CHAPTER 5

Neonatal melatonin treatment has favourable quantitative and qualitative influence on adult ovarian functions in the rat

Melatonin, the hormone of the pineal gland secreted during night in all vertebrates (Reiter, 1992; Cagnacci, 1996) is known to be involved in the modulation of seasonal reproduction in mammals (Bittman *et al.*, 1985; Hastings, 1989; Misztal *et al.*, 1996) and control of circadian and annual cyclicities (Bartness *et al.*, 1993; Reiter, 1993; Hardeland *et al.*, 1995). However, its role in reproduction of non-seasonal breeders is not that marked (Arendt, 1998). The reproductive system of adult rat is reported to be insensitive to exogenous melatonin (Reiter, 1980; Goldman *et al.*, 1981). Though there are a few reports of varying effects due to either pinealectomy or melatonin infusion in adult rats, a general concept of no major role for pineal has been the understanding (Reiter, 1980; Goldman *et al.*, 1981; Binkely, 1983). Immature rats have nevertheless been shown to be responsive to melatonin. Retardation of development of reproductive system in both male and female has been reported due to melatonin administration (Wurtman *et al.*, 1963; Motta *et al.*,

1967; Debeljuk, 1969; Kinson and Robinson, 1970; Kinson and Peat, 1971). Based on a number of experiments involving administration of melatonin at different periods in the immature stage, 20-40 days have been shown to be relatively more sensitive in terms of the hormone's inhibitory effect (Lang *et al.*, 1983, 1984).

Sexual development and maturation is a prolonged process commencing during the intrauterine life and mediated by the ontogeny of hypothalamus–pituitary-gonadal axis (HPG) (Chiappa and Fink, 1977). The role of the maternal pineal gland during pregnancy on the sexual function of offspring has been studied by Jarrige *et al.* (1987, 1990). Melatonin treatment during gestation in rat has been reported to delay sexual maturation of female offspring (Colmenero *et al.*, 1991). 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 (Diaz *et al.*, 1999). 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 (Weaver, 2000), 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 weight and testes weight in the period immediately

after melatonin treatment, more pronouncedly in the evening schedule (Patel and Ramachandran, 1992).

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.*, 2004). Since neonatal melatonin treatment has been seen 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.

MATERIAL AND METHODS:

Animals and Maintenance:

Healthy female laboratory rat neonates (Charles foster strain) were used for the present study. The animals were maintained at Sarabhai Research Center, with a constant temperature range of 21 ±2°C and a lighting regimen of LD 8:16 throughout the experimental period of 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 a few drops of ethanol and diluted in 0.9 % saline.

Experimental Protocol:

The experimental setup was divided into two groups of study.

Group I Control (C):

Female rat neonates were maintained in the laboratory till day 90 and served as controls. This consisted of 2 subgroups (as follows) of 10 animals each:

(i) Control rats (Maintained as such)

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

Group II Melatonin (MT):

Female rat neonates consisting of 10 animals were injected *i.p.* with 40μg melatonin/animal/day (N-Acetyl-5- hydroxytryptamine) (MT) from day 0 to day 21 postpartum, (procured from Sigma Chemical Co. USA) 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 anaesthesia and blood was collected by brachial venipuncture in epindorff 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 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 3μm 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; all the of 10-section volumes are summed to obtain an estimate of the total ovarian volume (in mm^3) (Plowchalk *et al.*, 1993; see Tilly, 2003). The total counts of different types of follicles were also 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 were assayed by ELISA using kit purchased from Glaxo (product code H -T_3H-0010 and H-T_4H-0010) and expressed as ng/ml of serum. Estradiol & Progesterone were assayed by using ELISA kit purchased from General Biologicals Corp, Taiwan and 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 analysis of variance (ANOVA) wherever applicable, at 95% confidence limit.

RESULTS:

Since there was no significant difference between the values of the two subgroups of controls, the data of only vehicle control is considered.

Body and Ovary Weight:

The body weight of melatonin treated animals was significantly less at all ages of study including at adult stage. However, there was no significant difference in absolute or relative ovarian weight (Table 5.1; Figs. 5.1a, 5.1b, 5.1c). The per day growth rate was significantly lower in melatonin treated rats compared to controls, with almost similar ovarian growth rate (Table 5.2; Figs. 5.2a, 5.2b).

