ISSN 0969-711X, Volume 37, Number 3

Endocr (2010) 37:479–488
DOI 10.1007/s12020-010-9331-7

ORIGINAL ARTICLE

Effect of intra-testicular melatonin injection on testicular functions, local and general immunity of a tropical rodent *Funambulus pennanti*

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Received: 17 November 2009 / Accepted: 29 March 2010 / Published online: 13 April 2010

Abstract Local antigonadotrophic action of melatonin in testes has never been correlated with local and general immune status of any rodent. Intra-testicular injection of melatonin (2.5 μg/50 μl) for 10 days (MI-10D) and 20 days (MI-20D) was given to young adult male of *Funambulus pennanti* and testicular androgen receptor (AR), androgen binding protein (ABP) expression, 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, and Mel1aR expression in thymus was checked along with general immune parameters. Further, immunohistochemical localization of Mel1aR in testes was done. Decreased AR, ABP expression, testes weight, 3β-HSD activity, testosterone level, and spermatogenesis but increased Mel1aR expression in thymus, immunoreactivity in testes, and testicular macrophages following injection was noted. Lymphatic tissue weight, leukocyte, lymphocyte count, lymphocyte proliferation in spleen, thymus, plasma melatonin, and IL-2 level increased in a duration-dependent manner following intra-testicular injection. Intra-testicular injection of melatonin decreased steroidogenesis by enhancing the primary effect of melatonin on Leydig cell endocrine function. Along with reduced circulatory testosterone production, an increase in testicular as well as general immunity was observed in a duration-dependent manner. Therefore, a local participation of melatonin in testes of *F. pennanti* to control testicular androgen production is suggested.

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Keywords Steroidogenesis · Androgen receptor · Testes · Melatonin receptor · Immunity · *Funambulus pennanti*

Introduction

The role of melatonin as a pro and antigonadotrophic substance in spontaneous and seasonal breeders has been repeatedly established [1, 2]. Previous reports in hamster suggest that melatonin can act directly on the testes to suppress luteinizing hormone (LH)-stimulated testosterone release by Leydig cells [3]. Melatonin receptors (Mel1aR) have also been located in immature rat Leydig cells [4, 5]. These results suggest that melatonin may directly act on Leydig cells to suppress the testosterone release by rat and hamster testes in vitro. However, a local application (in vivo) of melatonin, i.e., in testes and regulation of male reproduction in terms of spermatogenesis and steroidogenesis has never been investigated. Further, male contraception is also a potential area of research where the negative effect of local application of melatonin on spermatogenesis will be of high potential. Therefore, in the present study, we first planned to note the effects of intra-testicular injection of melatonin on steroidogenesis (androgen receptor (AR), androgen binding protein (ABP) expression, and 3β-hydroxysteroid dehydrogenase (3β-HSD) activity), spermatogenesis and localization of Mel1aR immunoreactivity in testes of a tropical squirrel and diurnal seasonal breeder, *Funambulus pennanti* during the reproductively active phase.

Melatonin acts on immune system and also modulates reproduction by acting at different level of the hypothalamo–pituitary–gonadal axis [6] of seasonal breeders

[7]. Sex steroids also modulate immune function, as their concentration varies during the breeding and the non-breeding seasons [8, 9]. Even before the importance of the thymus in immunity had been recognized, researchers noted thymic hypertrophy following gonadectomy, particularly in female animals [10]. Sex steroid hormones are likely mediators of seasonal patterns of immune function and diseases as well [8]. Consequently, receptors for estrogen and androgens have been found on lymphoid tissue suggesting that the gonadal steroid can act directly on immune organs to influence immune function [11]. Therefore, along with the first objective, we proposed to note the expression of AR and Mel1aR in testes and Mel1aR, AR expression in thymus for their role toward modulation of cell-mediated immunity after intra-testicular injection of melatonin. This may help to explain partially the reason behind the decreased seasonal reproductive activity with an increase in immunity.

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. Experiment was performed during reproductively active phase (April–June; temp. approx. max. 40°C and min. 37°C, humidity approx. 45%, and day length approx. 13:22 h). The squirrels, *F. pennanti*, were collected from the vicinity of Varanasi (Lat. 25°, 18′ N; Long. 83°, 1′E) in the first week of April. After 2 weeks of acclimation (equivalent to ambient condition), healthy young adult male squirrel of average weight 100 ± 10 g were randomly selected and divided into three groups having seven male squirrels in each. They were kept in wire net cages (25″ × 25″ × 30″ in size) during experiments and were maintained in a well-ventilated room exposed to ambient conditions. Squirrels were fed with soaked gram seeds (*Cicer arientium*), nuts, seasonal fruits/vegetables, and water ad libitum.

Experimental groups

Twenty-one young adult male squirrels were randomly assigned to three groups each containing seven animals, first group served as control saline treated in both the testes (CT), second group was given intra-testicular melatonin injection (2.5 μg/testes/100 g body wt) for 10 days (MI-10D), and the third group was treated for 20 days with intra-testicular melatonin injection (MI-20D) during evening hours (4:30–5:30 pm; 1.5 h before sunset) in both the testes. The middle part of the testes was selected for injection everyday. No local reaction (around the area of injection) was observed following intra-testicular injection. We have already reported 25 μg/100 μl as an immunoenhancing physiological dose when given in vivo to *F. pennanti* [12]; therefore, we selected lower doses of melatonin 0.25 and 2.5 μg/testes/100 g body wt for our intra-testicular experiment. We did not observed any significant effect following 0.25 μg dose injection in comparison to 2.5 μg dose. Thus, 2.5 μg/testes/100 g body wt was selected as it caused significant changes in all parameters studied.

