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NEONATAL EXPOSURE TO MELATONIN FAVOURS FOLLICULAR SURVIVAL AND INCREASES FECUNDITY IN THE FEMALE RAT

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SUMMARY

Rat neonates have been exposed to melatonin by i.p. injection of 40µg melatonin/ animal/day daily in the evening at 16:30 hrs from day 0 to day 21. The preweaning hypermelatonemia did not significantly influence either adult body weight or ovarian weight. The estrogen titre was reduced and progesterone titre increased in melatonin treated rats. Increased thyroid weight and elevated thyroid hormones levels were also recorded. The primordial and primary follicles were more in number with significantly lesser atretic follicles of all developmental stages. The first litter size was significantly greater *(11± 3 vs 8 ± 2)* in melatonin treated rats compared to controls. These results are interpreted in terms of protective action of neonatal melatonin against follicular apoptosis either directly or indirectly through thyroid hormones.

Key words : Follicular apoptosis; Melatonin; Progesterone; Thyroid hormone.

INTRODUCTION

Sexual development and maturation is a prolonged process commencing during the intrauterine life and mediated by the ontogeny of hypothalamus- pituitary-gonadal axis (HPG) (1). The role of the maternal pineal gland during preg nancy on the sexual function of offspring has been studied (2,3). Melatonin treatment during gestation in rats has been reported to delay sexual maturation of female offspring (4). A subsequent study by the same group has indicated that maternal melatonin is necessary for normal somatic growth and postnatal development of reproductive organs of the offspring (5). Since the influence of melatonin on the development of the reproductive system has been known to commence during the prenatal period and extend into the postnatal life (6), melatonin infusion either in the evening or in the morning in the infantile to prepubertal period (10-25 days) has been tested in our laboratory. This study showed decreased body and testes weight after melatonin treatment, more pronouncedly in the evening schedule (7).

Recent study from this laboratory on long term influence of neonatal melatonin administration has revealed favourable influence on body weight gain with increased germ cell number in the adult testis and a permanent hyposetting of the central set point of the neuroendocrine reproductive axis (Ramachandran *et al, communicated)*. Since neonatal melatonin treatment has shown to influence male gonadal structure and functions and adult neuroendocrine axis, in the present study, influence of evening melatonin infusion in the preweaning period (0-21 days) on adult body and ovarian weights, ovarian histoarchitechture and serum hormone profiles has been evaluated.

MATERIALS AND METHODS

Animals and maintenance

Healthy female laboratory rat neonates (Charles Foster strain) were used for the present study. The animals were maintained in Sarabhai Research Center, with a constant temperature range of 21 ± 2°C and under a lighting regimen of LD 8:16 throughout the experimental period of

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study. The animals were fed with standard diet (Amrut Rat Feed) and water *ad libitum*. The treatment was initiated on day '0' (day of birth) and terminated on day 21 postpartum.

Preparation of melatonin

Melatonin (N-acetyl 5-methoxytryptamine) procured from Sigma Co. USA was weighed and dissolved in required quantity of ethanol and diluted accordingly in 0.9 % saline.

Experimental protocol

The experimental setup was divided into two groups of study.

Group I Control (C) :Rat neonates (female) were maintained in the laboratory till day 90 and served as controls. This consisted of 2 subgroups (as follows) of 30 animals each:

(i) Control rats (Maintained as such)

(ii) Injected *i.p.* with vehicle (0.9% saline) at 16.30 hrs.

Group II Melatonin treatment :Rat neonates (female) were injected *i.p.* with 40 mg melatonin/animal/day from day 0 to day 21 postpartum, at 16.30 hrs.

Parameters and methods of evaluation

The treatment was discontinued from day 22 and the female animals were sacrificed at 22, 45 and 90 days of age, and various morphometric, gravimetric and histocytometric studies were carried out. The animals were sacrificed under mild anesthesia and blood was collected by brachial venipuncture in ependorff tubes. They were centrifuged at 4000 rpm and serum was collected and stored at -4°C. Later, these serum samples were utilized for assay of various hormones. The viscera was cut open and the ovaries were excised, blotted free of tissue fluids and weighed accurately in a Mettler balance. The absolute weight so obtained was converted in to relative weight and expressed as percentage of body weight. The ovaries were fixed in Bouin's fluid and processed for paraffin wax histology.

