CHAPTER 7

Simultaneous melatonin administration is able to resist the negative influence of neonatal hypothyroidism on folliculogenesis but not on body weight and ovarian weight and volume

Sexual development and reproductive maturation is a protracted event initiated during the foetal stage and influenced by the developing hypothalamic-pituitary-gonadal axis (HPG) (Chippa and Fink, 1977). Thyroid hormones are critically involved in embryonic and foetal development of vertebrates (Sullivan *et al.*, 1987). Both thyroid hormone deficiency and excess are known to affect the development and functions of reproductive system (Kalland *et al.*, 1978; Mattheji., 1995). Prepubertal hypothyroidism induced from the day of birth till day 40 postpartum in rats has been shown to result at 40 days in reduced body and ovarian weight, with the ovaries of such rats containing more secondary and less antral follicles, smaller non-atretic antral follicles and more atretic follicles with no corpora lutea compared to age-matched controls (Dijkstra *et al.*, 1996). Number of studies on postnatal hypothyroidism have reported retarded growth and physical development, delay in eye opening and teething, slow to respond to

general environment and also to have depressed body weight (Meisami, 1984; Tamasy *et al.*, 1984; kawada *et al.*, 1988; Akaike *et al.*, 1991; Madeira *et al.*, 1991, 1992; Akaike and Kato, 1997). Previous study on induced hypothyroidism during the neonatal period in the Charles foster strain of rats has recorded increased follicular atresia and reduced corpora lutea together with decreased secretion of estrogen and progesterone from the adult ovary (Chapter 2).

In terms of melatonin status, immature rats have been shown to be relatively more responsive than adults. The development of reproductive system of both male and female has shown retardation due to melatonin administration (Wurtman *et al.*, 1963; Motta *et al.*, 1967; Debeljuk, 1969; Kinson and Robinson, 1970; Kinson and Peat, 1971). Postnatal days 20-40 have been shown to be more critical in this inhibitory effect (Lang *et al.*, 1983, 1984). Since the influence of melatonin on the development of the reproductive system has been shown 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 this 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). In continuation, neonatal administration of melatonin was

studied on a long term basis on adult ovarian functions, which revealed increased numbers of various follicles and more than doubled number of corpora lutea with fewer atretic follicles and greater fecundity (Chapter 1).

Since postnatal hypothyroidism and hypermelatonemia have shown reverse set of effects, an attempt is made in the present study to evaluate the effect of simultaneous neonatal hypothyroidism and hypermelatonemia on adult ovarian functions.

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.

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 Hypothyroidism + Melatonin (HPOT+MT):

Female rat neonates consisting of 10 animals were subjected to transient hypothyroidism (HPOT) by feeding mothers with 0.1% 6-propyl 2-thiouracil (PTU) in drinking water from day 0 to day 21 post partum and the pups were simultaneously injected. with 40μg melatonin/animal/day i.p. (N-Acetyl-5- hydroxytryptamine) (MT) (procured from Sigma Chemical Co. USA) at 16.30 hrs.

Parameters and Methods of Evaluation:

As in chapter 5

Histology and Histometry:

As in chapter 5

Hormone Assays:

As in chapter 5

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 ovarian weight:

The body and ovarian weights of experimental rats were consistently lower at all ages of study from 22-90 days postpartum. Whereas the body weight at 90 days was 26% lesser than controls, the absolute and relative weights of ovary were respectively 37% and 18% lesser (Tables 7.1, 7.2; Figs. 7.1a, 7.1b, 7.1c, 7.2a, 7.2b).

Table 7.1: Chronological alteration showing body weight, absolute and relative ovary weight of control and melatonin treated hypothyroid 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
HPOT+MT	30.833[c] ±0.477	82.00[c] ±0.93	166.83[c] ±4.475	0.014[c] ±0.0012	0.026[c] ±0.0022	0.048[b] ±0.005	0.047 0.0043	0.031[c] 0.0022	0.028 0.0031

C – Control; HPOT+MT – Melatonin treated Hypothyroid

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

Table 7.2: Per day Body and Ovary Growth Rate (g/day) in Control and melatonin treated Hypothyroid 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
HPOT+MT	1.183	1.137	0.942	0.0001	0.00026	0.00024