Table 5.1: Chronological alteration showing body weight, absolute and relative ovary weight of control and melatonin treated female rats

Treatment	Body weight			Absolute Ovary weight			Relative Ovary weight		
	Age in Days			Age in Days			Age in Days		
	22	45	90	22	45	90	22	45	90
C	51.33 ±1.873	136.667 ±4.176	226.167 ±9.575	0.024 ±0.0003	0.062 ±0.0069	0.076 ±0.0074	0.046 ±0.0006	0.045 ±0.0015	0.034 ±0.0025
MT	46.667 ±0.843	129.333 ±2.917	194.833[a] ±6.66	0.018[c] ±0.0007	0.050 ±0.0045	0.074 ±0.0064	0.038[b] ±0.0023	0.040 ±0.0039	0.038 ±0.0023

C – Control; MT – Melatonin treated

Values expressed as Mean ± SEM of ten animals; [a] $p<0.01$; [b] $p<0.005$, [c] $p<0.0005$

Table 5.2: Per day Body and Ovary Growth Rate (g/day) in Control and Melatonin treated female rats

Treatment	Per Day Body Growth Rate			Per Day Ovary Growth Rate		
	Age in days			Age in days		
	0-22	22-45	45-90	0-22	22-45	45-90
C	2.055	1.896	1.000	0.00009	0.00084	0.00015
MT	1.706	1.907	0.727	0.00011	0.00072	0.00026

C – Control; MT – Melatonin treated

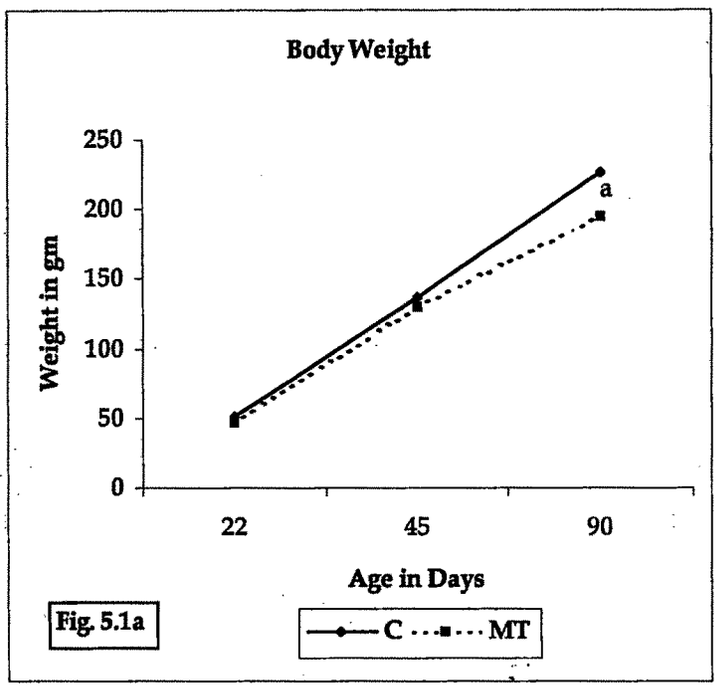


Fig. 5.1a

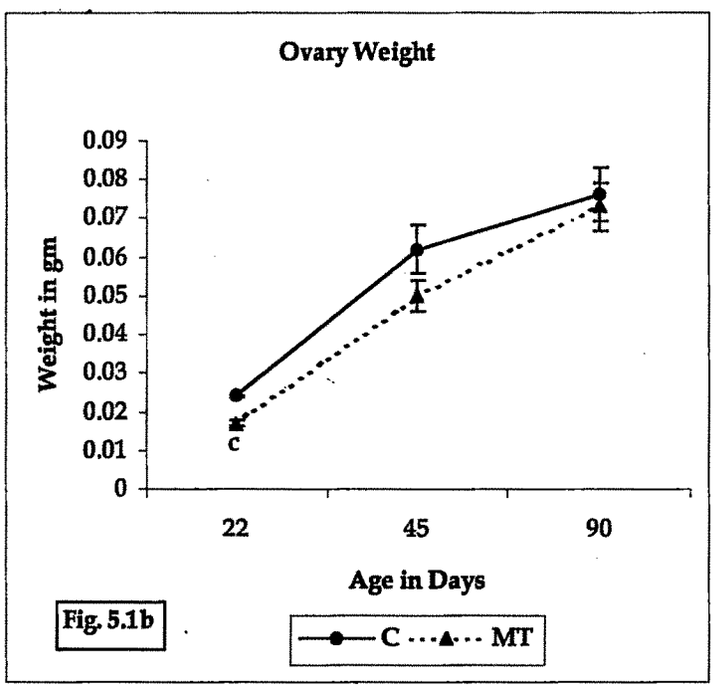


Fig. 5.1b

Figures 5.1a&b: Chronological alterations showing body and ovary (g) weight in control (C) and melatonin treated (MT) rats

Values expressed as Mean ± SEM of ten animals;