Sample collection

After 24 h of last injection, squirrels were weighed and killed by decapitation during night time between 8:00 pm and 10:00 pm. Trunk blood was collected in heparinized tubes and processed for total leukocyte count (TLC) and percent lymphocyte count (%LC) using Leishman's stain method [7]. Plasma was kept at −80°C till the radioimmunoassay (RIA) of testosterone was performed. Spleen, thymus, testes, and seminal vesicle were dissected out on ice, cleaned, and weighed on an electronic balance (Denver Instruments, Gottingen, Germany) to record relative spleen and thymus weight and then processed for the assay of blastogenic response in terms of percent stimulation ratio (%SR). Thymus and testes were processed for western blot analysis. Testes were also fixed in Bouin's fluid for histology and immunohistochemical analysis.

Blastogenic response in terms of percent stimulation ratio (%SR)

Blastogenic response was measured in terms of [^{3}H] thymidine uptake against stimulation by concanavalin-A of the splenocytes, details of which has been published elsewhere [7, 13]. Tissue culture medium RPMI-1640 and all other chemical were purchased from Sigma Chemicals, USA. Spleen and thymus were dissected out and processed for preparation of single cell suspensions. The number of cells was adjusted to 1×10^6 cells/ml in culture medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Two milliliters of cell suspension was placed in duplicate culture tubes and kept at 37°C in a 5% CO_2 incubator (Heraeus, Germany) for 72 h. Blastogenic response was measured in terms of [^{3}H] thymidine (BARC, India; specific activity 8.9 Ci/mM) uptake against stimulation by Concanavalin-A (Con A; T cell mitogen; SIGMA, USA) of the splenocytes and thymocytes [7, 14]:

$$\%SR = \frac{CPM\ with\ Con\ A}{CPM\ without\ Con\ A} \times 100.$$

Histology

After fixation in Bouin's fluid for 22 h, testes were paraffin embedded and cut into 6-μm sections. Deparafinized sections were stained using hematoxylin and eosin. Testes morphology was observed under microscope (Leica MPV-3, Germany). The morphometric analysis of spermatogenic activity and number of testicular macrophages were counted in sections randomly selected from each animal with the help of filar ocular micrometer (Webcon Pvt. Ltd. India)

3β-HSD estimation

3β-HSD enzyme assay was performed as it is located on smooth endoplasmic reticulum of Leydig cells and is responsible for the conversion of androstenediol to testosterone. 3β-HSD enzyme was assayed according to the method of Shivanandappa and Venkatesh [15] using testicular homogenate. Testes were pooled and homogenized to make 10% tissue homogenate in 0.1 M Tris–Cl buffer (pH 7.8). The homogenate was centrifuged at 12,000 × g at 4°C and the supernatant was used as the source of enzyme. The enzyme was assayed in 0.1 M Tris–Cl buffer (pH 7.8) containing 500 mM NAD, 100 mM DHEA as substrate, and enzyme (50 μl) in a total volume of 3.0 ml and incubated at 37°C for 1 h. The reaction was stopped by the addition of 2.0 ml of phthalate buffer (pH 3.0) and the absorbance was noted at 490 nm. The enzyme activity was calculated from the standard curve of NADH and expressed as nmol NADH formed /h/mg protein.

Immunohistochemical localization of melatonin receptor (Mel1aR)

For immunohistochemical localization of Mel1aR, testes were dissected out and kept in Bouin's fluid for 22 h. After dehydration, paraffin blocks were prepared and 6 μm thick transverse sections (T.S.) were cut and mounted on gelatin (1%) coated slides and deparaffinized. After rehydration, endogenous peroxide activity was blocked by 0.3% H_2O_2 in methanol for 30 min at room temperature (RT). Sections were washed thrice with phosphate buffer saline (PBS; 0.1 M NaH_2PO_4, Na_2HPO_4, NaCl; pH 7.4) and preincubated with horse blocking serum (1:100 in PBS; PK-6200, Vector laboratories, Burlinghame, CA) for 2 h. Then sections were incubated with primary antibody (Mel1aR; R-18, anti-rat; goat raised; Santa Cruz Biotech, USA, dilution 1:200) overnight at 4°C. Sections were washed thrice by PBS and were incubated with biotinylated secondary antibody (Vectastain ABC Universal kit; PK-6200, Vector laboratories, Burlinghame, CA, dilution 1:50). Sections were then washed with PBS and a pre-formed ABC reagent was conjugated to the free biotin of the secondary antibody. The antigens were visualized using the 0.03% peroxidase substrate 3, 3-diaminobenzidine (DAB, Sigma chemicals St. Louis, USA) in 0.01 M Tris–Cl (pH 7.6) and 0.1% H_2O_2 and counterstained with Ehrlich's hematoxylin. Sections were dehydrated and mounted with DPX. Finally, they are observed and photographed under Leitz-MPV-3 microscope (Germany). The negative controls were obtained by omitting the primary antibody and incubating the testes section with goat serum and counterstained with hematoxylin.

Western blot analysis of Mel1aR, androgen receptor (AR), and androgen binding protein (ABP)

We checked ABP expression as it is a testicular glycoprotein [16, 17] that binds androgens with high affinity [18] and transports them to the epididymis [16]. Testes and thymus homogenates were lysed in RIPA buffer (1% (v/v) NP-40, 0.1% (w/v) sodium dodecyl sulfate (SDS) in phosphate-buffered solution (PBS) containing aprotinin, sodium orthovanadate, and phenylmethylsulfonylfluoride (PMSF). Quantification of the protein was done by Bradford method [19]. For AR blot analysis aliquots containing 40 μg of protein from testes and 60 μg of protein from thymus were resolved by 10% (w/v) SDS-polyacrylamide gel electrophoresis, while 60 μg of protein from thymus was resolved by 12% (w/v) SDS-polyacrylamide gel electrophoresis for Mel1aR immunoblot analysis. Electrophoresis was followed by electrotransfer to a Nitrocellulose membrane (Bioscience, Keene NH, USA). Immunodetection was carried out by using anti-AR, anti-ABP, and anti Mel1aR antibodies (anti-AR, N-20, Santa Cruz Biotech, USA, diluted 1:250; rabbit ABP antiserum, diluted 1:200 was kind gift from Dr. Peter Petrusz, University of North Carolina, USA; anti Mel1aR, R-18, Santa Cruz Biotech, USA, diluted 1:200) and β-actin antibody (A-2228, Sigma-Aldrich Chemicals, St. Louis, USA, diluted 1:1,000) all were diluted in PBS containing 5% skimmed milk and 0.1% Tween-20 followed by horseradish peroxidase conjugated secondary antibody (donkey anti-rabbit IgG for AR and ABP antisera; diluted 1:10,000; donkey anti goat IgG for Mel1aR diluted 1:1,000 and donkey anti mouse IgG for β-actin, diluted 1:10,000), which were further, detected using Super Signal West Pico Chemiluminescent Substrate (# 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 and expressed as % control

value [20]. Each sample corresponds to tissue from a single animal and at least four gels corresponding to each subunit and experimental condition were analyzed. Details of the validation method for Mel1aR in *F. pennanti* have been performed as described and published elsewhere [21].