C – Control; HPOT+MT – Melatonin treated Hypothyroid

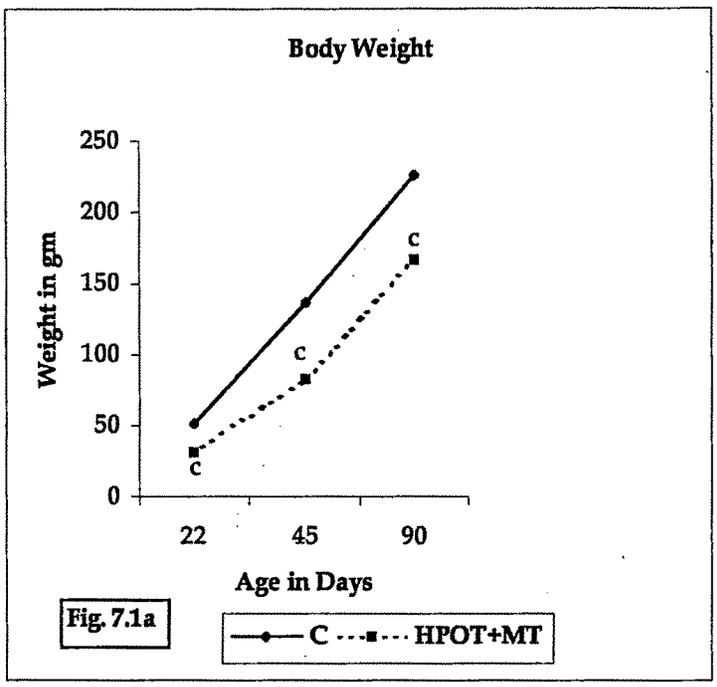


Fig. 7.1a

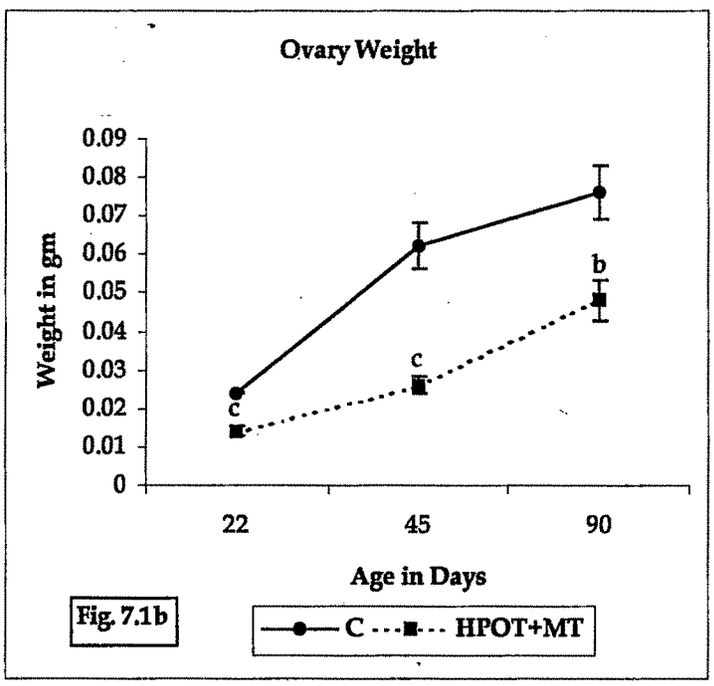
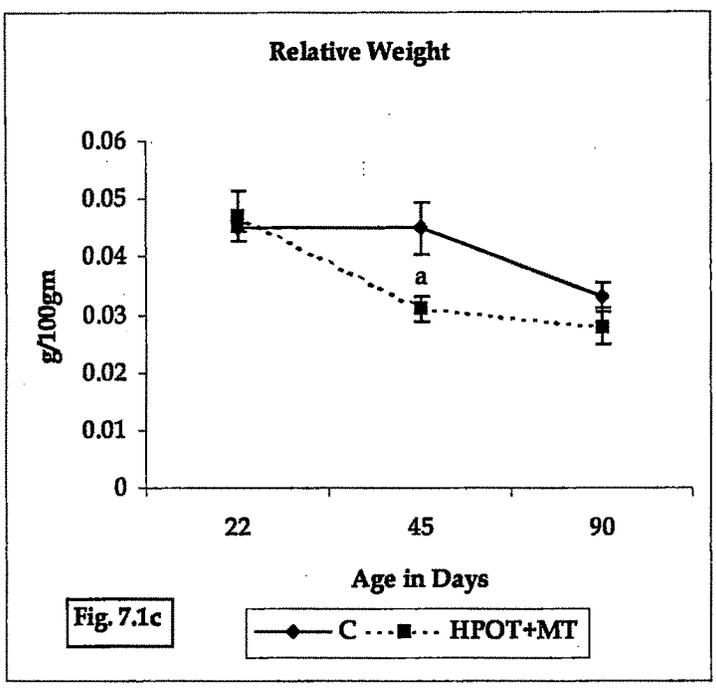


