

Melatonin in modulation of reproductive functions in the female Indian pygmy field mouse, *Mus terricolor*

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Abstract

The role of melatonin in control of seasonal reproduction is well established and it is known to have pro/antigonadotrophic effect in different species of small rodents. The Indian pygmy field mouse, *Mus terricolor* is a tropical, wild, nocturnal, short day breeder. Till date no report exists regarding the effects of melatonin on reproductive functions of this rodent, therefore, the present experiment was designed to study the melatonin-induced changes on the reproductive functions of female *M. terricolor*. Our study describes the effects of melatonin injections (25µg/100gm body weight) on the ovarian and uterine activity of *M. terricolor* during the reproductively active phase/RAP of its breeding cycle. The results suggest an antigonadotrophic action of melatonin in this rodent as deflected by decreased ovary and uterine weight, degenerative changes in the histology of the ovary and uterus and reduced plasma estradiol and progesterone. In case of short day breeders melatonin is known to act as a progonadotrophic hormone. But, exogenous melatonin administration at a dose of 25µg/100gm body weight during RAP suppressed the reproductive functions of this tiny rodent suggesting an antigonadotrophic property of melatonin at a higher threshold level. The results suggest a pivotal role of melatonin in adaptive strategy for the regulation of reproduction in seasonal breeders.

Keywords: Melatonin, Reproduction, *Mus terricolor*; Tropical rodent, Seasonal breeder

INTRODUCTION

Photoperiod is regarded to be the most reliable cue to time reproductive activity (Rosa and Bryant, 2003; Muteka *et al.*, 2006). The destination for the information about the day length is the pineal gland where melatonin is secreted. Pineal gland translates neural stimuli mediating photoperiod into hormonal stimuli melatonin. The daily duration and the level of melatonin secreted in the pineal regulates the secretion of gonadotropins /gonadal steroids and consequently reproduction in seasonal breeders (Heideman and Bronson, 1990; Frungieri *et al.*, 2005; Nakao *et al.*, 2008; Prendergast *et al.*, 2009). The role of pineal and melatonin has been well studied and reported in animals that breed during long day (hamsters: Hoffman and Reiter, 1965; voles: Farrar and Clarke, 1976; ferrets: Herbert *et al.*, 1978; white-footed mice: Glass and Lynch, 1981; Sp/Indian palm squirrel: Haldar and Saxena, 1990; Bisnupuri and Haldar, 2001; Yadav and

Haldar, 2009) and short day (sheep: Lincoln, 1979; Bittman *et al.*, 1983; deer: Plotka *et al.*, 1984). Turek *et al.* (1975) Turek and Pappas (1980) Petterborg (1986) and Maitra and Ray (2000) reported that melatonin when administered exogenously suppresses gonadal activity, and the effects are dose dependent.

The Several studies have reported the effects of melatonin on reproductive physiology of female animals. Wurtman (1963) for the first time reported in the that melatonin injections delay the spontaneous vaginal opening and cause highly significant reduction in ovarian and uterine weight and incidence of vaginal estrus. Many workers have reported disruption of ovarian cyclicity, reduction in weight of reproductive tract, gonadotropins and gonadal steroids after melatonin treatment (Bridges *et al.*, 1976; Tamarkin *et al.*, 1976; Reiter *et al.*, 1980; Richardson *et al.*, 1981; Vriend *et al.*, 1987). Melatonin receptor has been localized in ovary (Clemens *et al.*, 2001) and uterus (Zhao *et al.*, 2002), suggesting direct action of melatonin on these organs.

Mus terricolor, commonly known as Indian pygmy field mouse, is a small tropical wild rodent, found throughout South East Asia (Aplin *et al.*, 2003). *M. terricolor*, an important pest of rice and wheat fields, make burrows in the earthen dykes rose for holding water in the cultivated fields (Singh *et al.*, 2009). It has an earth colored body and a grey belly and tail slightly longer in proportion to the head and body length (Sharma, 1996; Aplin *et al.*, 2003; Singh *et al.*, 2009; Basu *et al.*, 2012; Basu and Singaravel, 2012). Singh *et al.* (2009) mentioned seasonal availability and speculated on seasonal reproduction, but did not give any evidence in favor of such theory. *M. terricolor* presents two peaks of annual gonadal cycle, one from October to January, and a second brief phase in April that is dependent on food availability (Data in communication). The evolutionary history and cytogenetics of *M.terricolor* have been explored in depth (Sharma, 1996; Singh *et al.*, 2009). The circadian sensitivity of this nocturnal rodent has been studied by Basu *et al.* (2012) and Basu and Singaravel, (2012). Till date no reports exist regarding the effect of melatonin administration on reproduction of small wild rodent. There fore, a the aim of the present study is to explore the effects of exogenous melatonin administration on the ovary and uterus of female *M. terricolor* during reproductively active phase, i.e., in the month of December.

