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ORIGINAL ARTICLE

Age dependent expression of melatonin membrane receptor (MT1, MT2) and its role in regulation of nitrosative stress in tropical rodent *Funambulus pennanti*

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Abstract
Age-dependent declining level of melatonin induces free radical load and thereby deteriorates immune function. However, reports are lacking about age-dependent melatonin membrane receptor (MT1 & MT2) expression, their role in regulation of reactive nitrogen species (RNS) and eventually how they affect immunity of a tropical rodent *F. pennanti*. We checked MT1R, MT2R and iNOS expression in lymphoid organs of young middle and old aged squirrels. Nitrite and nitrate ion concentration (NOx) in lymphoid organs, testes and plasma, lymphocyte proliferation and IL-2 level was recorded. Age-dependent decrease in MT1 and MT2 receptor expression, lymphocyte proliferation, IL-2 level and increased RNS in lymphoid organs, testes and plasma was observed with decreased circulatory melatonin. Androgen and AR expression was increased in middle-aged while declined in old-aged squirrels. Present study suggests that age associated immunosenescence is consequence of increased RNS which might have important relationship with melatonin membrane receptors in *F. pennanti*.

Keywords: *Aging, Melatonin, MT1R, MT2R, Nitrosative stress, Immune system*

Introduction

Aging is associated with a decline in immune function known as immunosenescence [1]. The 'Oxidative Theory of Aging' by Harman [2] states that reactive oxygen species (ROS) and nitrogen species (RNS) causes random damage to cells which includes impaired physiological functions and increased incidence of diseases with increased age, ultimately leading to mortality. Nitric oxide (NO) is an important mediator causing nitrosative stress during several physiological conditions like inflammation and found to be responsible for increased oxidative load with advancing age [3]. NO synthesis is caused by three isoforms of NO synthases (inducible, neuronal and endothelial) where inducible NOS2 (iNOS) is involved in mediating inflammatory stress. Inducible NOS produces very large, toxic amounts of NO in a sustained manner which is mainly considered to inhibit the expression of genes involved in cellular proliferation and growth.

The decline in the production of a number of hormones associated with aging such as, growth hormone (GH), estrogen, dehydroepiandrosterone and the pineal substance melatonin, have been proposed to play a significant role in immunomodulation [4]. Melatonin has been demonstrated to bear a general immunoenhancing effect in many animal species as well as in humans [5]. Serum melatonin concentrations are known to be affected by the age of the animal; secretion increases and becomes circadian in older infants, the peak nocturnal concentrations are highest at the ages from 1 to 3 years, after which they decline gradually [6]. Number of studies suggest that pinealectomy produces various pathological changes resembling senescence are reversed by the administration of a pineal extract or melatonin. The primary effect of aging on the immune system implies a functional decrease in T lymphocyte responsiveness [7]. However, most of the studies dealing with role of melatonin in age associated immunosenescence have

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been carried out in laboratory rodents however there exists an acute paucity of information in wild seasonal breeder where melatonin plays more significant role in terms of seasonality and reproduction. Further, the reason behind the declining melatonin level and immune responses with age in wild rodents are still unknown. Our objective was to explore whether there exist any relationship between the age dependent neuroendocrine control of melatonin secretion and modulation of immune responses in a wild tropical rodent *F. pennanti.*

Melatonin membrane receptors (MT1 & MT2) located on number of neural and non neural target organs including immunocompetent cells are reported to have role in modulating immune function [8]. Despite of the handful of reports regarding age dependent decrease in circulatory melatonin responsible for immunosenescence following increased oxidative stress there is an absolute scarcity of information about the age dependent role of melatonin membrane receptors MT1 and MT2 and their correlation with oxidative stress in the form of iNOS (RNS) in any seasonally breeding rodent.

We hypothesized that the decreased melatonin with age could possibly affect its membrane receptor (MT1 and MT2) expression on lymphoid organs, which in turn can possibly affect the compromised immune function. For the study, we have checked melatonin receptor MT1 and MT2 expression along with iNOS expression and lymphocyte proliferation in lymphoid organs (spleen and thymus) of young-, middle- and old aged *F. pennanti.*

Materials and methods

Maintenance of animals

All the experiments were conducted in accordance with institutional practice and within the framework of revised Animal (Specific Procedure) Act of 2007 of Govt. of India on Animal Welfare. Indian Palm squirrel *Funambulus pennanti* is a diurnal, seasonal (long day) breeder of Indian origin. They are semi-domestic in nature. Details of its habit, habitat and reproductive pattern have been published elsewhere [9]. The squirrels were collected from the vicinity of Varanasi (Lat. 25°, 18' N; Long. 83°, 1'E). After 2 weeks of acclimatization to laboratory conditions (equivalent to ambient condition) healthy young male squirrels (~180–200-day old), middle-aged (~16–18-months old) and old-aged (~2.5–3-year old) male squirrels were randomly selected and divided into three groups according to the experimental plan having five male squirrels in each group. The lifespan of squirrels is ~3–3.5 years in captivity. The age of squirrels were determined by measuring incisor length and cranium diameter as reported previously [10]. During the experiments, squirrels were maintained in individual wire net cages (25″ × 25″ × 30″ in size) in a well-ventilated room exposed to ambient conditions (ambient photoperiod ~12L(light):12D(dark), average day/night temperature ~35°C/30°C; humidity ~50%). Squirrels were fed with soaked gram seeds (*Cicer arietinum*), nuts, seasonal fruits/vegetables and water *ad libitum*.