Fig. 7.1b

Figures 7.1a&b: Chronological alterations showing body and ovary (g) weight in control (C) and melatonin treated hypothyroid (HPOT+MT) rats

Values expressed as Mean ± SEM of ten animals;

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



Figures 71.c: Chronological alterations showing relative (g/100g) ovary weight in control (C) and melatonin treated hypothyroid (HPOT+MT) rats

Values expressed as Mean ± SEM of ten animals;

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

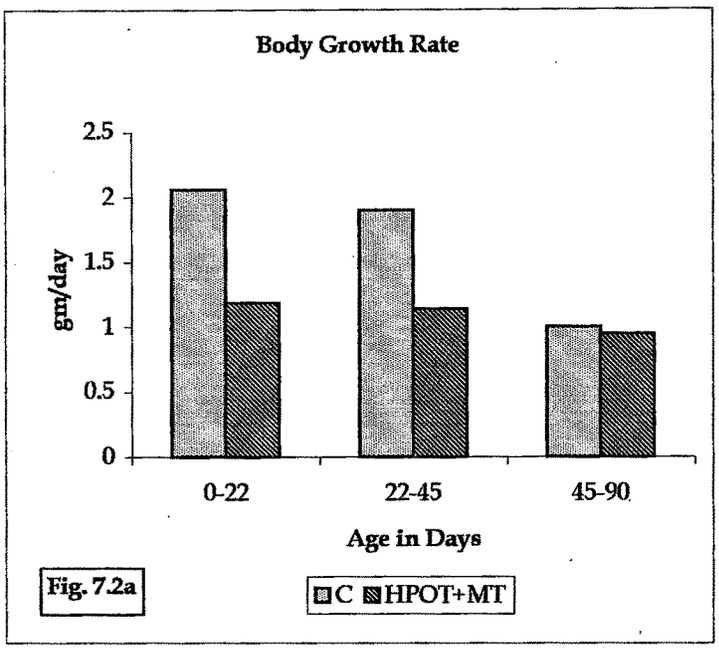


Fig. 7.2a

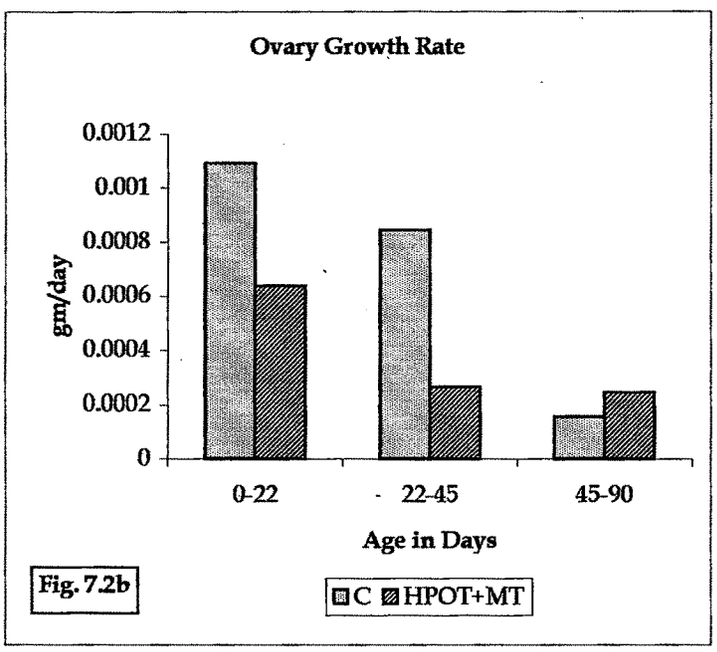


Fig. 7.2b

Figures 7.2a&b: Chronological alterations showing body and ovary (g/day) growth rate in control (C) and melatonin treated hypothyroid (HPOT+MT) rats

Ovarian Histology and Histometry:

The ovarian volume was significantly lower throughout, with a 34 % decrement in the adult stage at 90 days (Plate VIIA and VIIB). There was a generalized increase in the total number of follicles at 45 and 90 days. The increase in follicular number was of primordial and secondary follicles but with significant reduction in antral follicles and corpora lutea. The number of atretic follicles was significantly high in the experimental rats (Table 7.3).