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 of the animal. The mice were collected from the fields of vicinity of Varanasi (Lat. 25°, 18' N; Long. 83°, 1'E) India, following the methods as described earlier (Bardhan and Sharma, 2000; Singh *et al.*, 2009; Basu *et al.*, 2012; Basu and Singaravel, 2012).

After 2 weeks of acclimatization to laboratory conditions healthy young adult non-pregnant female mice of average weight 11 ± 1 g were randomly selected from the collected rodents and divided into two groups having six female mice in each. They were kept in commercial polypropylene cages during experiments and were maintained in a well-ventilated room exposed to ambient conditions ($27 \pm 2^\circ\text{C}$, with gentle ventilation). Mice were fed with commercial food pellets along with wheat, paddy/rice and water *ad libitum*.

Experimental groups

Our animal model is a keystone species as it serves as an important part of food web being food of different predatory and endangered birds such as white owl, etc. So in order to avoid any disbalance in the biodiversity by capturing more animals for our experimental purpose we restricted our study during reproductively active phase of the animal when the effects of melatonin can be well exhibited and studied.

First group (n=6) treated with normal (0.9%) ethanolic saline served as control, second group was given intra-peritoneal melatonin injection (25 µg/100 g body wt. Johnston and Zucker, 1980; Maitra and Ray, 2000; Ahmad and Halidar, 2010) for 15 days during evening hours (17:00-18:00 hrs, one hour before sunset).

Melatonin was purchased from Sigma-Aldrich Chemicals, St. Louis, MO, USA. Melatonin was first dissolved in few drops of ethanol and then diluted with normal saline upto the desired concentration and injected.

Sample collection

Twenty four hour after the last injection, the mice were weighed and sacrificed by over-anesthetization during night time between 8:00 pm and 10:00 pm. Trunk blood was collected directly from heart in heparinized tubes and plasma was collected then kept at -80°C till the ELISA for estradiol, progesterone (Biotron Diagnostics Inc. Hemet, California, USA) and melatonin (Uscn Life Science Inc. USA) was performed. Ovaries and uter in females were dissected out on ice, blotted free from blood, cleaned from extra tissue, and weighed on an electronic balance (Denver Instruments, Gottingen, Germany). Ovary and uterine horn of left side were fixed in Bouin's fluid for histology while that of right side were kept for biochemical estimations of ovarian cholesterol and uterine protein.

Histology

After fixation in Bouin's fluid, ovaries and uteri were processed for routine histological procedure. Some 6-µm sections were deparafinized, stained using Ehrlich's hematoxylin and Eosin. The stained sections of the tissues were observed under microscope (Leica MPV-3, Germany) and documented.

Biochemical Estimations

Ovarian cholesterol was estimated using commercial kit and manufacturer's protocol (Bio Lab Diagnostics, India). According to the manufacturer's protocol, the provided reagents were added in three set of test tubes i.e. Blank, Standard and Test samples. 1 ml of cholesterol reagent was added to all the three sets. 20µl of distilled water, standard reagent and test samples (homogenate) was added to Blank, Standard and Test samples respectively. After mixing well, all the test tubes were incubated at 37°C for 10 minutes and read at 510nm on Spectrophotometer (UV-200-RS, mrc, Israel). The protein content of the uterus was quantified using the Bradford method (1976).

Hormonal analysis

The plasma contents of estradiol, progesterone and melatonin in respective groups were

estimated using ELISA kit according to manufacturer's instruction.