Radioimmunoassay/RIA

The plasma contents of testosterone (Immunotech, France; intra & inter assay variation; 4.5 and 6%; sensitivity; 6 pg/ml, and recovery; 95%) and corticosterone (gift from NIHFW, New Delhi, India; intra and inter assay variation; 5.5 and 8.5%; sensitivity; 0.25 µg/dl, and recovery; 85%) in respective groups were estimated according to manufacturers instruction. The RIA of melatonin was performed following the method of Rollag and Niswender [22] using Guildhey antisera (Guildhey, Surrey, UK). Details of the method published elsewhere [23]. The validation of radioimmunoassay was performed as described earlier [23]. The intra and inter assay variation for melatonin was 9 and 15%. The sensitivity for melatonin RIA was 18–20 pg/ml and the recovery was 92%.

Technically, it was not feasible for us to perform the RIA/ELISA of LH and follicle stimulating hormone (FSH) because of the lack of specific antibody against our experimental model, i.e., Indian palm squirrel *F. pennanti*.

ELISA for IL-2

Sandwich ELISA was performed to quantify plasma level of IL-2 in all the three groups according to manufacturers instruction (Immunotech, France) Intra assay variation was between 3.3 and 7.2% and inter assay variation was between 6.2 and 8.2%; sensitivity; 5 pg/ml and recovery was between 80 and 132%.

Statistical analysis

Statistical analysis of the data was performed with one-way ANOVA followed by Student Newman–Keul's multiple range tests. The differences were considered significant when $P < 0.05$.

Results

Body weight

No significant difference was observed between saline treated control group and intra-testicular melatonin treated group for MI-10D and MI-20D (Table 1)

Lymphoid organs weight

Spleen showed significant ($P < 0.01$) increase in weight after 10 and 20 days of intra-testicular melatonin injection when compared to the control squirrels (Table 1). Contrasting with spleen, thymus showed significant ($P < 0.01$) increase in weight in MI-10D groups when compared with control groups but, thymus weight decreased in MI-20D groups when compared with MI-10D group (Table 1).

Testes and seminal vesicle weight

Testes and seminal vesicle showed significant ($P < 0.01$) decrease in weight following intra-testicular injection in both MI-10D and MI-20D treated groups when compared with control group (Table 1).

Total leukocyte and % leukocyte count (TLC and %LC)

We observed significant increase ($P < 0.01$) in TLC after 10-days of treatment (MI-10D) than untreated control squirrels. MI-20D group showed further, a significant increase in leukocyte count than control group ($P < 0.01$) (Table 1). Percent lymphocyte showed parallelism with that of TLC. MI-10D group showed significant ($P < 0.01$) increase in %LC than control group. MI-20D group

Table 1 Variation in body (in g), spleen and thymus weight (in g/100 g body weight), total leukocyte count (TLC; as cells/mm^3), % lymphocyte count (LC) after intra-testicular melatonin injection for 10 day (MI-10D) and 20 days (MI-20D)

Parameters	Control	MI-10D	MI-20D
Body weight	108 ± 5.6	100 ± 6	105 ± 4
Spleen weight	0.208 ± 0.0028	0.255 ± 0.039**	0.259 ± 0.016**
Thymus weight	0.104 ± 0.0184	0.215 ± 0.0104**	0.179 ± .0075**
Testes weight	1.53 ± 0.06	1.28 ± 0.049**	1.12 ± 0.0678**
Seminal vesicle wt.	0.355 ± 0.02	0.271 ± 0.013**	0.219 ± 0.01**
TLC (cells/mm^3)	5650 ± 420	6250 ± 700**	13200 ± 740**
%LC	15.8 ± 0.64	24.2 ± 1.44**	31.4 ± 2.08**

Values are mean ± SEM; $N = 7$; * $P < 0.05$; ** $P < 0.01$ control versus experimental group

Table 2 Variation in 3β-HSD activity (nmol NADH formed min^{-1} mg^{-1} protein), percent SR (%SR) in splenocytes and thymocytes, plasma testosterone level (ng/ml), corticosterone level (μg/dl), and plasma IL-2 level (pg/ml) after intra-testicular melatonin injection for 10 day (MI-10D) and 20 days (MI-20D)

Parameters	Control	MI-10D	MI-20D
3β-HSD activity	1.39 ± .072	0.81 ± 0.050**	0.585 ± 0.046**
%SR—splenocytes	114.2 ± 2.64	189.2 ± 6.96**	156.6 ± 9.92**
%SR—thymocytes	138 ± 3.6	356.2 ± 4**	258.4 ± 5.28**
Plasma testosterone	1.204 ± 0.1368	0.503 ± 0.07**	0.224 ± 0.030**
Plasma corticosterone	34 ± 4	35.8 ± 4.32	40.9 ± 4.48
Plasma melatonin	75 ± 4	120.8 ± 5.36**	142 ± 5.52**
IL-2 level in plasma	87.98 ± 4.016	138.78 ± 2.312**	159.772 ± 3.017**

Values are mean ± SEM; $N = 7$; * $P < 0.05$; ** $P < 0.01$ control versus experimental group

showed further increase in lymphocyte count ($P < 0.01$) (Table 1).