Table 7.3: Differential total follicular count in ovary of control and melatonin treated hypothyroid 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
HPOT+MT	22	678 ±36	523 ±29	417 ±24	73[c] ±05	-	166[c] ±21	0.41[c]
	45	452 ±23	239[b] ±19	298[a] ±17	93[b] ±09	-	209[c] ±18	0.50[c]
	90	339 ±16	177 ±13	159[c] ±09	58 ±03	42 ±04	221[c] ±26	0.87[c]

C – Control; HPOT+MT – Melatonin treated Hypothyroid

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

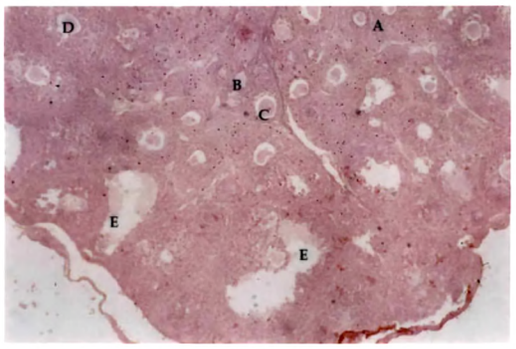
PLATE - VIIA

Figures **Photomicrographs of sections of Ovary of Control and melatonin treated hypothyroid rats**

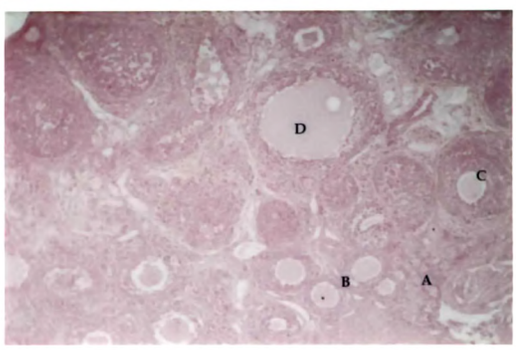
Sections of ovaries of 22 and 45 day old control rats showing less number of atretic follicles and number of primary, secondary and tertiary follicles compared to age-matched melatonin treated hypothyroid rats that shows more number of atretic follicles of all stages but less compared to HPOT rats.