Experiment

Male squirrels, *F. pennanti* were randomly selected and divided into three groups each containing five animals according to their age. Group I having five male young squirrels served as young-aged group (Y; weighing 60 ± 10 g, ~180–200-day old). In group II, middle-aged male squirrels were taken (MA; weighing 110 ± 10 g, ~16–18-month old). Group III had old-aged squirrels (OA; weighing 125 ± 10 g, ~2.5–3-year old).

Sample collection

The squirrels were weighed and sacrificed by decapitation after deep ether anesthesia. All the sacrifices were made during the night time between (20:00 h–22:00 h, IST). The trunk blood was collected in heparinized tubes and centrifuged at 3000 rpm for 20 min at 4°C. Plasma was kept at −20°C till the hormonal and biochemical estimations were performed. Spleen and thymus was dissected out on ice, weighed and processed for the assay of blastogenic response in terms of lymphocyte proliferation, and also, a part of it was kept for immunoblot analysis and biochemical estimations.

Western blot analysis of melatonin membrane receptor (MT1 and MT2), androgen receptor (AR) and inducible nitric oxide synthase (NOS2 or iNOS)

Western blot was done as published elsewhere [11]. Spleen, thymus and testes was homogenized and lysed in lysis buffer (RIPA buffer containing aprotinin, sodium orthovanadate and phenylmethylsulfonylfluoride (PMSF)) and quantified by Bradford method [12]. Aliquots containing 60 μg protein for melatonin receptor subtypes (MT1R and MT2R) androgen receptor (AR) and 100 μg for inducible nitric oxide synthase (iNOS) were resolved with 12% (for Mel receptor), 10% (for AR) and 8% for (iNOS) SDS–polyacrylamide gel electrophoresis, respectively, followed by electrotransfer to nitrocellulose membrane (Bioscience, Keene NH, USA). Immunodetection was carried out by using melatonin receptor antibodies (MT1R, R-18, & MT2R, T-18, Santa Cruz Biotech, USA, diluted 1:200), androgen receptor antibody (AR, N-20, Santa Cruz Biotech, USA, diluted 1:250), iNOS antibody (NOS2, M-19; sc-650, Santa Cruz Biotech, USA, diluted 1:100) and β-actin

antibody (A-2228, Sigma–Aldrich Chemicals, St. Louis, USA, diluted 1:1000) all were diluted in phosphate buffer saline (PBS; 0.1 M NaH_2PO4, Na_2HPO4, NaCl; pH 7.4) containing 5% skimmed milk and 0.1% Tween-20 followed by horseradish peroxidase conjugated secondary antibody (donkey anti-goat IgG, for MT1, MT2 and iNOS; donkey anti-rabbit IgG for androgen receptor and donkey anti-mouse IgG for β-actin, diluted 1:10 000), which were further, detected using Super Signal West Pico Chemiluminescent Substrate (P-34080, Thermo Scientific, Rockford, USA). Bands were quantified by measurement of optical density using Scion Image Analysis Software (Scion Corporation, MD, USA). Values were expressed as the ratio of the density of the specific signal to β-actin signal [11].

MTT assay for lymphocyte proliferation

Cell-mediated immune function was assessed by measuring splenocytre/thymocyte proliferation in response to the T-cell specific mitogen, Concanavalin-A (Con-A), using a colorimetric assay based on the reduction of tetrazolium salt Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) [13]. Spleen and thymus of squirrels were removed in sterile condition and a single-cell suspension was prepared by mincing and grinding them between sterile frosted glass slides. Erythrocytes were lysed by hypotonic shock using equal volume of cold ammonium chloride-Tris buffer (tris hydroxymethylene aminomethane, SRL, Mumbai, India); 0.5% Tris buffer and 0.84% NH_4Cl mixed in 1:10 ratio; pH 7.2). This single cell suspension along with ice-cold culture medium (RPMI-1640 supplemented with 1% penicillin (5000 U/mL), streptomycin (100 μg/mL), 1% L-glutamine (2 mM/mL), 0.1% 2-mercaptoethanol (5×10^{-2} M/mL) and 10% heat-inactivated fetal calf serum). The resulting cell suspension was washed three times and splenocyte/thymocyte counts and viability were determined with a hemacytometer and trypan blue exclusion method. Viable cells (which exceeded 95%) were adjusted to 1×10^7 cells/mL by dilution with culture medium, and 100 μL aliquots of each cell suspension were added to the wells of sterile flat-bottom 96-well culture plates. Concanavalin-A (Sigma–Aldrich, St. Louis, USA) was added to the culture medium at the concentration of 10 μg/mL. Fifty microliters of mitogen was added to the wells of the plate, which would contain the spleen/thymus cell suspensions to yield a final volume of 150 μL/well (each in duplicate). Finally, 50 μL of complete medium was added to make the final volume of 200 μL/well (each in duplicate). Plates were incubated at 37°C with 5% CO_2 for 69 h prior to addition of 10 μL of MTT (SRL, Bombay, India; 5 mg/mL in phosphate-buffered saline) per well. Plates were then incubated at 37°C with 5% CO_2 for an additional 4 h. At 72 h, 150 μL of acidified propanol (0.04 mol/L HCL in isopropanol) was added to each culture, and the optical density (OD) of each well was determined with a microplate reader (ELx-800, Biotek Instruments, Winooski VT, USA) equipped with a 570 nm wavelength filter. Mean OD values for each set of duplicates were used in subsequent statistical analyses. Response was calculated as percent stimulation index representing the ratio of absorbance of mitogen stimulated cultures to control cultures.