$^{a}p<0.01$; $^{b}p<0.005$, $^{c}p<0.0005$

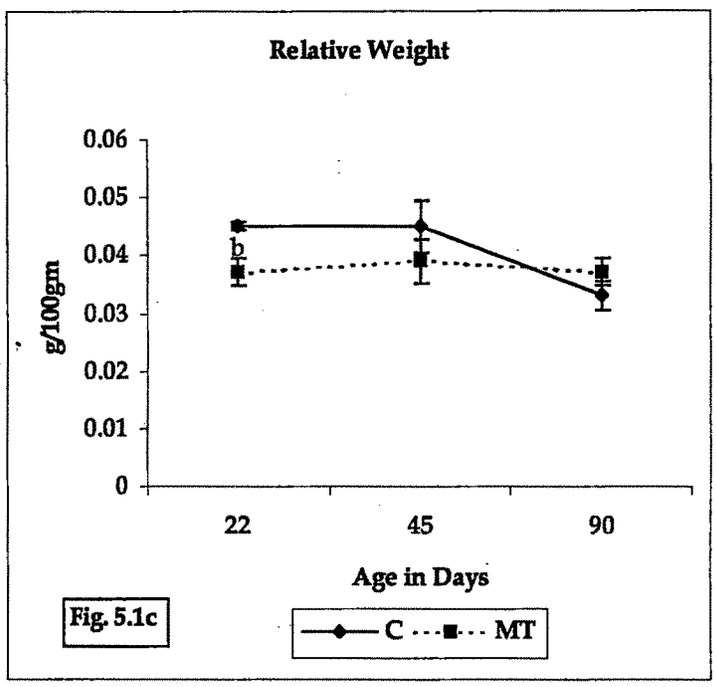
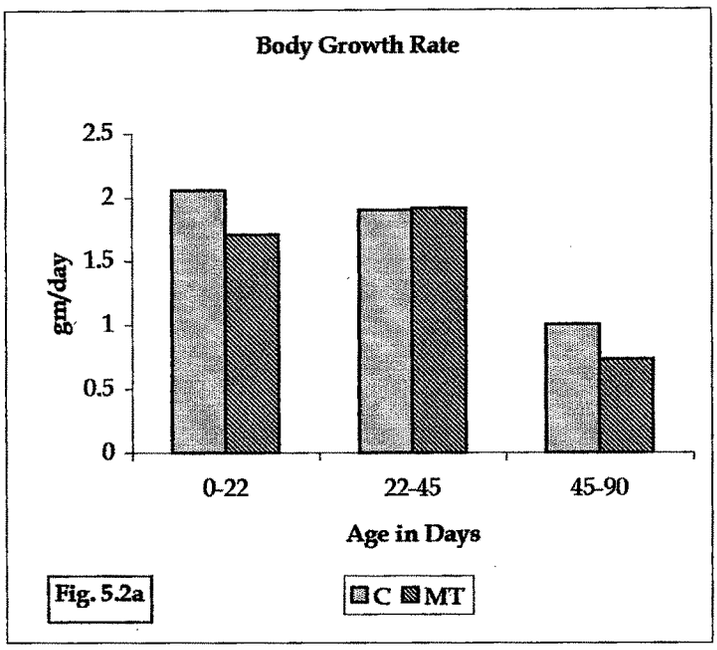
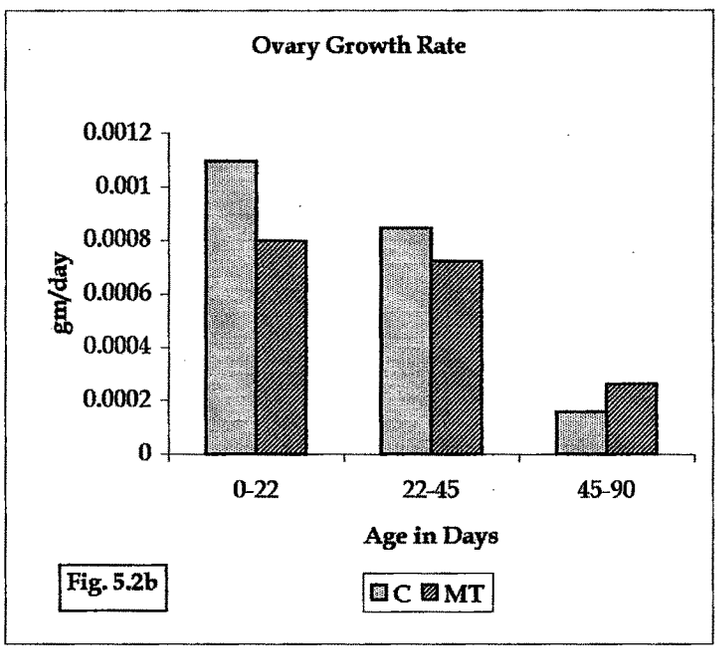


Figure 5.1c: Chronological alterations showing relative (g/100g) ovary weight in control (C) and melatonin treated (MT) rats

Values expressed as Mean ± SEM of ten animals;

$^{a}p<0.01$; $^{b}p<0.005$, $^{c}p<0.0005$





Figures 5.2a&b: Chronological alterations showing body and ovary (g/day) growth rate in control (C) and melatonin treated (MT) rats

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 (Plate VA and VB). Though there was no difference in ovarian volume, a differential count of various follicles has revealed significantly higher numbers of primordial, primary, preantral and antral follicles. In the 90 day old ovary, there was almost double the number of corporal lutea in melatonin treated rats compared to controls. A count of atretic follicles has also shown significantly lesser number in melatonin treated rats (Table 5.3).