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

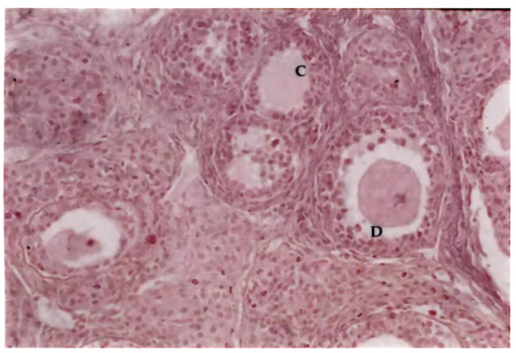
PLATE - VIIA



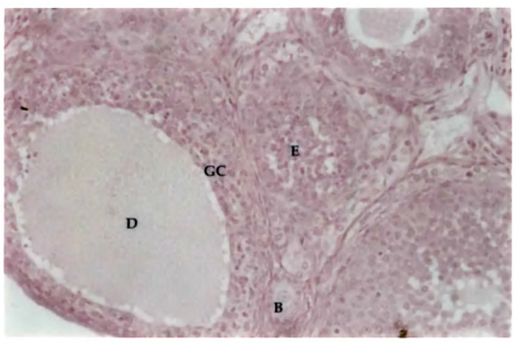
C 22 10x



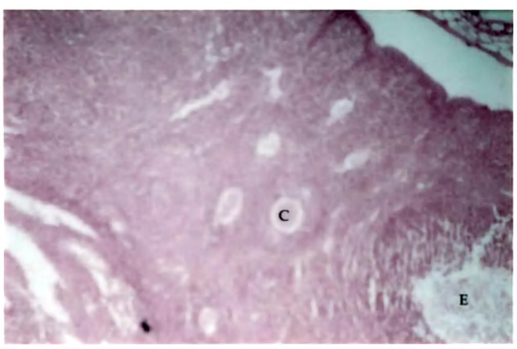
HPOT + MT 22 10x



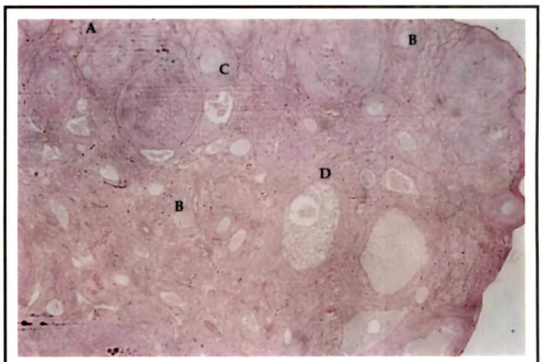
C 22 40x



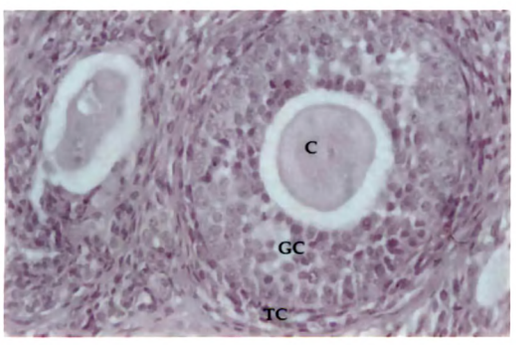
HPOT + MT 22 40x



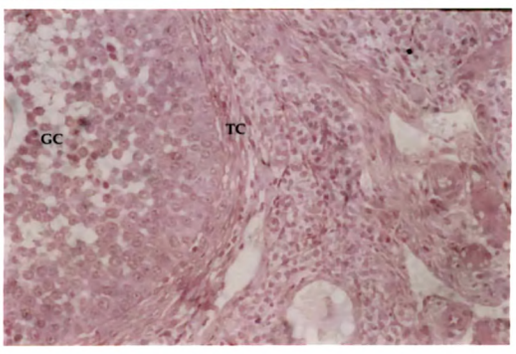
C 45 10x



HPOT + MT 45 10x



C 45 40x



HPOT + MT 45 40x

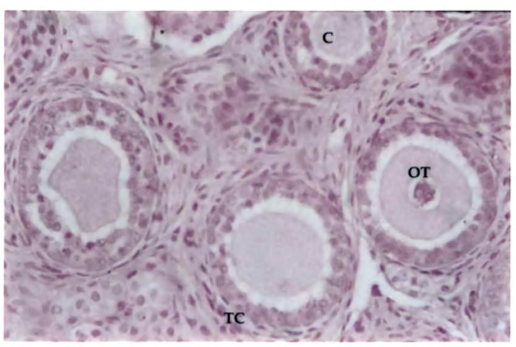
PLATE - VIIB

Figures **Photomicrographs of sections of Ovary of Control and melatonin treated hypothyroid rats**

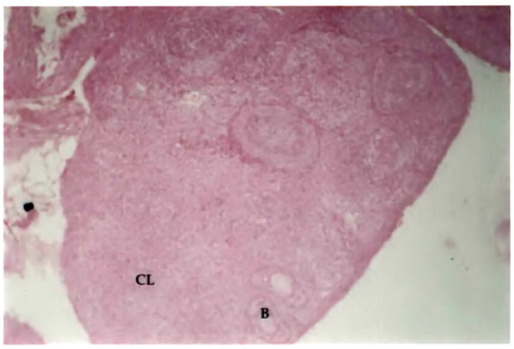
Sections of ovaries of 90 day old control rats showing more number of primary, secondary and tertiary follicles atretic follicles compared to age-matched melatonin treated hypothyroid rats that show more number of atretic follicles of all stages but less compared to HPOT rats.