Blastogenic response/%SR

The changes in cellular immunity (T-helper cells) in splenocytes and thymocytes were noted following mitogen Concanavalin-A challenge. We observed significant increase ($P < 0.01$) in percent stimulation ratio of splenocytes of squirrels treated with intra-testicular melatonin for 10 and 20 days in comparison with control squirrels while, thymocytes showed an increase only in MI-10D group ($P < 0.01$). Longer treatment of 20 days (MI-20D) further complemented the proliferation of cells when compared to control squirrels ($P < 0.01$) (Table 2).

Histological observation

The testes of control squirrel exhibited normal histological features of reproductively active phase. Closely packed seminiferous tubules (342 μm ± 17.6), lumen filled with sperms, various stages of spermatogenic activity with successive stages of transformation of spermatogonia into spermatozoa were observed (Fig. 1a). Degenerative changes including disorganized, elongated, and reduced diameters of seminiferous tubules with vacuoles (v), and exfoliation of germ cells, presence of giant cells, reduced spermatogenesis, and detachment of inter-tubular connective tissue were observed in testicular sections of intra-testicular melatonin injected squirrels (Fig. 1b, c). The diameter of seminiferous tubules and Leydig cells reduced following intra-testicular injection (159 μm ± 11.2) of melatonin in a duration-dependent manner when compared with control testes (Table 3). Number of macrophages also increased following intra-testicular injection of melatonin (Table 3).

3β-HSD activity

There was a significant decrease ($P < 0.01$) in testicular 3β-HSD enzyme activity in both the treated groups (MI-10D and MI-20D) in comparison to control group. The decrease in the 3β-HSD activity observed with an increase in the duration of melatonin treatment (Table 2).

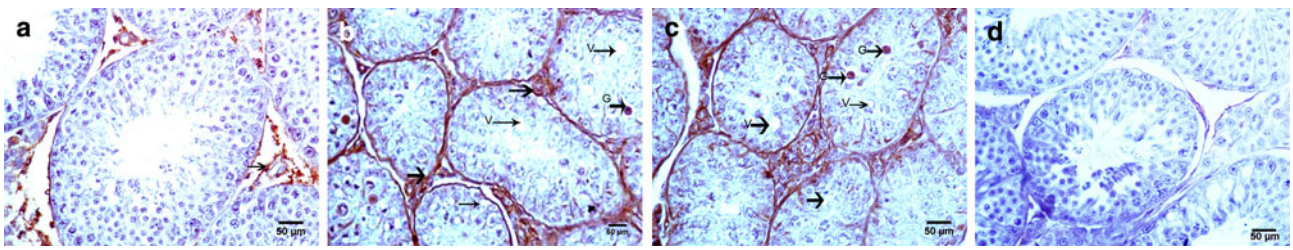

Fig. 1 **a** Histology of testes after intratesticular saline injection. Note the active testes presenting complete stages of spermatogenesis and spermatozoa. *Black arrows* show the areas of Mel1aR immunopositivity. **b** Histology of testes following 10 days of intratesticular melatonin injections (MI-10D). Note the complete inactive testes showing decrease in spermatogenesis, elongated and reduced seminiferous tubule diameter with some giant cells. *Black arrows* were showing immunopositivity on Leydig cells, peritubular myoid cells, endothelial lining of blood vessels in interstitial zone. "V"—vacuolation and "G"—giant cells. **c** Histomorphology of testes after 20 days of intratesticular melatonin injection (MI-20D). *Black arrows* showing the immunopositivity for Mel1aR in different regions of testes as mentioned for Fig. 1b. **d** Control section of testes showing no immunoreactivity

Table 3 Seminiferous tubular diameter (in μm), Leydig cell diameter (in μm), and number of macrophages per seminiferous tubule after intra-testicular melatonin injection for 10 day (MI-10D) and 20 days (MI-20D)

Parameters	Control	MI-10D	MI-20D
Seminiferous tubule diameter (μm)	342 ± 17.6	239.8 ± 6.24**	159 ± 11.2**
Leydig cell diameter (μm)	19.2 ± 1.12	14.5 ± 1.28**	10.5 ± 1.28**
Macrophages/seminiferous tubule	2–4	7–10	12–15

Values are mean ± SEM; ** $P < 0.01$ control versus experimental group

Hormonal analysis

We observed significantly decreased ($P < 0.01$) peripheral plasma testosterone level in MI-10D group when compared with control groups. When melatonin treatment was extended for 20 days (MI 20D), it further suppressed the testosterone level compared to the control and 10 days (MI 10D) treated groups ($P < 0.01$). There was no significant difference observed in peripheral corticosterone level in both the groups when compared with control group. Peripheral melatonin level was increased in a duration-dependent manner (Table 2).

Plasma IL-2

Plasma IL-2 level increased significantly in MI 10D and MI 20D groups. MI 20D showed maximum increase ($P < 0.01$) when compared with control group ($P < 0.01$) (Table 2).

Immunohistochemistry

Immunoreactivity for Mel1aR was observed in the Leydig cells of the testes of control squirrel (Fig. 1a). Following 10 days of intra-testicular melatonin injection, high immunoreactivity was observed on the cells surrounding basement membrane of the seminiferous tubules (peritubular myoid cells), Leydig cells, endothelial lining of the blood vessels when compared with control testes (Fig. 1b). Complete arrest of spermatogenesis was observed following 10 and 20 days of intra-testicular melatonin injection in a duration-dependent manner. After 20 days of melatonin injection, stronger immunoreactivity was observed in the above-mentioned areas (Fig. 1c). No immunoreactivity was observed in control testes sections processed by omitting primary antibody and incubated with goat serum (Fig. 1d).

Western blot analysis

We detected AR proteins as a single band between 100 and 120 kDa (when compared with standard marker protein; # SM-1841; FERMENTAS INC. Maryland, USA), in both testes and thymus. AR expression in thymus decreased significantly ($P < 0.01$) following 10 days treatment (MI 10D); while in the MI 20D group, it decreased more significantly ($P < 0.01$) (Fig. 2).