ELISA for melatonin

The assay for melatonin was performed according to the manufacturer's instruction given on the kit (Uscn Life Science Inc. USA). The intra- and inter- assay variation was <10 and <12% respectively. The sensitivity was 4.68 pg/mL and recovery percentage was between 90-115. All reagents, samples and standards were prepared according to standard protocols. 50µl standard and sample were added to the respective wells followed by 50 µl of Detection reagent A, followed by gentle shaking and incubation for one hour at 37° C, followed by aspiration and washing, thrice. 100 µl of Detection reagent B was subsequently added and the mixture was incubated at 37° C for 30 minutes, followed by washing and addition of 90 µl of substrate solution and incubation at 37° C for 15-25 minutes. Finally, 50 µl of stop solution was added and read at 450 nm.

ELISA for Estradiol and Progesterone

The ELISA kits for estimation of plasma estradiol and progesterone were purchased from Biotron Diagnostics Inc. Hemet, California, USA. According to the manufacturer's instruction, 25 µl of standard, control and samples were added in each well of ELISA plate followed by 100 µl of enzyme conjugate solution. The wells were then incubated with mild shaking at room temperature for two hours. The wells were then aspirated and washed three times with wash solution. Then, 100 µl of the TMB chromogenic solution (substrate) was added to each well and plate was incubated at room temperature for 30 minutes in dark. Finally, 100 µl of stop solution was added in each well and absorbance was recorded at 450 nm. Intra and inter assay variations were less than 5% and 14% respectively. The assay was carried out in triplicate.

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 statistically significant when $P \leq 0.05$.

RESULTS

Body weight

No significant difference was observed between saline-treated control group and melatonin-treated group clearly supporting that selected dose for experiment was physiological and that the animals were maintained properly in healthy condition as in nature with sufficient food and water (Fig. 1).

Weight of reproductive organs

There was a significant reduction in the relative weight of ovary and uterus of melatonin-treated group as compared with control (Fig. 2 and 3 respectively).

Biochemical estimations

We observed a significant increase in the content of ovarian cholesterol in melatonin-treated group as compared with the control group (Fig. 4) while the content of protein was significantly reduced in melatonin-treated group as compared with the control group (Fig. 5).

Hormonal analysis

Significantly decreased peripheral plasma estradiol and progesterone level was noted in

melatonin-treated group when compared with the control group (Fig. 6 and 7 respectively). Peripheral melatonin level was increased in melatonin-treated group as compared with control group (Fig. 8).

Histological observations

The transverse section of uterus of vehicle-treated females showed a normal endometrial histology (Fig. 9). It had a well-developed endometrium, narrow lumen and a large number of proliferated endometrial glands while the endometrium of melatonin-treated females had atrophied condition and hence the lumen was wide with less number of endometrial glands without any proliferation (Fig. 10).

The ovaries of the saline treated female *M. terricolor* showed several corpora lutea and antral follicles (Fig. 11) while melatonin-treated mice showed extensive degenerative changes in the ovary. The sections showed primordial, primary and secondary follicles. In melatonin-treated mice ovary several pyknotic nuclei were present in granulosa cell layer and no signs of ovulation, were observed, i.e., corpora lutea were completely absent (Fig. 12).

DISCUSSION

Melatonin modulates the reproductive-physiology of seasonally breeding mammals (Tamarkin *et al.*, 1976; Yadav and Haldar, 2009; Reiter, 2009; Ahmad and Haldar, 2010). The earlier reports suggest melatonin is neither progonadotrophic nor antigonadotrophic *per se*, but, it is the changing duration of the “nocturnal melatonin surge” that conveys the information about the time of the year to the animals for the hypothalmo-pituitary-gonadal axis to act accordingly and fine-tune their reproductive activity according to season a new aspect of neuroendocrine regulation of reproduction in seasonally breeding rodents.

Our study describes for the first time the effects of melatonin injections on the ovarian and uterine activity of a wild, tropical, nocturnal, short day breeding rodent *M. terricolor* during the reproductively active phase of its annual reproductive cycle. In the present study the antigonadotrophic action of melatonin in female *Mus terricolor* is depicted by a significant reduction in ovarian and uterine weight, suppressed steroidogenesis as evident by increased ovarian cholesterol (ovarian cholesterol could be a marker of low steroidogenesis as it is one of the major components required for the process of steroidogenesis) and reduced plasma estradiol as well as progesterone which further led to altered histological conditions of ovary and uterus. Wurtman (1963) in rats, Vaughan *et al.* (1976) in mice, Young Lai (1978) in hamsters, Margollis and Lynch (1981) in *Peromyscus leucopus*; Reiter *et al.* (1980) in rats, and Tamura *et al.* (1998) in rabbits; reported significant reduction in weight of reproductive organs and steroidogenesis after melatonin administration. The reduced steroidogenesis might be due to the action of melatonin on multiple sites, i.e., hypothalamus (reducing GnRH) and pituitary (reducing FSH and LH) thereby ovarian synthesis of E₂ (Vriend *et al.*, 1987; Chan *et al.*, 1995).