Histology and histometry

Ovaries were fixed immediately in Bouin's fluid and processed for histological studies. Paraffin sections of 3 mm thickness were cut on a microtome and stained with Haematoxylin-Eosin (HE). For morphometry and enumeration of ovarian follicles, homologous cross sections of entire ovary showing better area of vision were chosen. The section area is calculated by integrating the area inside the traced perimeter and volume is calculated by multiplying by the section thickness. The section volume is multiplied by 10 (to account for the number of sections skipped) to give the "10 section" volume; sum all of the 10-section volumes to obtain an estimate of the total ovarian volume (in mm^3) (8,9). The total counts of different types of follicles were made.

Hormone assays

The blood for hormone assays was collected from the brachial vein under mild anesthesia before sacrificing the animals. T_3 and T_4 assayed by using ELISA kit purchased from Medix Biochemica Oy Ab, Finland and estradiol *and* progesterone were assayed by using ELISA kit purchased from General Biologicals Corp, Taiwan and is expressed as ng/ml of serum.

Statistical analysis

All data are expressed as mean ± SEM. The data were analyzed by Student's 't' test and two-way analysis of variance (ANOVA) wherever applicable, at 95% confidence limit.

RESULTS

Body and ovary weight

The body weight of melatonin treated animals was significantly less at all ages of study including adult stage. However, there was no significant difference in absolute or relative ovarian weight (Table 1).

Histology and histometry

In general, the ovary of melatonin treated animals showed greater population of follicles at all ages of study compared to corresponding controls. Though there was no difference in ovarian volume, a differential count of various follicles has revealed significantly higher

Table 1: Chronological alteration showing body weight, absolute and relative ovary weight of control and melatonin treated female rats

Groups	Body weight Age in days			Absolute ovary weight Age in days			Relative ovary weight Age in days		
	22	45	90	22	45	90	22	45	90
C	51.33	136.67	226.17	0.024	0.06	0.076	0.046	0.045	0.034
	±1.873	±4.176	±9.575	±0.0003	±0.0069	±0.0074	±0.0006	±0.0015	±0.0025
MT	46.67	129.333	194.833	0.018	0.050	0.074	0.038	0.040	0.038
	±0.843	±2.917	±6.66[a]	±0.0007[c]	±0.0045	±0.0064	±0.0023[b]	±0.0039	±0.0023

C - Control; MT - Melatonin treated

Values expressed as Mean ± SEM of six animals.

[a]$p < 0.01$, [b]$p < 0.005$, $p<0.0005$

Table 2: Differential total follicular count in ovary of control and melatonin treated female rats

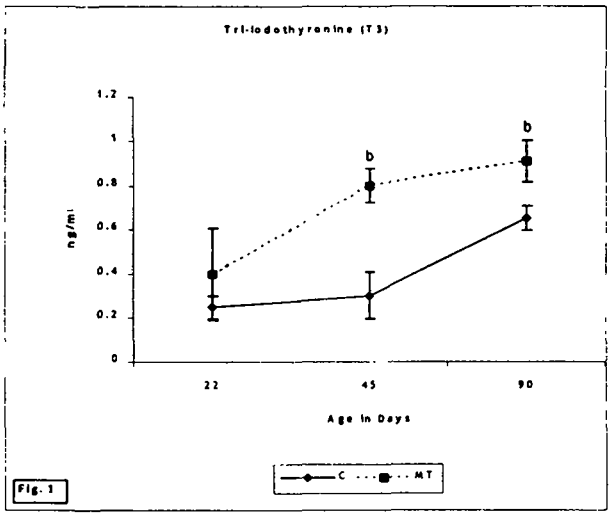
Groups	Age in days	Follicle type						Ovarian volume (mm^3)
		Primordial	Primary	Secondary	Antral	CL	Atretic	
C	22	821±48	531±25	350±20	181±04	-	10±0.8	0.58
	45	426±17	162±11	245±10	142±08	-	29±02	0.68
	90	300±13	168±08	96±04	72±05	48±05	36±03	1.31
MT	22	891[b]±42	391±19[b]	452[b]±12	183[a]±05	-	05±0.4[c]	0.45[a]
	45	446±15	229±10[b]	277±28	166±11	-	09±0.1[c]	0.88[b]
	90	373[b]±15	217±12[b]	108±04	96[b]±04	72[b]±06	13±01[c]	1.29