Total nitrite and nitrate (NOx) concentration for NO estimation

Total nitrite and nitrate concentration, an indication of NO synthesis, was measured in plasma, spleen, thymus and testes following the method of Sastry et al. [14] and Fiore et al. [15]. Briefly, for plasma nitrite and nitrate concentration, blood was collected in a heparinized tube and centrifuged at 3000 rpm for 20 min. to separate plasma. For tissue, 5% tissue homogenate (5%, w/v, spleen, thymus and testes) was prepared in 0.01 M phosphate buffer, pH = 7.4. To 100 μL of each sample (plasma and spleen/thymus homogenate) or standard (KNO_3), 400 μL of carbonate buffer was added followed by a small amount (0.15 g) of activated copper–cadmium alloy filings and incubated at room temperature with thorough shaking. The reaction was stopped by the addition of 100 μL of 0.35 M NaOH followed by 120 mM $ZnSO_4$ solution under vortex and allowed to stand for 10 min. Tubes were then centrifuged at 8000 rpm for 10 min. Aliquots (100 μL each) of clear supernatant were transferred into the wells of a microplate (in quadruplicate), and Griess reagent (50 μL of 1% sulphanilamide prepared in 2.5% orthophosphoric acid and 50 μL of 0.1% N-naphthyl ethylenediamine prepared in distilled H_2O) was added to it. After 10 min, the absorbance was read at 545 nm in an enzyme linked immunosorbent assay (ELISA) reader (ELx-800, Biotek Instruments, Winooski VT, USA). A standard graph was plotted against different concentrations (0, 20, 40, 60, 80 and 100 μM) of KNO_3.

IL-2 production and analysis in culture supernatant

IL-2 activity was estimated by the activated splenocytes/thymocytes proliferation. Spleen/thymus of squirrels was removed in sterile condition. Splenocyte/thymocytes were isolated by the routine method as explained above. Cells were suspended in RPMI-1640 medium at a concentration of 1×10^7 cells/mL. Then, 100 μL of suspension, 100 μL of Con-A with final concentration of 100 μg/mL and 800 μL RPMI-1640 medium were added to each well of 24-well culture plate, respectively, final volume being 1 mL. The cultures were centrifuged (500 × g, 10 min.) following incubation at 37°C, 5% CO_2 for 48 h. The

supernatants were collected, and preserved at −20°C for IL-2 estimation [16].

ELISA for IL-2

Sandwich ELISA was performed to quantify IL-2 level in culture supernatants and plasma collected from all the groups according to manufacturer's instruction (Immunotech, France). Intra-assay variation was between 3.3 and 7.2%, inter-assay variation was between 6.2 and 8.2%, sensitivity, 5 pg/mL and recovery was between 80 and 132%.

Radioimmunoassay of testosterone and melatonin

Radioimmunoassay of testosterone was performed according to the manufacturer's instruction (Immunotech, France). The intra- and inter-assay variation was 14.8 and 15% respectively. The sensitivity was in between 0.025 and 20 ng/mL and recovery percentage was between 91 and 117. The RIA of melatonin was performed following the method of Rollag and Niswender [17] using Guildhey antisera (Guildhey, Surrey, UK). Details of the method and validation of radioimmunoassay was performed and published elsewhere [18]. The intra- and inter-assay variation for melatonin was 9 and 15% respectively. The sensitivity for melatonin RIA was 18–20 pg/mL. The recovery of melatonin RIA was 92%.

Statistical analysis

All statistical analyses were performed using SPSS version 17.0. Statistical analysis of the data was performed with one-way ANOVA followed by multiple comparisons by the Duncan's multiple range tests. Values are expressed as means ± SE. A p-value of <0.05 was considered statistically significant.

Results

Western blot analysis

Melatonin membrane receptor MT1 and MT2 expression. A significant ($p < 0.01$) decrease in MT1 and MT2 receptor expression was observed in age-dependent manner in both spleen and thymus. Middle-(MA) and old-aged (OA) squirrels showed significant ($p < 0.01$) decrease in MT1 and MT2 receptor expression in both spleen and thymus when compared with young squirrels (Y) (Figure 1A and B).

iNOS expression. iNOS expression in spleen (Figure 2A), thymus (Figure 2B) and testes (Figure 3A) of young-, middle- and old-aged squirrels increased significantly ($p < 0.01$). Further, middle- and old-age group showed significant ($p < 0.01$) increase in iNOS expression when compared with young squirrels (Figures 2A, B and 3A).

AR expression. We noted AR expression in testes of all the three groups. Middle-aged squirrels showed significant increase in AR expression while significant ($p < 0.01$) decrease in old-aged squirrels was noted when compared with young squirrels. Further, old-aged squirrels showed significant ($p < 0.01$) decrease in testicular AR expression when compared with middle-aged group (Figure 3B).

Nitrite and nitrate ion (NOx) estimation. Age-dependent increase in nitrite/nitrate ions (NOx) in plasma, (Figure 4A) spleen, thymus and testes (Figure 4B) of squirrels was noted. Further, middle-and old-aged squirrels showed significant ($p < 0.01$) increase in NOx concentration when compared to the young squirrels (Figure 4A and B).