The classical target for melatonin action is hypothalamic-pituitary axis. Fraschini *et al.* (1968) was the first one to report that the antigonadal action of melatonin is due to its action on hypothalamus. Later several workers reported the involvement of hypothalamus in melatonin action. Lang *et al.*, (1983) found decreased GnRH level due to melatonin action. Kao and Weisz (1977), Petterborg and Paull (1984), and Glass and Knotts, (1987) observed modulation of

storage and secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus by melatonin *in vitro* or *in vivo*. Different mechanisms have been proposed that explain melatonin's inhibitory effect on GnRH. The pulsatile pattern of GnRH release, which results in the intermittent release of gonadotrophic hormones from the pituitary, has a critical importance for reproductive functions but the factors responsible for this release pattern are not known. Calcium is a second messenger involved in GnRH release. The increase in Ca^{2+} resulting from melatonin treatment may impair pulsatile GnRH release, which may cause inactivation in gonadotrophic cells in pituitary. Melatonin may act directly by affecting the hypothalamic functions by inhibitory regulation of gonadotropin releasing hormone neurons (Roy *et al.*, 2001). Melatonin may also be involved in the control of GnRH gene expression and secretion (Roy and Belsham, 2002). Thus by inhibiting GnRH secretion and by altering its pulsatile secretion melatonin might have inhibitory effect on release of gonadotropins.

Martin and Klein, (1976) reported the first direct melatonin action on the pituitary. Melatonin inhibited the GnRH-induced LH release by neonatal rat anterior pituitary cells *in vitro* (Martin and Klein, 1976) or hamster pituitary *in vivo* (Wun *et al.*, 1986). Other reports suggest that daily properly timed melatonin injections to females inhibit reproduction by reducing the ability of pituitary to secrete the gonadotropins (FSH and LH) thereby reduce the circulating gonadotrophin level (Tamarkin *et al.*, 1976; Voordouw *et al.*, 1992). The measurement of LH and FSH is required to support above statement but LH and FSH measurement of this wild rodent can not be done due to lack of specific antibody.

Further, intraperitoneal evening injections of melatonin led to a significant increase in ovarian cholesterol (a key component in steroidogenesis) and significantly reduced the plasma estradiol and plasma progesterone. Our data gets support from previous reports (Voordouw *et al.*, 1992 ; Chan *et al.*, 1995) . Vriend *et al.*, (1987) reported that melatonin injections disrupted the normal pattern of gonadotropin secretion and resulted in atrophy of uterus and vagina. These changes were accompanied with depressed serum and pituitary prolactin and depressed level of estradiol. Chan *et al.*, (1995) reported an increased incidence of follicular atresia in the groups treated with melatonin (MEL), methoxytryptamine (MTA) and methoxytryptiphol (MTP). Treatment with melatonin (MEL), methoxytryptamine (MTA) and methoxytryptiphol (MTP) also resulted in lower plasma levels of estradiol-17 β and progesterone. Voordouw *et al.*, (1992) observed a significant decrease in LH, estradiol and progesterone in women following melatonin administration either alone or in combination with a synthetic progestin, consequently this combination was shown to be an effective oral contraceptive. In mammals melatonin modulates physiological functions through activation of at least two pharmacological and molecularly distinct receptors, the MT1 and MT2 (Masana and Dubcovich, 2001). Cohen *et al.*, (1978) first reported [3H] melatonin binding sites in cytoplasmic fractions of hamster, rat and human ovaries. Later Yie *et al.*, (1995), and Clemens *et al.*, (2001) detected melatonin binding sites in ovarian tissue using 2-[¹²⁵I] Iodomelatonin. Brzezinski *et al.*, (1987), and Ronnberg *et al.*, (1990) found melatonin in ovarian follicular fluid suggesting a direct effect of this hormone in ovarian function. Woo *et al.*, (2001) also suggested a direct role of melatonin in regulating ovarian function. The above findings suggest a direct action of melatonin on ovary and thereby on the mechanism for regulating the steroidogenesis. This in turn might have reduced the estrogen level

in the circulation of our animal model.