Table 5.3: Differential total follicular count in ovary of control and melatonin treated female rats

Treatment	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[b] ±19	452[b] ±12	183[a] ±05	-	05[c] ±0.4	0.45[a]
	45	446 ±15	229[b] ±10	277 ±28	166 ±11	-	09[c] ±0.1	0.88[b]
	90	373[b] ±15	217[b] ±12	108 ±04	96[b] ±04	72[b] ±06	13[c] ±01	1.29

C – Control; MT – Melatonin treated; CL – Corpus Lutea

Values expressed as Mean ± SEM of ten animals; [a] $p<0.01$; [b] $p<0.005$, [c] $p<0.0005$

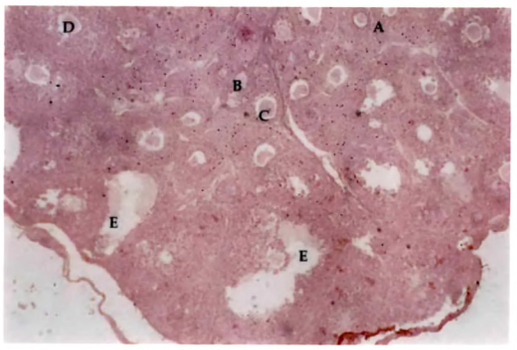
PLATE - VA

Figures: **Photomicrographs of sections of Ovary of Control and Melatonin treated rats**

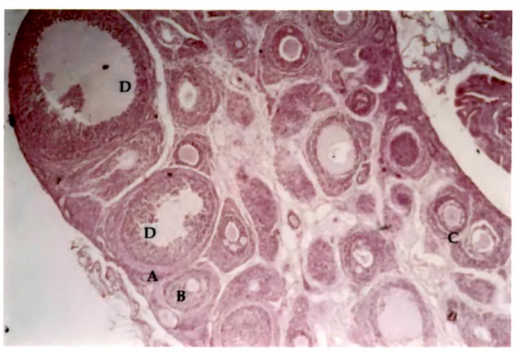
Sections of ovaries of 22 and 45 day old control rats showing more number of atretic follicles compared to age matched treated groups that shows more number of pre-antral follicles and fewer atretic follicles.

A – Primordial follicle, **B** – Primary follicle, **C** - Secondary follicle, **D** – Antral follicle, **E** – Atretic follicle, **GC** – Granulosa cells, **TC** – Thecal cells, **OT** – Oocyte