Androgen binding protein was detected as a single major band between 45 and 50 kDa in the testes, which precisely corresponds to the predicted molecular mass of the receptor, i.e., 47 kDa [24, 25]. AR percent expression in testes decreased significantly ($P < 0.01$) after 10 days treatment (MI-10D) and a further decrease was noted following treatment for 20 days (MI-20D) ($P < 0.01$) when compared with the control group (Fig. 3). ABP expression pattern was similar to that of AR and exhibited a significant decrease ($P < 0.01$) in percent expression following MI 10D treatment, which was more significant ($P < 0.01$) following MI 20D group (Fig. 4).

We detected Mel1aR as a single major band between 35 and 40 kDa in thymus, which has already been reported by

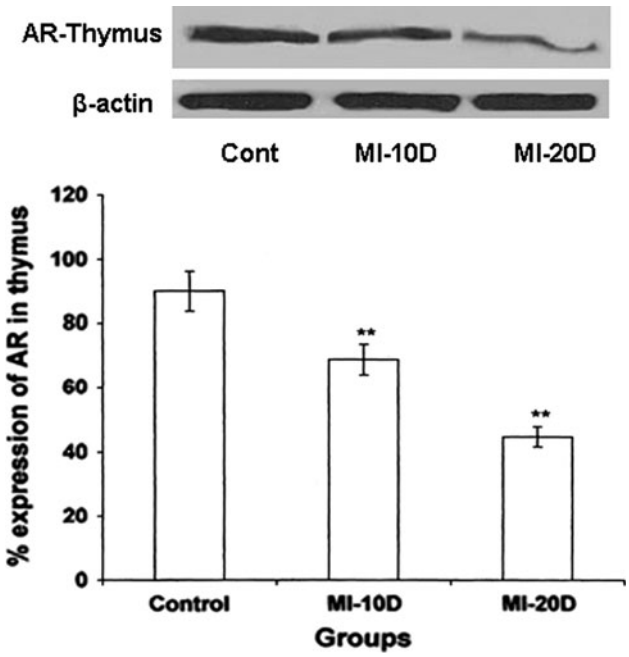

Fig. 2 Western blot analysis of androgen receptor (AR) expression in thymus of *F. pennanti*. β-Actin expression was used as loading control. *Lower panel* shows the % expression of receptor following Scion image analysis. Values are means ± SEM, $N = 4$. Significant of difference saline injected control versus MI-10D and MI-20D. * $P < 0.05$ and ** $P < 0.01$

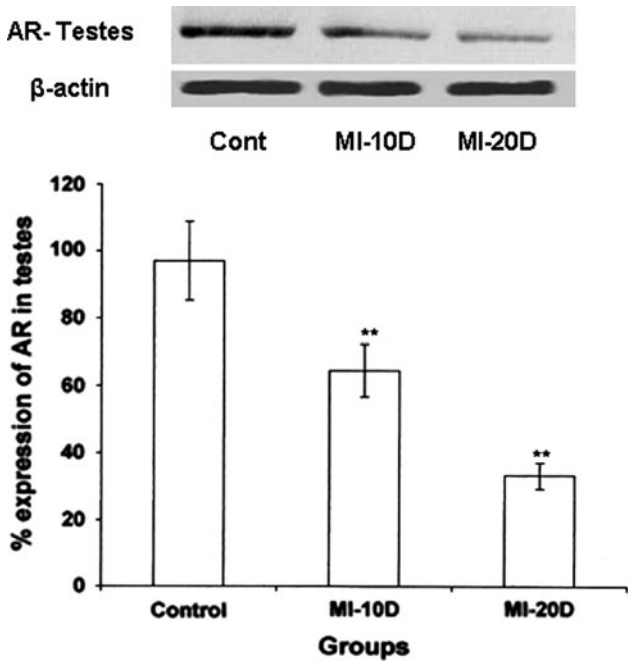

Fig. 3 Western blot analysis of androgen receptor (AR) expression in testes of *F. pennanti*. *β*-actin expression was used as loading control. Lower panel shows the % expression of receptor following Scion image analysis. Values are means ± SEM, $N = 4$. Significant of difference saline injected control versus MI-10D and MI-20D. * $P < 0.05$ and ** $P < 0.01$

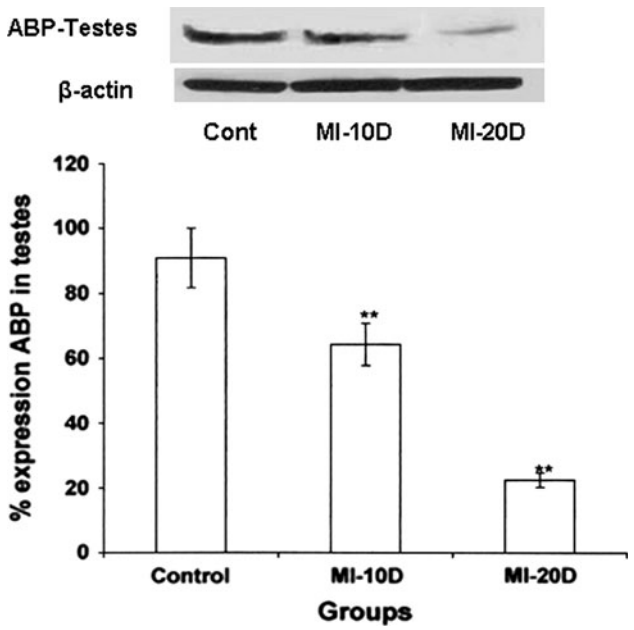

Fig. 4 Western blot analysis of androgen binding protein (ABP) expression in testes of *F. pennanti*. *β*-Actin expression was used as loading control. *Lower panel* shows the % expression of receptor following Scion image analysis. Values are means ± SEM, $N = 4$. Significant of difference saline injected control versus MI-10D and MI-20D. * $P < 0.05$ and ** $P < 0.01$

us in the lymphoid organs of *F. pennanti* [21]. Mel1aR expression in thymus was significantly increased ($P < 0.01$) in duration-dependent manner following intra-testicular melatonin injection (Fig. 5).