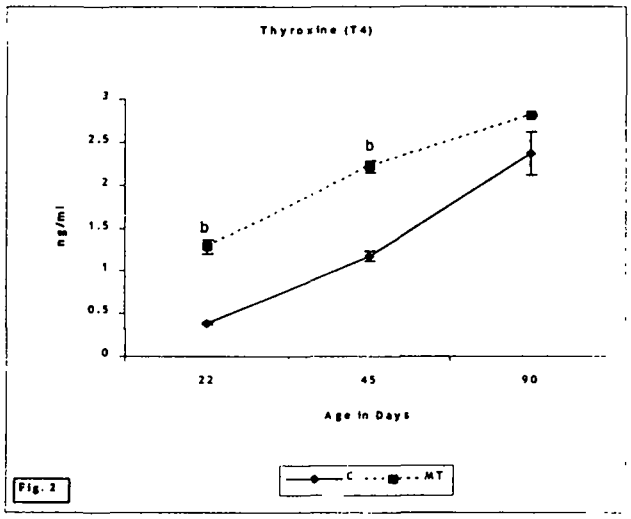
C - Control; MT - Melatonin treated; CL - Corpora lutea

Values are expressed as Mean ± SEM of six animals.

[a]$p<0.01$; [b]$p<0.005$; [c]$p<0.0005$

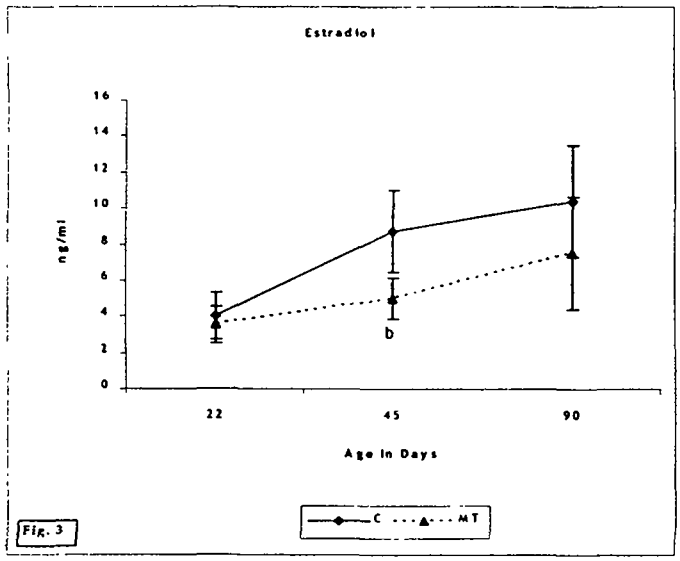
numbers of primordial, primary, preantral and antral follicles. In the 90 day old ovary, there was almost double the number of corpora lutea in melatonin treated rats compared to controls. A count of atretic follicles has also shown significantly lesser number in melatonin treated rats (Table 2).

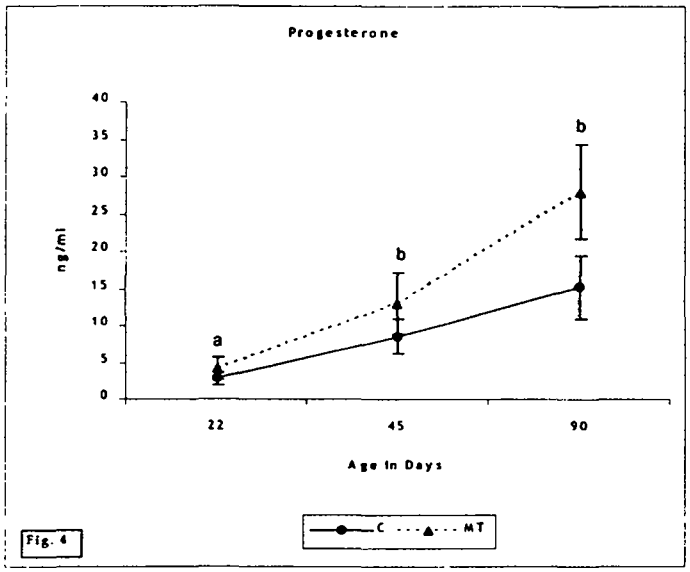




Figures 1 and 2: Chronological alterations showing serum T3 and T4 (ng/ml) levels in control (C) and melatonin (MT) treated rats

Values are expressed as Mean ± SEM of four samples; $*p< 0.05$; $**p< 0.001$





Figures 3 and 4:Chronological alterations showing serum estradiol and progesterone (ng/ml) levels in control (C) and melatonin (MT) treated rats

Values expressed as Mean ± SEM of four samples; $*p < 0.05$; $**p < 0.001$

Serum hormone profile

The circulating titre of estrogen was significantly less and that of progesterone significantly high in melatonin treated rats of all ages (Fig 3 and 4). Both circulating T_3 and T_4 levels were also significantly higher in melatonin treated rats (Fig 1 and 2).