RIA of melatonin and testosterone. Age-dependent decrease in circulatory level of melatonin was observed in young-, middle- and old-aged squirrels (Figure 5A). Middle- and old-aged squirrels showed significant ($p < 0.01$) decrease in plasma melatonin level when compared to young squirrels. Plasma testosterone presented similar pattern to that of AR expression in testes. Middle-aged group presented significant ($p < 0.01$) increase while old-aged squirrels showed significant ($p < 0.01$) decrease in circulatory testosterone level when compared with young squirrels (Figure 5B).

Lymphocyte proliferation index of spleen and thymus. Lymphocyte proliferation in terms of percent stimulation index was noted following the challenge by a T-cell specific mitogen, Con-A to splenocytes and thymocytes. Splenocyte and thymocyte proliferation showed significant ($p < 0.01$) decrease in age-dependent manner in all the three groups. Middle- and old-aged squirrels showed significant ($p < 0.01$) decline in splenocytes and thymocyte proliferation index when compared with young squirrels (Figure 6A).

ELISA of IL-2 in plasma and culture supernatants of splenocytes/thymocytes. IL-2 level followed the pattern of lymphocyte proliferation. A significant ($p < 0.01$) decrease in age-dependent manner was noted in culture supernatant and plasma of all the three groups. Further, middle- and old-aged squirrels showed significant ($p < 0.01$) decrease in IL-2 level of culture supernatant and plasma when compared with young squirrels (Figure 6B and C).

Discussion

The present study demonstrates age-dependent expression of melatonin membrane receptor (MT1 and MT2) and iNOS in lymphoid organs and their

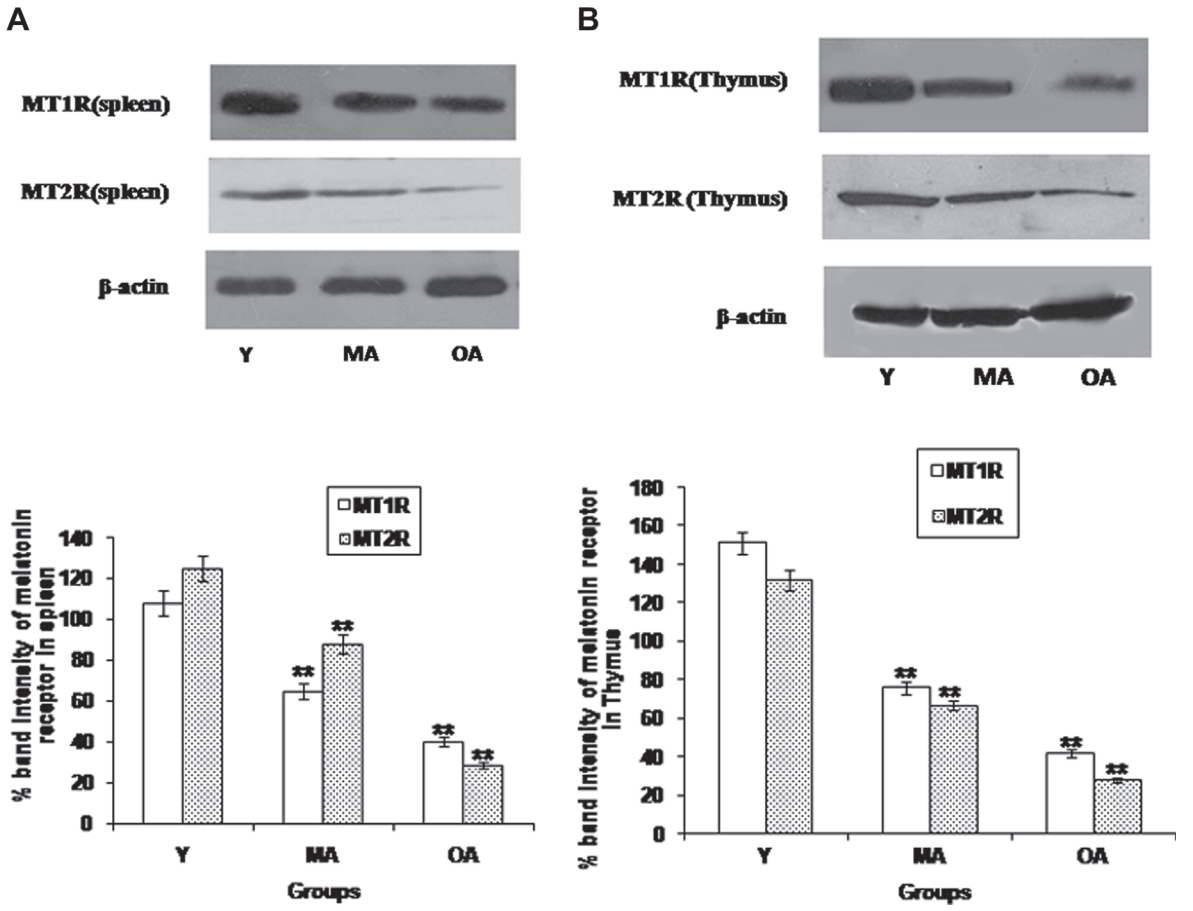

Figure 1(A, B). Western blot analysis of MT1 and MT2 receptor expression in spleen and thymus of young-(Y), middle aged-(MA) and old-aged (OA) *F. pennanti.* β-actin expression was used as loading control. Histogram (lower panel) shows the % expression of receptor following Scion image analysis. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $^{**}p < 0.01$, Y vs. MA and OA.

possible correlation with immune function of young middle- and old-aged tropical squirrels. With advancing age and diminishing circulatory melatonin level, the MT1 and MT2 receptor expression decreased while iNOS expression along with nitrite/nitrate ion concentration increased in spleen and thymus. Further, we have noted declined immune response with increasing age in terms of lymphocyte proliferation and IL-2 level in *F. pennanti*. Earlier report from our lab suggests that melatonin regulates its own membrane receptor on lymphoid organs, which in turn affects immune function of *F. pennanti* [19]. However, the present study revealed that age-related increase in nitrosative stress and reciprocal suppression of immune function has a strong association with melatonin membrane receptor (MT1 and MT2), which could be an important aspect to study in the field of melatonin physiology.