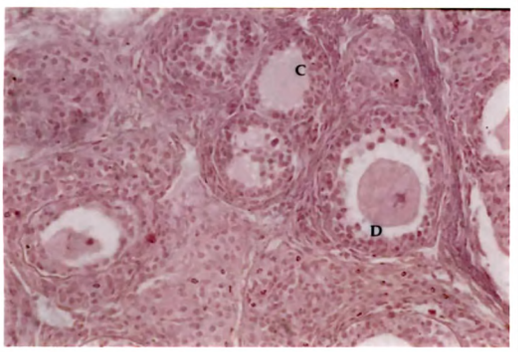
PLATE - VA



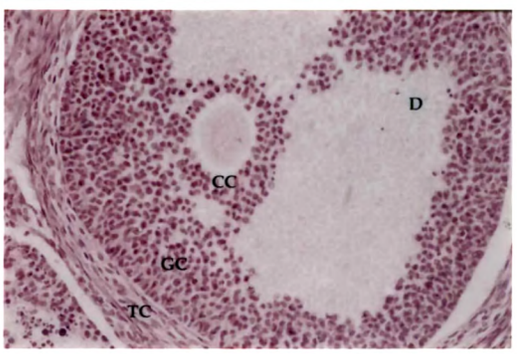
C 22 10x



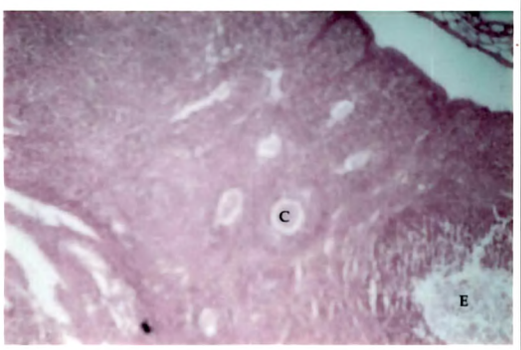
M 22 10x



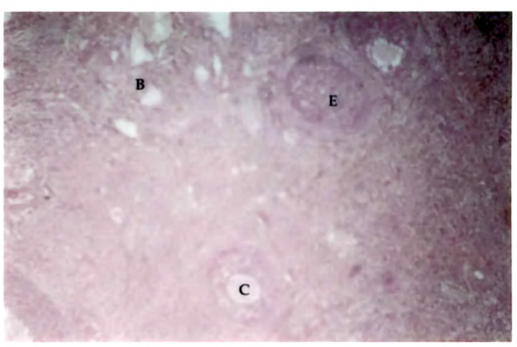
C 22 40x



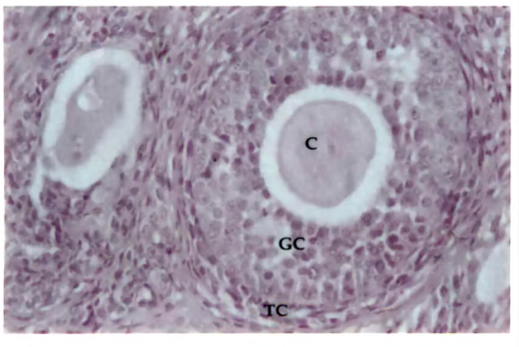
MT 22 40x



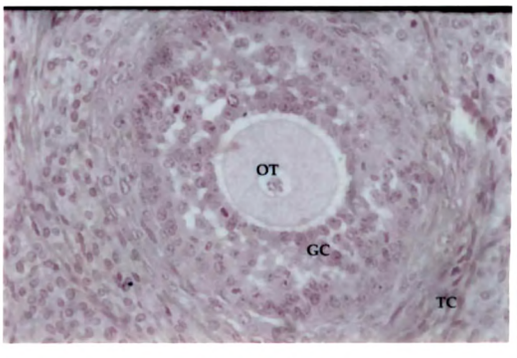
C 45 10x



M 45 10x



C 45 40x



M 45 40x

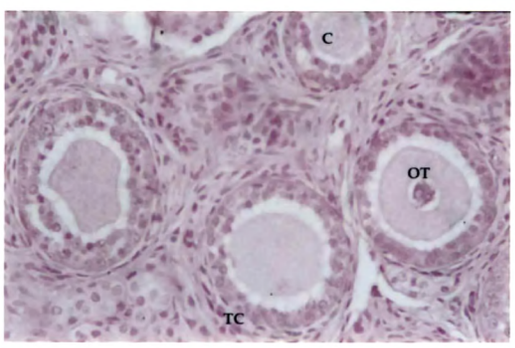
PLATE - VB

Figures: **Photomicrographs of sections of Ovary of Control and Melatonin treated rats**

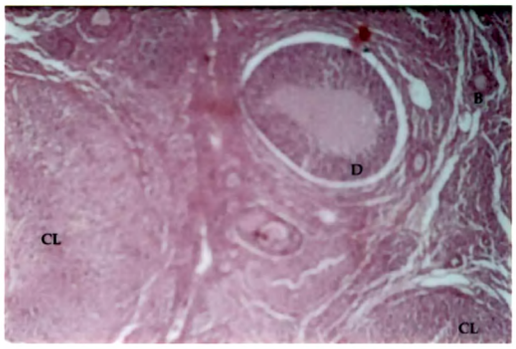
Sections of ovary of 90 day old control and melatonin treated rats showing more number of secondary and tertiary follicles in melatonin treated rats compared to controls.

A – Primordial follicle, B – Primary follicle, C - Secondary follicle, D – Antral follicle, E – Atretic follicle, GC – Granulosa cells, TC – Thecal cells, OT – Oocyte

