses of PHZ.

Chronic administration of PHZ in both high doses (0.5 mg and 1 mg/100 g b.wt.) produced significant oxidative damages as observed by high TBARS level, greater percentage of spleen cell death, and highly suppressed SOD activity during reproductively active phase (RAP) of the squirrel, whereas low dose of PHZ (0.025 mg/100 g b.wt.) resulted in nonsignificant increase in toxicity. To note the protection being provided by melatonin and vitamin E on splenic damages induced by 1 mg PHZ/100 g b.wt., we carried out another experiment with melatonin cotreatment (i.e., 0.5 mg/100 g b.wt.) and compared its effectiveness with vitamin E cotreatment (1 mg/100 g b.wt.). As shown in the present study, the combined treatment of vitamin E and PHZ showed equal protection as that of melatonin and PHZ in terms of cell death, splenomegaly, and leukocyte count, but vitamin E cotreatment was comparatively less effective in terms of TBARS level and had no difference when compared for their effectiveness on SOD activity. This might be due to differences in vitamin E lipophilic nature, unlike melatonin's amphiphilic nature. During its radical-scavenging action, vitamin E becomes a radical and loses its antioxidant activity and needs vitamin C to restore the properties, while the antioxidant action of melatonin involves donation of two electrons thereby ensuring that it does not become a free radical. Unlike other antioxidants, the metabolites of melatonin such as N^1 -acetyl- N^2 -formyl-5-methoxykynuramine (AFMK) and N -acetyl-5-methoxykynuramine (AMK) are themselves very potent free radical scavengers. Further, melatonin's one molecule is sufficient

to 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). Hence, in our study, the high dose of melatonin (0.5 mg/100 g b.wt.) for 7 days prevented the rise in oxidative damages of spleen to a greater extent than vitamin E action against higher doses of PHZ administration. SODs catalyze dismutation of O_2^- to O_2 and H_2O_2 . We found a decrease in SOD activity following PHZ administration indicating proper free radical load. Melatonin administration elevated enzyme activity of SOD in splenic tissue of *F. pennanti* as observed previously by Antolin et al. (1996), Liu and Ng (2000), Mayo et al. (2002) in hamsters and rats.

The extent of radical-induced damage is determined by the activity of free radical scavengers (Tan et al., 2003). The role of melatonin in stimulating the protective activity of antioxidant enzymes was measured by ABTS radical cation reduction as reported previously (Bartosz and Bartosz, 1999; Herraiz and Galisteo, 2004). Presence of high level of free radicals in PHZ-treated splenic tissue showed a delay in ABTS $^+$ radical cation scavenging rate by antioxidants. Presence of less free radical in both melatonin- and vitamin E-treated tissues showed comparatively increased ABTS percentage inhibition as determined by the degree of ABTS decolorization, which is directly proportional to the scavenging activities of antioxidants present in the system. Our results showed the melatonin and vitamin E as effective scavengers of ABTS radical cation. However, ABTS scavenging potency continued for longer duration in PHZ group cotreated with melatonin (0.5 mg/100 g b.wt.) with a sudden rise observed in scavenging activity of antioxidants after 1 min. But PHZ group cotreated with vitamin E (1 mg/100 g b.wt.) showed quite fast ABTS scavenging potency during 1 min, which decreased after 1 min and showed lower antioxidant activity when compared with

melatonin and PHZ group F [Fig. 2(f)]. The reason for this relatively short duration of scavenging activity might be because of the interaction of one ABTS radical cation with one vitamin E molecule while on the other hand, neutralization of four ABTS radical cations by one melatonin molecule leading to the higher scavenging rate in PHZ plus melatonin-receiving group F.

Melatonin seems to be an integral part of the immune system, exerting direct and/or indirect stimulatory effects on both cellular and humoral immune responses and neoplastic growth (Fraschini et al., 1998; Skwarlo-Sonta, 2002; Singh and Haldar 2005). It is known to counteract the immunosuppression induced by acute stress (Maestroni et al., 1988; Maestroni and Conti, 1989; Haldar et al., 2004). It has antiproliferative role in numerous cancer cell lines (Blask et al., 1997; Petranka et al., 1999; Shiu et al., 2000; Treeck et al., 2006). It regulates cellular functions through intracellular second messengers such as cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG), inositol phosphate (IP₃), calcium ion (Ca²⁺). Nonreceptor-mediated free radical scavenging action of melatonin is also reported (Reiter, 1995) in directly neutralizing the effect of reactive intermediates generated within the cell. Melatonin act directly on immune tissue via receptors on spleen cells to enhance their proliferative ability and thus immune function (Drazen et al., 2001).

The AO-EB-stained splenic cells showed cell shrinkage, surface blebbing, and chromatin condensation, which provide as a morphological profile of an apoptotic cell (Fig. 3). Melatonin-mediated activation of scavenging action and modulation of other antioxidative enzyme responses might have prevented leaking of chemical balances from the cell thus causing less apoptotic death of splenocytes in group F [Fig. 2(d)]. Hence, protection of lipids, proteins, and DNA from harmful effects of free radicals by melatonin, as previously noted by Reiter and Tan (2003) and Sainz et al. (2003), might also be responsible for immunomodulation.

CONCLUSION

Thus, the results of our study proposes immunomodulatory role of melatonin in splenic physiology and thereby regulation of immune status of tropical seasonal breeder, *Funambulus pennanti*. We suggest that when the squirrels are healthy and reproductively active, introduction of PHZ is stressful and induces free radical load of spleen. Such a situation can be improved with melatonin or vitamin E administration to a greater extent.

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