The free radical theory of aging [2] suggests that aging is the result of the failure of various protective mechanisms to counteract the damage induced by ROS/RNS radicals. Moreover, alterations in melatonin levels are involved in aging and age-related diseases [20]. Melatonin has been found to be a highly potent antioxidant and free radical scavenger [21,22], which increases survival and reduces the severity of debilitating diseases [20]. We observed decreasing trend in melatonin level with age being lowest in old squirrels and highest in young squirrels. Interestingly, increasing level of nitrite/nitrate ion was recorded in plasma with advancing age which is in accordance with the previous reports suggesting an inverse relationship between circulatory melatonin and free radicals.

We observed significant decrease in melatonin receptor MT1 and MT2 expression in both spleen and thymus of middle-and old-aged squirrels. However, recent study by Sanchez-Hidalgo et al., [23] reported significant decrease in MT1 receptor expression in middle-age spleen but found no significant change in thymus. The inconsistent result obtained in case of thymus may be a tissue specific regulation of melatonin receptor expression, which forms the key aspect involved in development, maintenance and function of the thymus. This differential regulation of the expression pattern could be a consequence of the different natural and geographical habitat to which the rat and wild tropical squirrel are exposed to. Further, the obtained varying results could be the outcome of ever changing signals of melatonin which are profound in case of seasonally breeding wild animals [24,25].

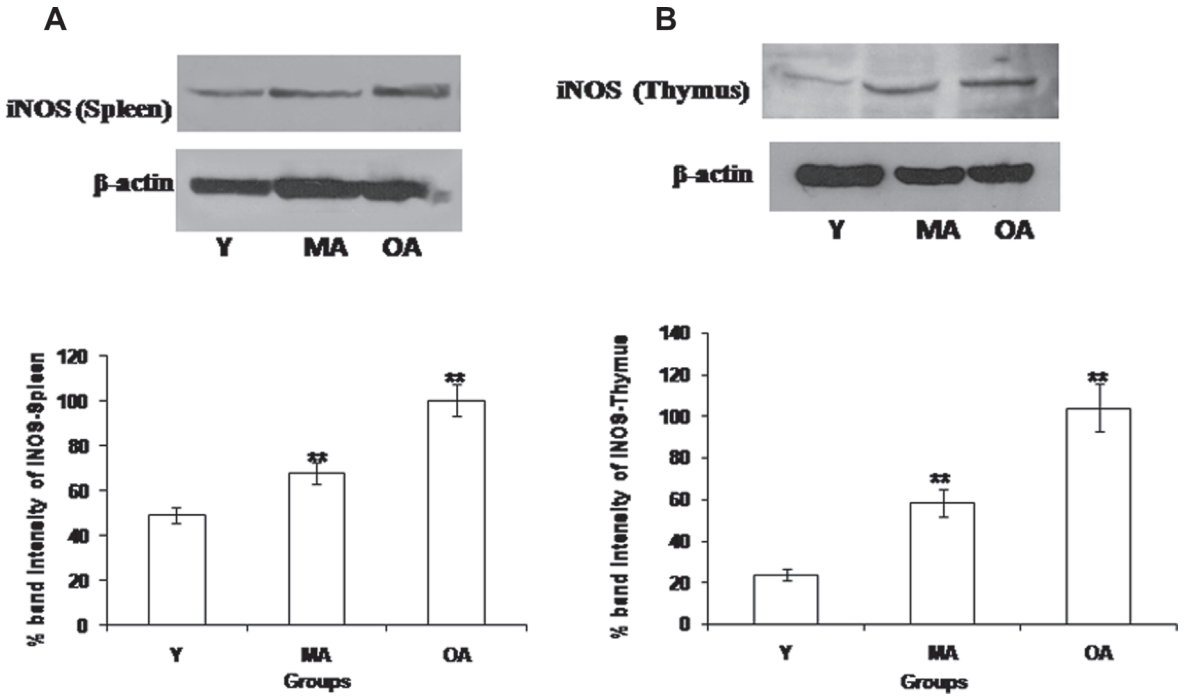

Figure 2(A, B). Western blot analysis of iNOS expression in spleen and thymus of young (Y), middle-aged (MA) and old-aged (OA) *F. pennanti.* β-actin expression was used as loading control. Histogram (lower panel) shows the % expression of receptor following Scion image analysis. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $^{**}p < 0.01$, Y vs. MA and OA.