DISCUSSION

In the present study, neonatal melatonin administration creating a hypermelatonemic state has shown a favourable influence on the adult ovarian functions marked by significantly increased number of follicles and greater fecundity of such rats. There are no studies on these lines involving melatonin excess during neonatal period. Neonatal melatonin excess does not seem to influence gonadal growth as in the present study, no significant difference in ovarian growth or final adult ovarian weight was recorded. Similarly, a previous study on neonatal melatonin treatment to male pups has also failed to show any significant difference in adult testes weight (Ramachandran *et al. (communicated)*. However, in the present study a differential effect in the body weight was recorded following neonatal melatonin treatment. In the present study, adult females weighed lesser than controls and in the above study of Ramachandran *et al. (communicated)* adult males weighed heavier than controls. Since in the above study, a possible long term positive resetting of the hypothalamo-pituitary-growth hormone axis was inferred as a possible cause in the light of known ability of melatonin to induce elevation of growth hormone level (10, 11), in the present case, it may be deemed to be due to a possible negative resetting. This might suggest a sexual difference on the influence of neonatal melatonin on the growth hormone axis as similar sexual difference with reference to the reproductive hormone axis postnatally was shown due to prenatal melatonin administration (3). Though there is no difference in the ovarian weight neonatal melatonin treatment has influenced ovarian functions. The histoarchitecture of the ovary at 22, 45 and 90 days of age has revealed increased numbers of follicle types in the melatonin treated rats. Since there is significant increase in primordial, primary, secondary and antral follicles, neonatal melatonin administration seems to have a favourable influence on the survival of follicles on a long-term basis. In recent times involvement of melatonin on ovarian functions has been increasingly realized. In this context, Lee *et al.* (12) have shown expression of melatonin receptor gene in the granuiosa cells of developing female mice. Woo *et al* (13) have demonstrated a direct role for melatonin in regulating ovarian functions by way of producting praam-lion, LH receptor expression as well as GnRH and GnRH receptor gene expression through melatonin receptors in human granulosa and thecal cells.

Apart from the presence of significantly higher number of follicles, the ovaries of melatonin treated rats in the present study have also shown significantly lesser number of atretic follicles and more number of corpora lutea suggesting increased follicular survival by decreased apoptosis. Interestingly, melatonin has been shown to improve the quality of oocytes by preventing degeneration as well as by preventing intra-follicular lipid peroxidation in the human ovary (14). In another study, melatonin was also shown to exert radioprotective action on ovarian follicles in a-irradiated mice and the degree of this protection was found to be concentration related (15). In all the above studies, the protective action of melatonin has been demonstrated by simultaneous / continuous presence of melatonin. As against that in the present study, the protective action of melatonin has been realized on long-term basis much after the cessation of administration of melatonin. This is a novel observation and the mechanisms of this long lasting protection afforded by neonatal melatonin administration remain a matter of conjecture. The possible explanations could be a permanent genetic reprogramming with reference to ovarian survival / apoptotic factors and/or permanent resetting of neuroendocrine ovarian hormonal axis. With reference to the former, follicular survival or

apoptosis is regulated by a number of hormones, growth factors and cytokines, which in turn activate several sub-programmes involving many genes (16). The second possibility of altered neuroendocrine reproductive axis is validated by the presently observed decreased estrogen:progesterone ratio and the increased thyroid hormone titres. In this connection, Brzezinski *et al.* (17) have suggested a role for melatonin in the intra ovarian control of progesterone production in the human ovary. Overall, it can be concluded from the present studies that neonatal hypermelatonemia has a favourable influence on adult ovarian functions marked by higher follicular survival and ovulation of more number of ova and significant higher number of corpora lutea and fecundity.

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