In addition to alteration in ovarian function melatonin equally controls uterine functions. Zhao *et al.*, (2002) showed the presence of melatonin receptors in the rat uterine endometrium suggesting that melatonin may act directly on the MT1 receptors in the antimesometrial stromal cells to inhibit their proliferation. This action is supposed to be mediated through a pertussis toxin sensitive adenylate cyclase coupled G (i) protein. In the present study melatonin injections altered the histology of uterus and decreased the protein content in the uterus, probably the presence of melatonin receptors in the uterus might be acting directly and regulating the endometrial vascular permeability and decidualization.

Our animal model *M.terricolor* breeds during the winter. In tropical countries like India days are short in winter and peripheral melatonin remains high. In our experiment when we exogenously administered melatonin at a dose of 25µg/100gm body weight, it showed an antigonadotrophic effect and suppressed the reproductive functions of this rodent. Progonadotrophic action of melatonin is known in short day breeders. During short photoperiod (winter) induced melatonin level maintains a threshold concentration in *vivo* that could not suppress the reproduction. But, when melatonin was given exogenously during above condition, it increased/disturbed the threshold level and hence an antigonadotrophic action of melatonin has been reflected. It can thus be inferred that it is the hypothalamo-hypophyseal region which measures the threshold sensitivity for melatonin in this small tropical rodent and thus maintain its adaptive strategies for reproduction. Available reports suggest that direct melatonin action may occur in more than one reproductive tissue, it can act from the level of hypothalamus to the levels of reproductive tract as the receptors for melatonin have been located in the SCN in the brain, pars tuberalis in the pituitary and reproductive tissues (Pang *et al.*, 1998). Thus it can be inferred that melatonin may act at the level of hypothalamus, pituitary, gonads and reproductive tract in this rodent to control its reproduction. Further, the experiments are in progress to show the presence and expression of melatonin receptors on above mentioned neuroendocrine axis.

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FIGURE LEGENDS

Fig. 1 Histogram representing effect of exogenous melatonin injections on body weight of *M.terricolor*. Values are expressed as mean, vertical bar on histograms represents \pm SEM, N = 6. Significance of difference; *P < 0.05, **P < 0.01.

Fig. 2 Histogram representing effect of exogenous melatonin injections on relative ovary weight of *M.terricolor*. Values are expressed as mean, vertical bar on histograms represents \pm SEM, N = 6. Significance of difference; *P < 0.05, **P < 0.01.

- Fig. 3 Histogram representing effect of exogenous melatonin injections on relative uterus weight of *M.terricolor*. Values are expressed as mean, vertical bar on histograms represents \pm SEM, N=6. Significance of difference; *P<0.05, **P<0.01.
- Fig. 4 Histogram representing effect of exogenous melatonin injections on ovarian cholesterol of *M.terricolor*. Values are expressed as mean, vertical bar on histograms represents \pm SEM, N=6. Significance of difference; *P<0.05, **P<0.01.
- Fig. 5 Histogram representing effect of exogenous melatonin injections on uterine protein of *M.terricolor*. Values are expressed as mean, vertical bar on histograms represents \pm SEM, N=6. Significance of difference; *P<0.05, **P<0.01.
- Fig. 6 Histogram representing effect of exogenous melatonin injections on plasma estradiol of *M.terricolor*. Values are expressed as mean, vertical bar on histograms represents \pm SEM, N=6. Significance of difference; *P<0.05, **P<0.01.
- Fig. 7 Histogram representing effect of exogenous melatonin injections on plasma progesterone of *M.terricolor*. Values are expressed as mean, vertical bar on histograms represents \pm SEM, N=6. Significance of difference; *P<0.05, **P<0.01.
- Fig. 8 Histogram representing effect of exogenous melatonin injections on plasma melatonin of *M.terricolor*. Values are expressed as mean, vertical bar on histograms represents \pm SEM, N=6. Significance of difference; *P<0.05, **P<0.01.
- Fig. 9 Transverse section of the uterus (stained with haematoxylin-eosin) of *M. terricolor* . following saline injection. Note the proliferated endometrial glands. Figure shown in 40X magnification.
- Fig. 10 Transverse section of the uterus (stained with haematoxylin-eosin) of *M. terricolor* showing histological changes following administration of melatonin. Note the non-proliferated endometrial glands. Figure shown in 40X magnification.
- Fig. 11 A and B Transverse sections of the ovaries (stained with haematoxylin-eosin) of *M. terricolor* following saline injection. Figures shown in 10X and 40X magnification.
- Fig. 12 Transverse section of the ovary (stained with haematoxylin-eosin) of *M. terricolor* showing histological changes following administration of melatonin.
- (A) Section of the ovary. Figure shown in 10X magnification.
- (B) Granulosae cells (GCs) showing pyknotic nuclei (Py). Figure shown in 40X magnification.
- (C) Figure 12 (B) in enlarged view Figure shown in 100X magnification.