Discussion

In seasonal breeding rodents, testicular activity depends largely upon the effect of the pineal-derived melatonin, secreted during darkness, mainly by acting at the level of the brain and pituitary or during the period of sensitivity of the reproductive system to melatonin [1, 26]. The present study describes the effects of intra-testicular injection of melatonin (in both MI-10D and MI-20D groups), which reduced steroidogenesis and spermatogenesis on one hand and enhanced the testicular as well as general immunity on the other. Further, no report exists to explain the possible role of local injection of melatonin in regulation of androgen secretion and its receptor (AR) expression in gonad and lymphoid organ. The possibility of diffusion of intra-testicular injected melatonin in general circulation cannot be ruled out. We wanted to suggest that the selected dose may act through autocrine/paracrine regulation (decrease) of testicular steroidogenesis thereby improving local as well as general immunity. Our results suggest that injection of melatonin interfered directly with endocrine function of testes by acting upon Leydig cells to reduce steroidogenesis (as reflected by the reduced plasma

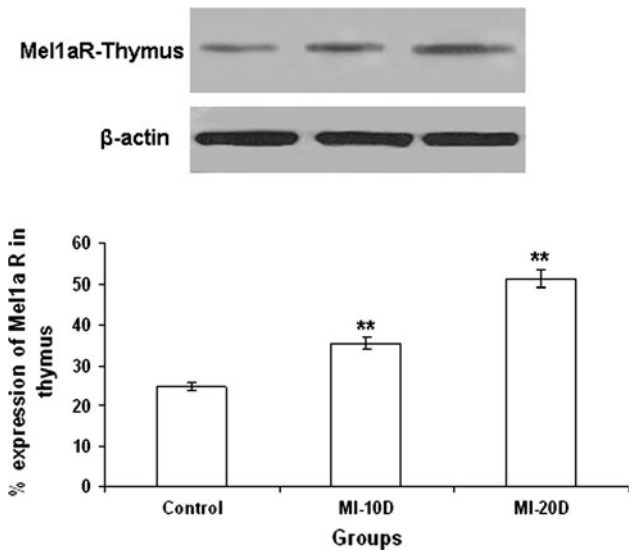

Fig. 5 Western blot analysis of melatonin receptor (Mel1aR) expression in thymus of *F. pennanti*. *β*-Actin expression was used as loading control. *Lower panel* shows the % expression of receptor following Scion image analysis. Values are means ± SEM, $N = 4$. Significant of difference saline injected control versus MI-10D and MI-20D. * $P < 0.05$ and ** $P < 0.01$

testosterone level and 3β-HSD activity) and spermatogenic activity (in terms of reduction in the diameters of seminiferous tubules, no. of Leydig cells, and an inhibition of spermatogenesis) when compared with the saline injected control squirrels. Previous reports of melatonin injection (i.p.) on downregulation of StAR and key steroidogenic enzymes 3β-HSD, and 17β-HSD expression following melatonin treatment in rat [2, 27, 28] support our present study. The decrease in 3 β-HSD enzyme activity noted by us is due to an effect of (i) melatonin on enzyme activity itself or (ii) on its gene and protein expression could be an interesting point for future elaboration.

Reports exist demonstrating the atrophy of the testes and the accessory sex organs with daily injection (i.p.) of melatonin [29–32]. Intra-testicular melatonin injection also significantly decreased the weight of the seminal vesicle, an androgen-dependent organ supporting our data of decreased peripheral level of testosterone. This result supported the effects noted earlier following melatonin injection in rat [27]. There is a possibility that this effect might be due to the high circulatory level of melatonin, which diffused out from the testes into the circulation and inhibited the HPG axis.

Our study also explored the possible effect of intratesticular melatonin injection on androgen level and its receptor (AR) expression in gonad. Intra-testicular melatonin injection decreased the expression of AR and ABP at translational level in the testes suggesting that the effect being independent of hypothalamo–hypophyseal axis. Besides its local inhibitory effect melatonin via circulation might have reached the hypothalamus–pituitary complex to inhibit gonadotropin release and subsequently reduce gonadal function [1]. Such an indirect effect of melatonin administration cannot be excluded. For lack of the specific antibody against LH, FSH for this squirrel, we are unable to submit that this action is hypothalamo–hypophyseal independent/dependent. Recent reports suggest that testosterone regulates spermatogenesis via AR expression in testicular somatic cells, namely Sertoli cells, peritubular myoid cells, and Leydig cells [33, 34]. We observed reduced steroidogenesis and spermatogenesis as evident by the decreased circulatory testosterone, decreased expression of AR, ABP within the testis following intratesticular melatonin injection, which could have produced significant impact upon local and general immune status of squirrels. Therefore, the present study could be taken as an interface between reproductive and immune status in the context of tropical seasonally breeding rodents.

In an extension, we are also reporting immunolocalization of Mel1aR type in squirrel testes to propose that melatonin might be acting directly through its receptor present in testes for local modulation of steroidogenesis. Mel1aR immunoreactivity was confined mostly on the membranes of Leydig cells, as demonstrated earlier by using iodomelatonin binding assay method [35, 36]. The comparatively less expression of Mel1aR on the Leydig cells in control testes might be due to high circulatory testosterone level. Intra-testicular melatonin injection by reducing the peripheral testosterone level (due to local action on Leydig cells and also via media of circulation to HPG axis) might have released Mel1aR from the inhibition of testosterone and hence, a strong immunoreactivity was observed in experimental testes as also observed by us under annual study of Mel1aR expression and melatonin/testosterone level in *F. pennanti* (Ahmad, 2009, unpublished PhD Thesis, BHU, India).

Along with the negative effect on the spermatogenesis and steroidogenesis of testes in *F. pennanti*, intra-testicular melatonin injection also caused increase in general immune status as recorded by the observed changes in immune parameters (weight of lymphoid organs, TLC, %LC, %SR) and a high number of testicular macrophages as an indicator of increased local immunity. Highest immunity was noted in MI-20D treated group suggesting that melatonin-induced decrease in testosterone level might have increased cell-mediated immunity. Our previous experimentation with testosterone as well as melatonin administration in vivo also suggests an inverse relationship between those two hormones in immune regulation [7]. This is the first report suggesting that local injection of melatonin not only increased testicular (local) immunity but also the general immunity.