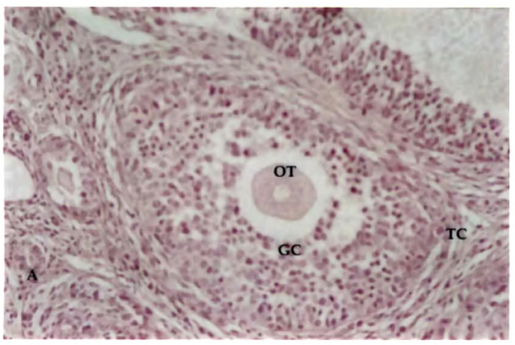
PLATE - VB



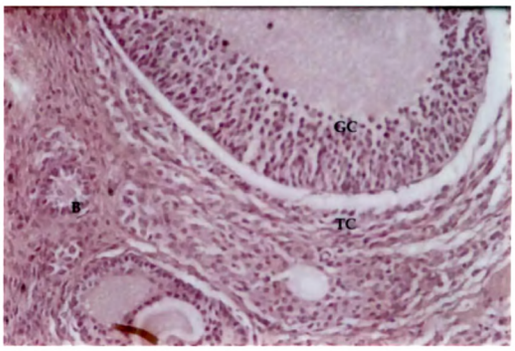
C 90 10x



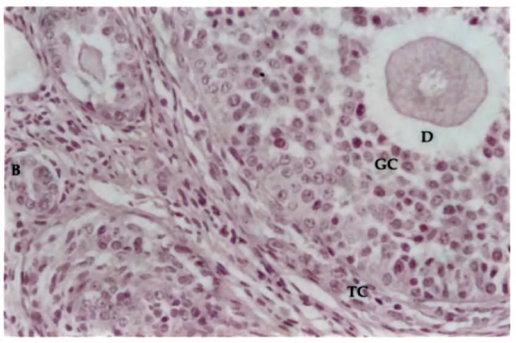
MT 90 10x



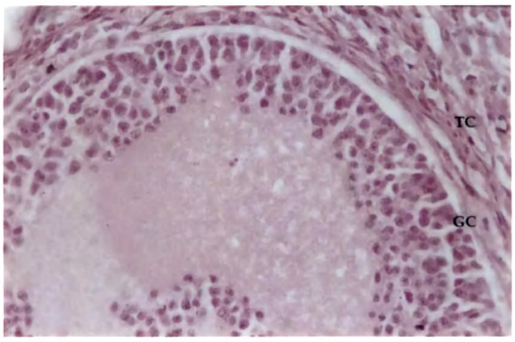
C 90 25x



MT 90 25x



C 90 40x



MT 90 40x

Serum Hormone Profile:

The circulating titre of estrogen was significantly less and that of progesterone significantly high in melatonin treated rats of all ages. Both circulating T3 and T4 levels were also significantly higher in melatonin treated rats (Tables 5.4 & 5.5; Figs. 5.4a, 5.4b, 5.5a, 5.5b). In order to avoid the contradiction due to differential levels of estrogen and progesterone during the estrous cycle, the serum levels of only those animals which were in late diestrous or early proestrous on the day of sacrifice were assayed and the values represented are on an average of 3-6 animals. Serum T4 and T3 levels were assayed due to the fact that our previous studies have shown a definite influence of neonatal melatonin administration to these levels not only during the treatment or the post-treatment but even in the adult stage as a long-term effect (Ramachandran *et al.*, 2004). Moreover, recent studies have shown that thyroid hormones have definite influence on ovarian development in the postnatal periods (Hapon *et al.*, 2003).

Table 5.4: Serum hormone profiles of T3 and T4 (ng/ml) in control and melatonin treated female rats

Treatment	T3			T4		
	Age in Days			Age in Days		
	22	45	90	22	45	90
C	0.251 ±0.051	0.303 ±0.107	0.653 ±0.053	0.38 ±0.013	1.170 ±0.061	2.368 ±1.131
MT	0.400 ±0.206	0.800[b] ±0.074	0.910 ±0.095	1.282[c] ±0.084	2.212[c] ±0.068	2.805 ±0.015

C - Control; MT - Melatonin

Values expressed as Mean ± SEM of ten animals;

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

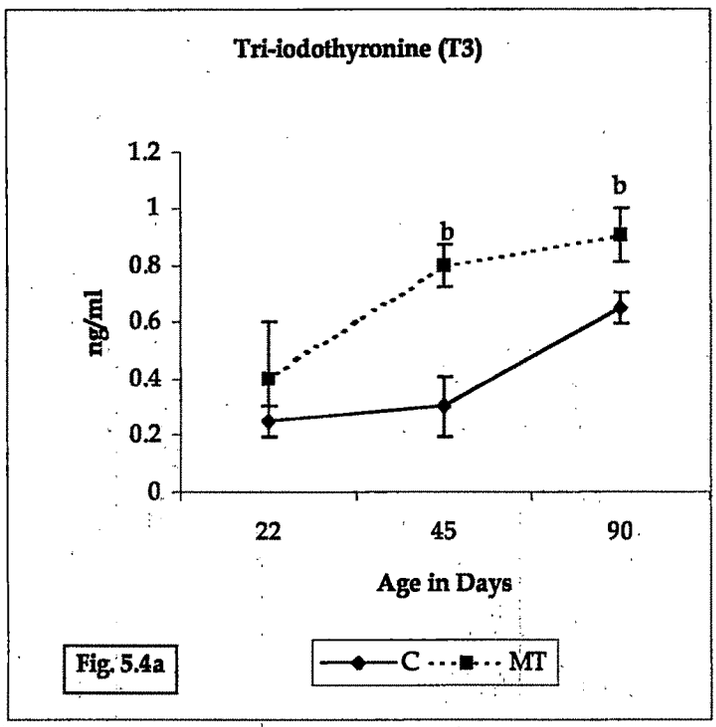
Table 5.5: Serum hormone profiles of Estradiol and Progesterone (ng/ml) in control and melatonin treated female rats