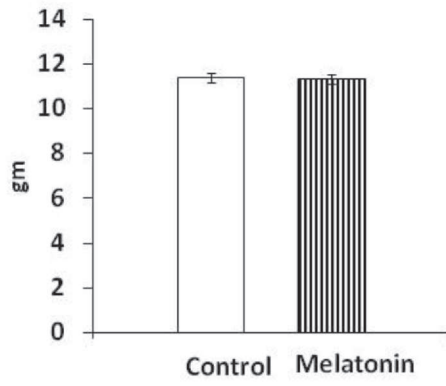


Fig.1

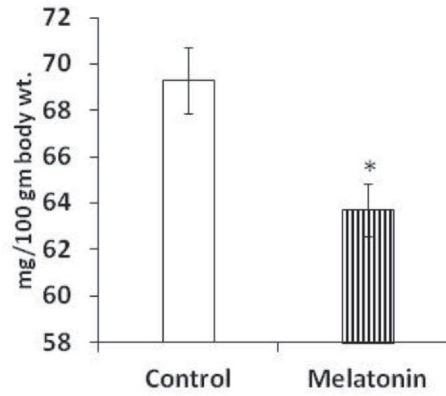


Fig.2

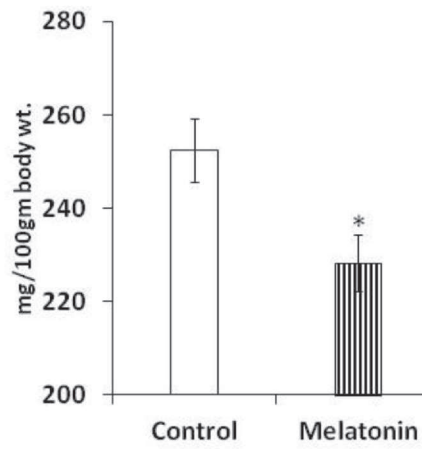


Fig.3

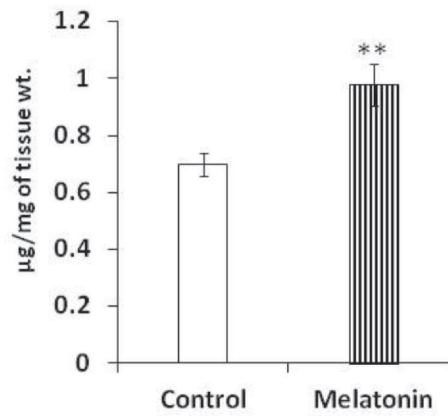


Fig.4

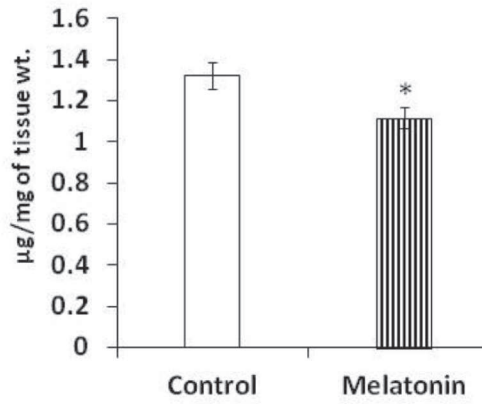


Fig.5

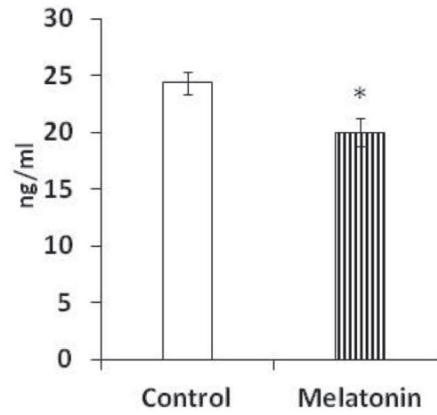


Fig.6

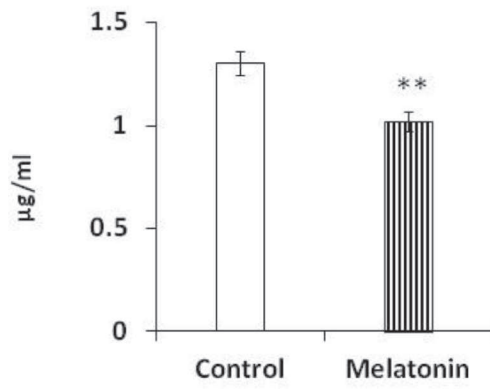


Fig.7

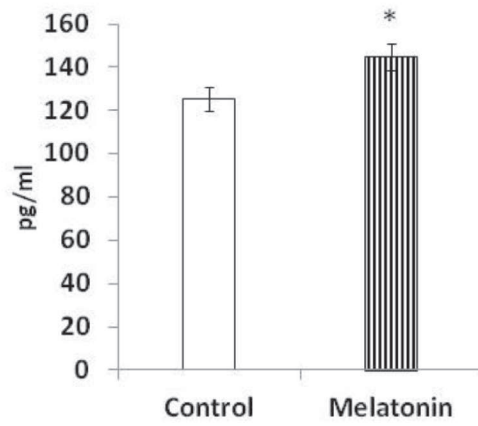


Fig.8