Interestingly, in adult Indian palm squirrels, an active thymus has been reported [13] and a strong negative relation between androgen thymus activity and their cumulative effect on cell-mediated immunity in tropical rodents is already known [7]. We report here the AR expression in the thymus of adult palm squirrel and found significant decrease in AR expression in thymus following both MI-10D and MI-20D treatments of melatonin. With decrease in AR expression in thymus, a simultaneous increase in Mel1aR expression has been observed in a duration-dependent manner. This decrease in androgen level, AR expression and increase in Mel1aR expression could be responsible for the abrupt increase in thymus weight in the MI-10D group and a significant increase in cellular immunity in terms of blastogenic response of thymocytes as well as splenocytes. This might have triggered a wave of proliferation in T-helper cells which could be responsible for increase in cytokine level, i.e., IL-2 level in plasma in both MI-10D and MI-20D group of squirrels. The present study suggests a "trade off" relation between the expression of AR and Mel1aR in thymus, which could be the reason of increase in cell-mediated immunity following intra-testicular melatonin injection.

Melatonin has been proposed to regulate the immune system by affecting cytokine production of immunocompetent cells [37]. The observed increase in plasma IL-2 level of MI-10D and MI-20D squirrels could be attributed to the increased production of Th-1 cells in thymus and spleen following intratesticular melatonin injection, which reached them via circulation. Our result is in line with classical reports of melatonin as enhancer of the production of IL-2, IFN-γ, and IL-6 by cultured human mononuclear cells [38]. Increase in testicular macrophage might have increased the intra-testicular interleukin production, which reduced steroidogenesis. Thus, the increase in macrophage number in the testicular interstitium, which is a part of immune system, supports our suggestion of positive local immunomodulation by melatonin.

The possibility of experimental stress following injections etc. has been ruled out as no significant difference in plasma corticosterone level was observed between control saline treated and MI-10D, MI-20D group squirrels. There is a possibility that melatonin may be synthesized within the testes [39] as the interstitial cells produce high levels of serotonin [40, 41]. This possibility led us to propose a systemic effect of melatonin via Mel1aR receptor. This pathway might be inhibiting the Leydig cell function and thereby testicular regression in this squirrel. Increased Mel1aR and decreased AR expression in thymus also points toward systemic effect of melatonin in modulating local and general immunity. The observed effect was duration dependent; therefore, a local endocrine action of melatonin injection may be proposed.

In conclusion, our findings demonstrate the existence of a melatonergic system in the *F. pennanti* testis that acts in an autocrine manner to modulate testicular steroidogenesis, which affects over-all physiology, thereby causing an increase in thymic Mel1aR expression and a decrease in thymic AR expression in a systemic manner, which could be the first report of its kind in any tropical seasonal breeder.

Acknowledgments Authors thank to Department of Science & Technology, New Delhi, Indian Council of Medical Research, New Delhi, for financial support as Junior Research Fellow to Raise Ahmad. Instrument gift by Alexander von Humboldt Foundation, Bonn, Germany, is gratefully acknowledged.

References

1. R.J. Reiter, Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr. Rev. **12**, 151–180 (1991)
2. M.B. Frungieri, A. Mayerhofer, K. Zitta, O.P. Pignataro, R.S. Calandra, S.I. Gonzalez-Calvar, Direct effect of melatonin on Syrian hamster testes: melatonin subtype 1a receptors, inhibition of androgen production, and interaction with the local corticotropin-releasing hormone system. Endocrinology **146**, 1541–1552 (2005)
3. M. Niedziela, A. Lerchil, E. Nieschlag, Direct effects of the pineal hormone melatonin on testosterone synthesis of Leydig cells in Djungarian hamsters (Phodopus sungorus) in vitro. Neurosci. Lett. **201**, 247–250 (1995)
4. T.B. Ng, L.L. Lo, Inhibitory actions of pineal indoles on steroidogenesis in isolated rat Leydig cells. J. Pineal Res. **5**, 229–243 (1988)
5. S. Valenti, M. Giusti, R. Guido, G. Giordano, Melatonin receptors are present in adult rat Leydig cells and are coupled through a pertussis toxin-sensitive G-protein. Eur. J. Endocrinol. **136**, 633–639 (1997)
6. R.C. Gaillard, E. Spinedi, Sex and stress-steroids interactions and the immune system: evidence for a neuroendocrine-immunological sexual dimorphism. Domest. Anim. Endocrinol. **15**, 345–352 (1998)
7. R. Ahmad, C. Haldar, Photoperiod-testicular-immune interaction in a seasonal breeder Indian palm squirrel Funambulus pennanti during the reproductively inactive and active phases. J. Neuroendocrinol. **21**, 2–9 (2009)
8. R.J. Nelson, G.E. Demas, Seasonal changes in immune function. Q. Rev. Biol. **71**, 511–548 (1996)
9. R.J. Nelson, G.E. Demas, S.L. Klein, Photoperiodic mediation of seasonal breeding and immune function in rodents: a multifactorial approach. Integr. Comput. Biol. **38**, 226–237 (1998)
10. D. Eidinger, T.J. Garrett, Studies of the regulatory effects of the sex hormones on antibody formation and stem cell differentiation. J. Exp. Med. **136**, 1098–1116 (1972)
11. I.G. Barr, K.W. Pyke, P. Pearce, B.H. Toh, J.W. Funder, Thymic sensitivity to sex hormones develops post natally, an in vivo and an in vitro study. J. Immunol. **132**, 1095–1099 (1984)
12. C. Haldar, S. Rai, R. Singh, Melatonin blocks dexamethasone-induced immunosuppression in a seasonally breeding rodent Indian palm squirrel, *Funambulus pennanti*. Steroids **69**, 367–377 (2004)
13. C. Haldar, R. Singh, Pineal modulation of thymus and immune function in a seasonally breeding tropical rodent, *Funambulus pennanti*. J. Exp. Zool. **289**, 90–98 (2001)
14. J.L. Pauly, J.E. Sokal, A simplified technique for in vitro studies of lymphocytes reactivity. Proc. Soc. Exp. Biol. Med. **140**, 40–44 (1972)
15. T. Shivanandappa, S. Venkatesh, A colorimetric assay method for 3-beta-hydroxy-delta 5-steroid dehydrogenase. Anal. Biochem. **254**, 57–61 (1997)
16. F.S. French, E.M. Ritzen, A high affinity androgen-binding protein (ABP) in rat testis: evidence for secretion into efferent duct fluid and absorption by epididymis. Endocrinology **93**, 88–95 (1973)
17. B.J. Danzo, B.C. Eller, M.C. Orgebin-Crist, Studies on the site of origin of the androgen binding protein present in epididymal cytosol from mature intact rabbits. Steroids **24**, 107–122 (1974)
18. U. Westphal, Steroid-protein interactions II. Monogr. Endocrinol. **27**, 198–301 (1986)
19. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254 (1976)
20. O. Treeck, C. Haldar, O. Ortmann, Antiestrogens modulate MT1 melatonin receptor expression in breast and ovarian cancer cell lines. Oncol. Rep. **15**, 231–235 (2006)
21. R. Ahmad, C. Haldar, Photoperiodic regulation of melatonin receptor MT1 & MT2 expression dynamics in spleen and thymus of a tropical rodent Funambulus pennanti during reproductively active and inactive phase. Chronobiol. Int. (2009) (in press)
22. M.D. Rollag, G.D. Niswender, Radioimmunoassay of melatonin in sheep exposed to different light regimes. Endocrinology **98**, 482–488 (1976)