The marked decrease in MT1 and MT2 receptor expression in age-dependent manner in lymphoid organs of *F. pennanti*, could be taken as major factor involved in reducing lymphocyte proliferation of middle- and old-aged squirrels. The T cell responsiveness to *in vitro* mitogen (Con-A) stimulus was greatly diminished in aged squirrels which may be due to the reduction in IL-2 secretion as a function of aging

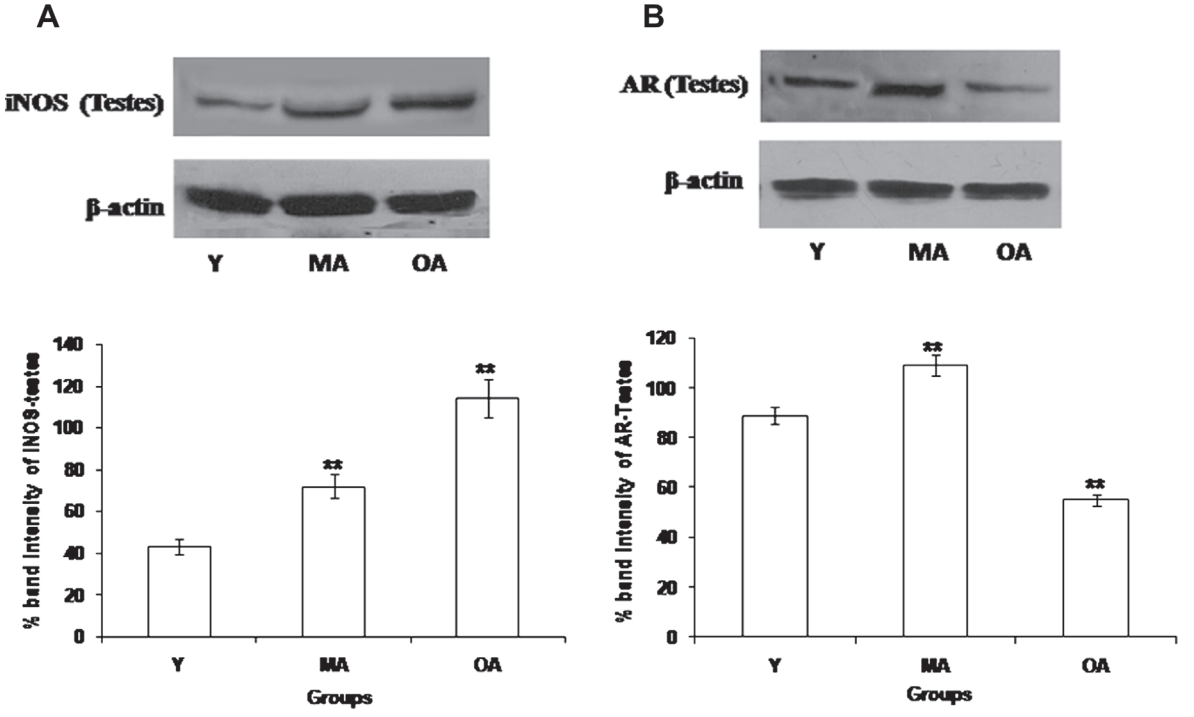

Figure 3A. Western blot analysis of iNOS expression in testes of young (Y), middle aged (MA) and old aged (OA) *F. pennanti.* β-Actin expression was used as loading control. Histogram (lower panel) shows the % expression of receptor following Scion image analysis. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $p < 0.01$, Y vs. MA and OA. B Western blot analysis of AR expression in testes of young (Y), middle aged (MA) and old aged (OA) *F. pennanti.* β-Actin expression was used as loading control. Histogram (lower panel) shows the % expression of receptor following Scion image analysis. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $^{**}p < 0.01$, Y vs. MA and OA.

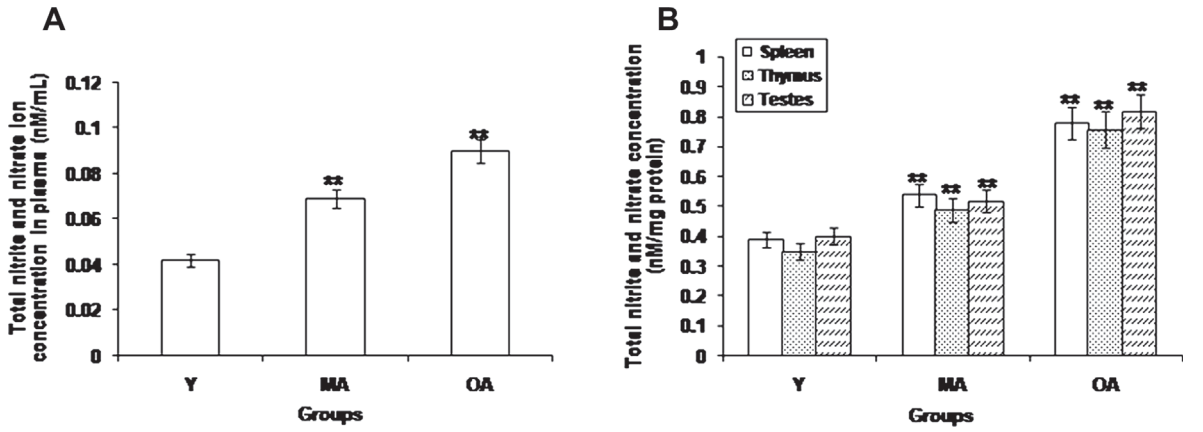

Figure 4A. Total nitrite and nitrate concentration in plasma of young (Y), middle aged (MA) and old aged (OA) *F. pennanti*. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $^{**}p < 0.01$, Y vs MA and OA. B Total nitrite and nitrate concentration in spleen, thymus and testes of young (Y), middle aged (MA) and old aged (OA) *F. pennanti*. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $^{**}p < 0.01$, Y vs. MA and OA.