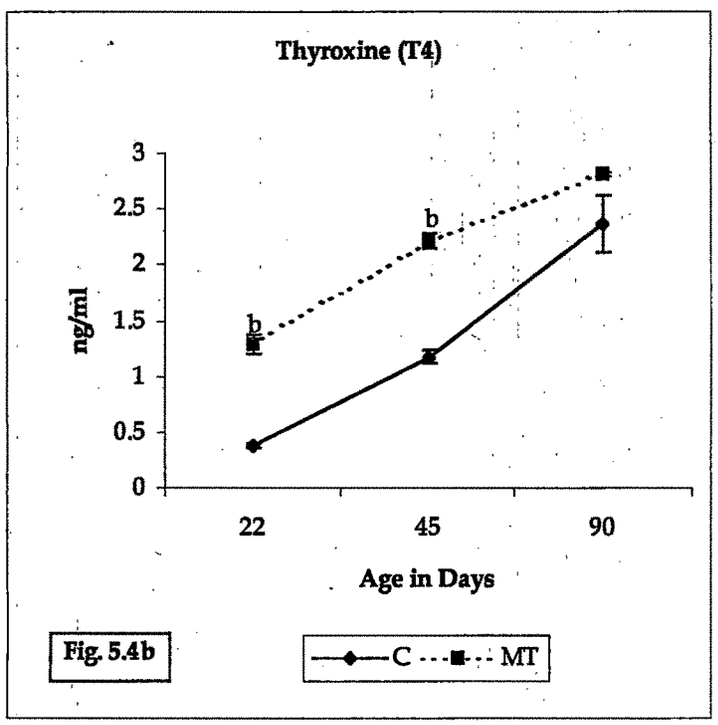
Treatment	Estradiol			Progesterone		
	Age in Days			Age in Days		
	22	45	90	22	45	90
C	4.02 ±1.30	8.73 ±2.55	10.39 ±3.10	2.75 ±0.88	8.55 ±2.41	15.19 ±4.36
MT	3.53[b] ±1.01	5.00[b] ±1.12	7.52 ±2.00	4.07 ±1.5	13.0 ±4.11	27.96 ±6.33

C - Control; MT - Melatonin

Values expressed as Mean ± SEM of 3-6 animals;

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





Figures 5.4a&b: Chronological alterations showing serum T3 and T4 (ng/ml) levels in control (C) and melatonin treated (MT) rats

Values expressed as Mean ± SEM of ten animals;

$^{a}p<0.01$; $^{b}p<0.005$, $^{c}p<0.0005$

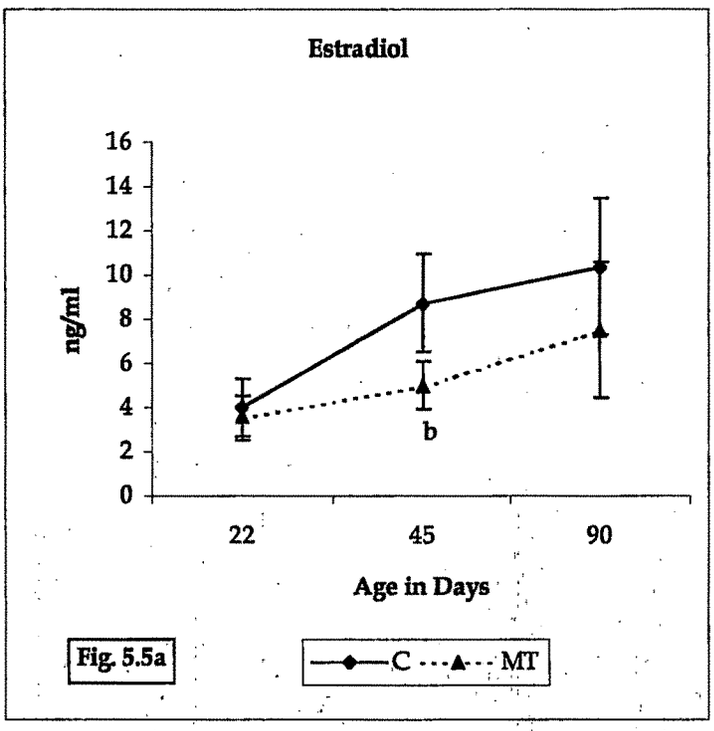


Fig. 5.5a

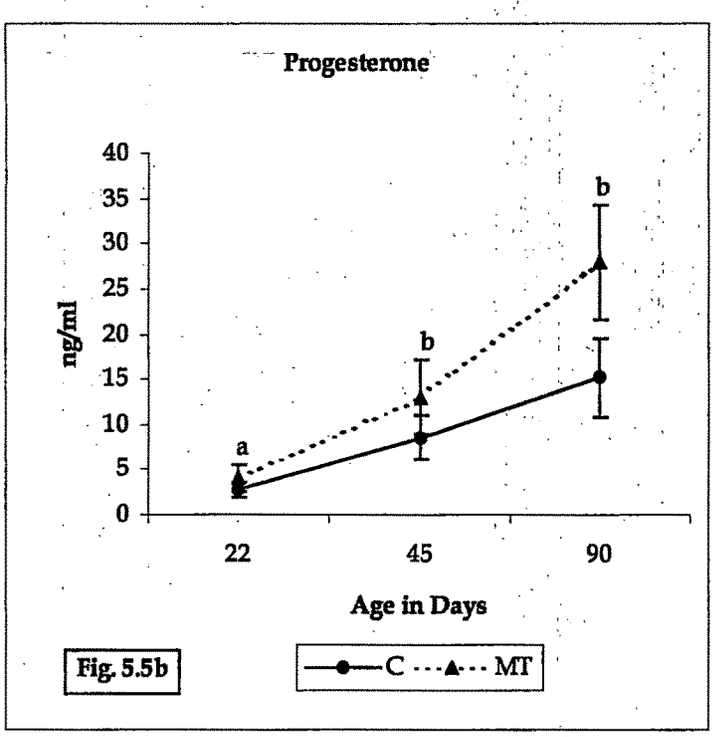


Fig. 5.5b

Figures 5.4a&b: Chronological alterations showing serum Estradiol and Progesterone (ng/ml) levels in control (C) and melatonin treated (MT) rats