Melatonin in modulation of reproductive functions in the female Indian pygmy field mouse, *Mus terricolor*

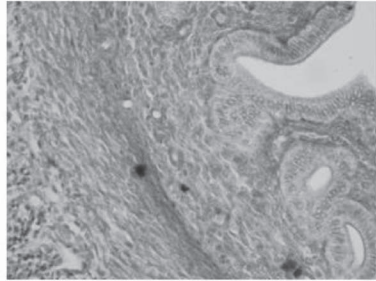


Fig.9

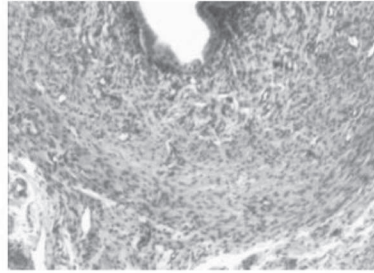


Fig.10

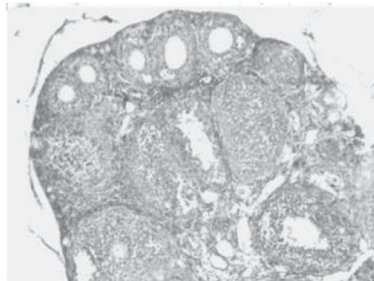


Fig.11A

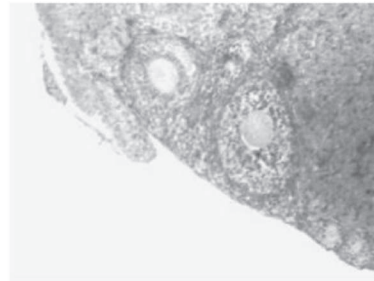


Fig.11B

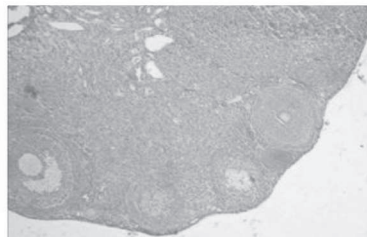


Fig.12A

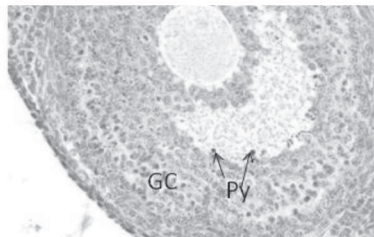


Fig.12B

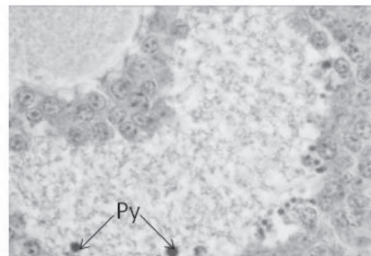


Fig.12C