[26,27]. The age-associated decline in immune function is characterized by a decrease in the functional activity of NK cells, granulocytes and macrophages [28]. Besides causing changes in innate immunity, aging is associated with changes in cellular and humoral immunity [29,30]. Similarly, we found decreased level of IL-2 *in vivo* and *in vitro* condition with increasing age, which represents reduced ability of innate and adaptive immune system to counter the increasing RNS load in aged squirrels.

Although melatonin displays NO-scavenging properties [21,31], the effect to reduce nitric oxide (NO) levels mainly depends on its ability to inhibit the expression of iNOS [32–34]. Therefore, we checked iNOS expression in lymphoid organs to find out the impact of reduced circulatory melatonin level with age. We found increased iNOS expression with age in spleen, thymus and testes of squirrels which suggest that reduced level of melatonin was unable to counteract the elevating level of RNS in middle- and old-aged squirrels. The major effect of increased RNS in lymphoid organs as well as plasma is the compromised immune function in terms of reduced IL-2 level and lymphocyte proliferation with increasing age. Our study reports a clear cut age-dependent, trade-off relationship between melatonin membrane receptor expression and iNOS in lymphoid organs which could be the possible reason behind compromised immune function in squirrels.

NO function is an important mediator in action of hormones and neurotransmitters which are vital for the regulation of reproduction [35]. Nitric oxide acts as a possible mediator of testicular actions such as stress and inflammation. More specifically, the application of NO donors reduced testicular testosterone (T) production *in vivo* [36] as well as *in vitro* [37]. We have also checked testicular NOx, iNOS and correlated with AR expression in testes and circulatory testosterone. NOx and iNOS expression increased in age-dependent manner and showed inverse relationship with circulatory androgen and AR expression in testes. Our results were in line with the previously reported inverse relationship between NOx levels in plasma or testes and T content observed during the

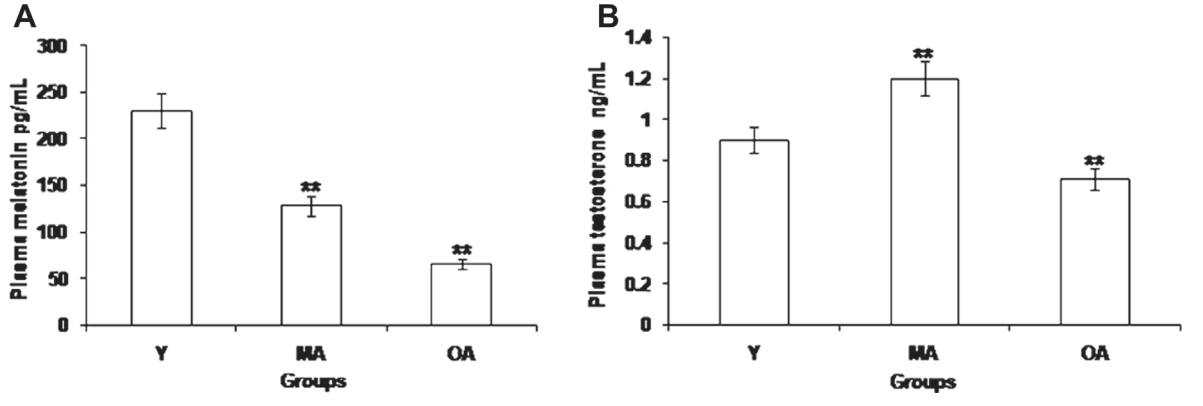

Figure 5(A, B). Circulatory levels of plasma melatonin (pg/mL) and testosterone (ng/mL) in young (Y), middle aged (MA) and old aged (OA) *F. pennanti*. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $^{**}p < 0.01$, Y vs. MA and OA.