Values expressed as Mean ± SEM of 3-6 animals;

$^{a}p<0.01$; $^{b}p<0.005$, $^{c}p<0.0005$

DISCUSSION:

In the present study, neonatal melatonin (MT) 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. Though there are no studies on these lines involving melatonin excess during neonatal period, effect of melatonin excess during foetal/prenatal stage has been studied in the rat (Diaz *et al.*, 1995). This study showed on altered neonatal hormonal status more particularly with reference to LH and prolactin. A sexual difference was also indicated by the elevated LH levels till the prepubertal period in female offsprings born to melatonin treated mothers and a decreased LH level in male offsprings. The authors had concluded that both pinealectomy of the mother or melatonin treatment could affect foetal development and influence the postnatal ontogeny of the hormones involved in the neuroendocrine reproductive axis in developing rats.

Another study involving maternal melatonin treatment or pinealectomy during gestation has also indicated the requirement of maternal melatonin for normal somatic growth and postnatal development of the reproductive organs of the offsprings (Diaz *et al.*, 1999). In contrast to foetal melatonin excess, neonatal melatonin excess does not seem to influence gonadal growth as in the present

study no significant difference in ovarian growth or final adult 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.*, 2004). However, a differential effect of neonatal melatonin on body weight gain and ultimate adult body weight is recorded, as in the present study; adult females weighed lesser than controls and in the above study of Ramachandran *et al.* (2004) 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 (Mckneown *et al.*, 1975; Vriend *et al.*, 1990), 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 (Diaz *et al.*, 1995; Diaz *et al.*, 2000).

Though there is no difference in the ovarian weight, neonatal MT treatment has nevertheless a favourable influence on ovarian functions. The histoarchitechture of the ovary studied post treatment at 22, 45 and 90 days of age has revealed increased

numbers of all follicle types in the melatonin treated rats. Since there is significant increase in primordial, primary, secondary and antral follicles, neonatal MT seems to have a favourable influence on the survival of follicles on a long-term basis. In recent times involvement of MT on ovarian functions has been increasingly realized. In this context, Lee *et al.* (2001) have shown expression of melatonin receptor gene in the granulosa cells of developing female mice. The expression has been shown to be high in all developmental follicles except primordial and atretic and, based on these observations they have concluded that MT has a pivotal role in folliculogenesis. Woo *et al.* (2001) have demonstrated a direct role for melatonin in regulating ovarian functions by way of progesterone production, 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 (Takasaki *et al.*, 2003). In another study, melatonin was also shown to exert

connection, Brzezinski *et al.* (1992) have suggested a role for melatonin in the intra-ovarian control of progesterone production in the human ovary. Further evidence is provided by the work of Faigon *et al.* (1982) showing melatonin injections in neonates disrupting LH negative feedback response and diminished LH induced steroid release, of Johnston *et al.* (2003) reporting altered sensitivity to GnRH induced FSH and LH release due to neonatal melatonin administration and of Nakamura *et al.* (2003) of an increased progesterone production of follicles in response to melatonin. Based on recent reports on thyroid hormones favouring follicular growth (Jiang et al., 2000) it is also inferable that the presently recoreded increased thyroid hormone levels in response to eonatal melatonin, may also provide a favourable environment for follicular growth and reduce the degree of follicular apoptosis. 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 indicated by the significantly higher number of corpora lutea and fecundity of melatonin treated female rats.

SUMMARY:

The long-term effects of neonatal hypermelatonemia on adult ovarian weight and functions and serum hormone profiles have been studied in Charles Foster strain of rats. The neonates were administered melatonin (MT) *i.p.* (40 μg/animal/day) in the evening from day 0 to day 21 post-partum. On post-partum days 22, 45 and 90 days of age, the serum titres of T3, T4, estradiol and progesterone and ovarian weight and histoarchitechture were assessed. Though there was no difference in adult ovary weight between control and MT treated rats, there was an increase in number of follicles with significantly higher number of antral follicles and corpora lutea in MT rats. The number of atretic follicles showed a significant decrement. It is concluded from these observations that neonatal melatonin excess increases progesterone secretion and decreases estrogen secretion and affords protection to follicles from apoptosis thereby increasing the follicular number and corpora lutea.