23. S. Dubey, C. Haldar, Environmental factors and annual Harderian–pineal–gonadal interrelationship in Indian jungle bush quail, *Perdicula asiatica*. Gen. Comp. Endocrinol. **106**, 17–22 (1997)
24. D.R. Joseph, D.A. O'Brien, P.M. Sullivan, M. Becchis, J.K. Tsuruta, P. Petrusz, Overexpression of androgen-binding protein/sex hormone-binding globulin in male transgenic mice: tissue distribution and phenotypic disorders. Biol. Reprod. **56**, 21–32 (1997)
25. D.A. Jeyaraj, G. Grossman, P. Petrusz, Altered bioavailability of testosterone in androgen-binding protein-transgenic mice. Steroids **70**, 704–714 (2005)
26. G.A. Lincoln, Neuroendocrine regulation of seasonal gonadotrophin and prolactin rhythms: lessons from the Soay ram model. Reprod. Suppl. **59**, 131–147 (2002)
27. S.K. Maitra, A.K. Ray, Role of light in the mediation of acute effects of a single afternoon melatonin injection on steroidogenic activity of testis in the rat. J. Biosci. **25**, 253–256 (2000)
28. S. Valenti, M. Giusti, Melatonin participates in the control of testosterone secretion from rat testis: an overview of our experience. Ann. N. Y. Acad. Sci. **966**, 284–289 (2001)
29. L. Tamarkin, W. Westrom, A. Hamill, B.D. Goldman, Effect of melatonin on the reproductive systems of male and female Syrian hamsters: a diurnal rhythm in sensitivity to melatonin. Endocrinology **99**, 1534–1541 (1976)
30. L. Tamarkin, C.W. Hollister, N.G. Lefebvre, B.D. Goldman, Melatonin induction of gonadal quiescence in pinealectomized Syrian hamsters. Science **198**, 935–947 (1977)
31. P. Pevet, C. Haldar, T. Ocal, Effect of 5-methoxytryptophan and 5-methoxytryptamine on the reproductive system of the male golden hamster. J. Neural Transm. **51**, 303–311 (1981)
32. T.B. Ng, W.Y. Chan, Action of pineal indoleamines on the reproductive systems of the male C 57 mouse and golden hamster. J Neural Transm. Gen. Sect. **93**, 87–98 (1993)
33. M. Welsh, P.T.K. Saunders, N. Atanassova, R.M. Sharpe, L.B. Smith, Androgen action via testicular peritubular myoid cells is essential for male fertility. FASEB J. **23**, 4218–4230 (2009)
34. R.S. Wang, S. Yeh, C.R. Tzeng, C. Chang, Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocrinol. Rev. **30**, 119–132 (2009)
35. H. Vera, M. Tijmes, L.E. Valladares, Melatonin and testicular function: characterization of binding sites for 2-[125I]-iodomelatonin in immature rat testes. Steroids **62**, 226–229 (1997)
36. S. Valenti, L. Fazzuoli, G. Giordano, M. Giusti, Changes in binding of iodomelatonin to membranes of Leydig cells and steroidogenesis after prolonged in vitro exposure to melatonin. Int. J. Androl. **24**, 80–86 (2001)
37. E. Kuhlwein, M. Irwin, Melatonin modulation of lymphocyte proliferation and Th1/Th2 cytokine expression. J. Neuroimmunol. **117**, 51–57 (2001)
38. S. Garcia-Maurino, M.G. Gonzalez-Haba, J.R. Calvo, M. Rafii-El-Idrissi, V. Sanchez-Margalet, R. Goberna, J.M. Gurrero, Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating CD4+ cells: a possible nuclear receptor-mediated mechanism involving T helper type1 lymphocytes and monocytes. J. Immunol. **159**, 574–581 (1997)
39. M. Tijmes, R. Pedraza, L. Valladares, Melatonin in the rat testes: evidence of local synthesis. Steroids **61**, 65–68 (1996)
40. J.C. Tinajero, A. Fabbri, M.L. Dufau, Regulation of corticotropin-releasing factor secretion from Leydig cells by serotonin. Endocrinology **130**, 1780–1788 (1992)
41. J.C. Tinajero, A. Fabbri, M.L. Dufau, Serotonergic inhibition of rat Leydig cell function by propranolol. Endocrinology **133**, 257–264 (1993)