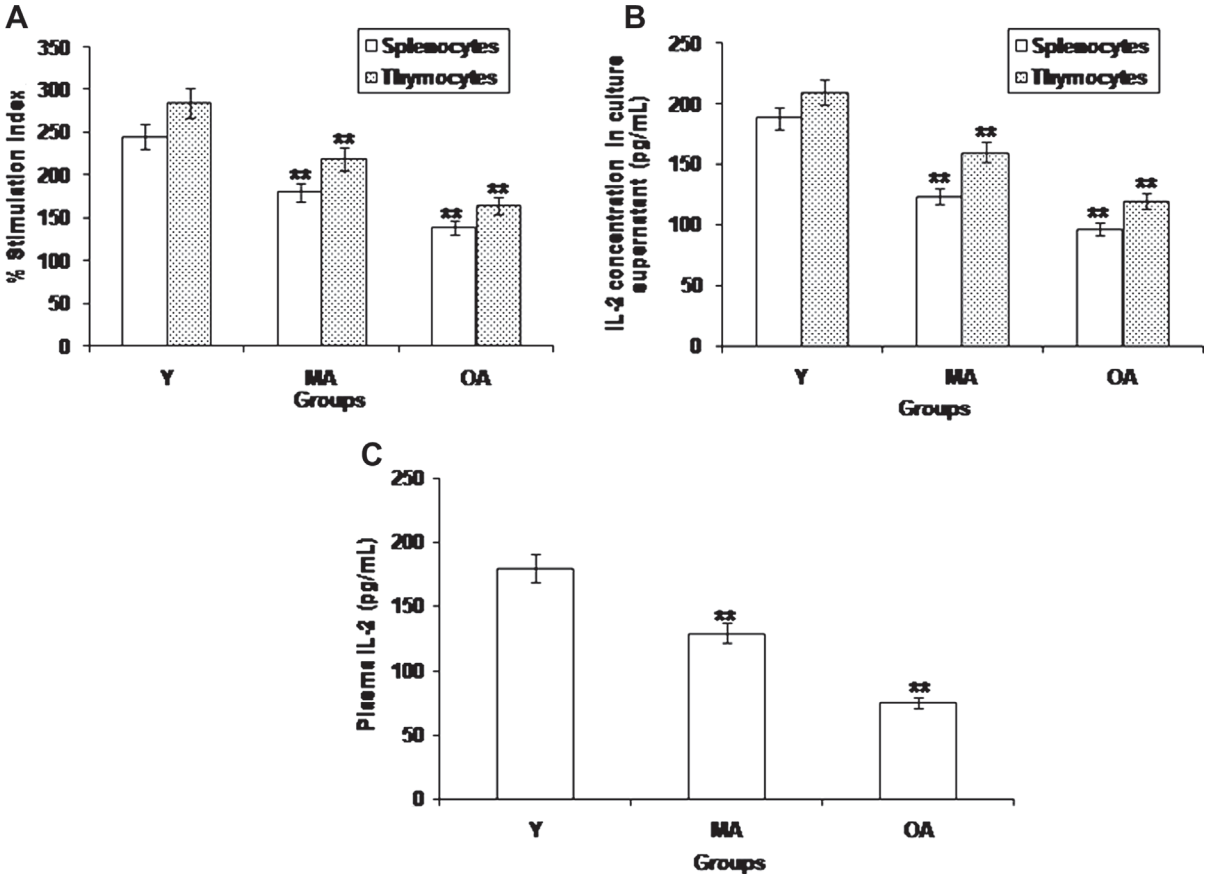

Figure 6A. Blastogenic response in terms of % stimulation index of splenocytes and thymocytes in young- (Y), middle- (MA) and old-aged (OA) *F. pennanti*. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $^{**}p < 0.01$, Y vs. MA and OA. B *In vitro* IL-2 levels in stimulated splenocyte and thymocyte culture supernatant of young- (Y), middle- (MA) and old-aged (OA) *F. pennanti*. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $^{**}p < 0.01$; Y vs. MA and OA. C *In vivo* IL-2 levels in blood plasma of young- (Y), middle-aged (MA) and old-aged (OA) *F. pennanti*. Values are expressed as mean, bars represent ± SEM, N = 5. Significance of difference $^{**}p < 0.01$; Y vs. MA and OA.

inflammatory response. NO has been shown to reduce T production *in vivo* [36] and to directly suppress Leydig cells function *in vitro* [37,38]. Similarly, in our study NO acted as a free radical molecule and enhanced nitrosative stress with increasing age which might have declined testosterone and AR in the old-aged squirrels.

Interestingly, middle-aged squirrels showed high NOx, AR and testosterone at the same time. It could be explained on the basis that testicular NO signalling pathway is involved in regulation of Leydig cell steroidogenesis [36–37,39]. Further, in middle-aged squirrels testosterone could also be responsible for up-regulation of iNOS activity as reported in rat [40]. Therefore, in middle-aged squirrels, NO and androgen level is maintaining reproductive physiology, while in old-aged squirrels, NO is more of acting as a free radical and is responsible for increased iNOS and NOx level in plasma, which might be hampering reproductive physiology as evidenced by reduced androgen and AR expression.

Since, testicular macrophages reside in close proximity to Leydig cells in the interstitial tissue [41], they have been hypothesized to act as a source of NO, thereby modulating Leydig cell steroidogenesis under conditions of immune activation [42,43]. The harmful effects of NO are mediated by biologically activated molecules produced by the reaction of NO with the superoxide anion yielding $ONOO^-$ and peroxynitrous acid (ONOOH) causing molecular damage to a variety of tissues [44]. We found an age-dependent increase in iNOS expression and nitrite/nitrate ion concentration in testes of *F. pennanti* and recorded increase in reproductive aging with increasing age. This increase of NO markers in testes with age could also be the major reason behind the reduced reproductive function. Increased testicular iNOS expression in middle- and old-aged squirrel presented an inverse relationship with MT1 and MT2 R expression in lymphoid organs, which could be taken as another factor involved in suppressing immune function in these squirrels. The present study marks the relationship between nitrosative stress and melatonin membrane receptor (MT1 and MT2) in wild tropical rodents. Our study gets strong support from earlier reports stating the role of melatonin in regulating antioxidative enzymes via its membrane and nuclear receptors [45], while the report from Kilic et al. [46]

proposes no direct relationship between the melatonin receptor and iNOS expression in mice brain, which is a neuronal tissue and not a lymphatic one, suggesting an existence of a tissue specific regulation of iNOS expression.

In summary, diminishing levels of melatonin with progressing age usually subsides the sensitivity and responsiveness of the target tissues (lymphoid organs) to the melatonin due to decrease in the expression of membrane-bound melatonin receptor, aggravating the likelihood of nitrosative stress and, thereby, compromising the immune function in a tropical seasonal breeder *F. pennanti*. The age related decrease in the expression of melatonin receptor may be considered as a generalized consequence of deterioration of the physiological functions with aging [23]. In nutshell, the present study suggest that apart from declining level of melatonin and increasing oxidative stress with age, there also exists a possible role of melatonin membrane receptor. Therefore, more precise studies are required in order to decipher the specific role of melatonin membrane receptors in age associated immune disorders marked by the enhanced oxidative stress.

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Declaration of interest

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