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

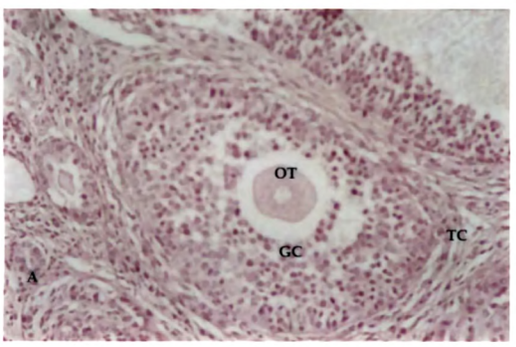
PLATE - VIIB



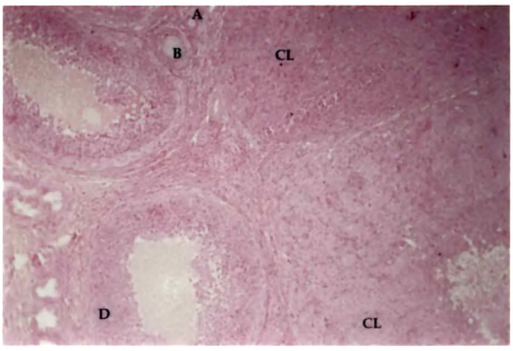
C 90 10x



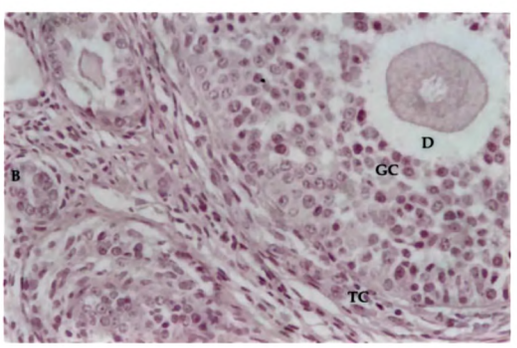
HOPT + MT 90 10x



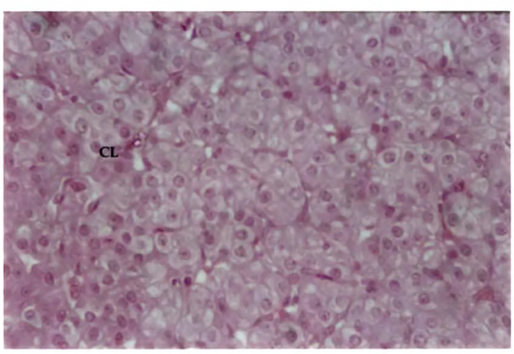
C 90 25x



HOPT + MT 90 25x



C 90 40x



HOPT + MT 90 40x

Serum Hormone Profile:

Both estrogen and progesterone titres were lower than in controls at 45 and 90 days though, in the immediate post-treatment period at 22 days the levels were higher than in controls. Both T3 and T4 levels were significantly higher at all ages in the experimental rats compared to controls (Tables 7.4 & 7.5; Figs. 7.4a, 7.4b, 7.5a, 7.5b). 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.

Table 7.4: Serum T3 and T4 (ng/ml) levels of control and melatonin treated hypothyroid 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 ±0.255
HPOT+MT	0.396 ±0.018	0.550 ±0.068	0.698 ±0.037	12.023[c] ±0.37	26.88[c] ±2.663	32.44[c] ±0.085

C – Control; HPOT+MT – Melatonin treated Hypothyroid

Values expressed as Mean ± SEM of ten animals;

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

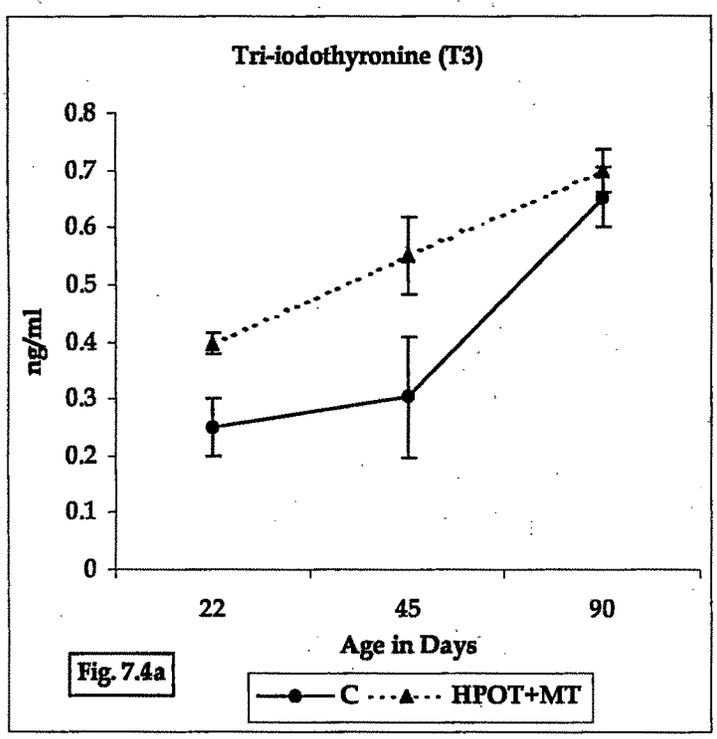
Table 7.5: Serum Estradiol and Progesterone (ng/ml) levels of control and melatonin treated hypothyroid female rats

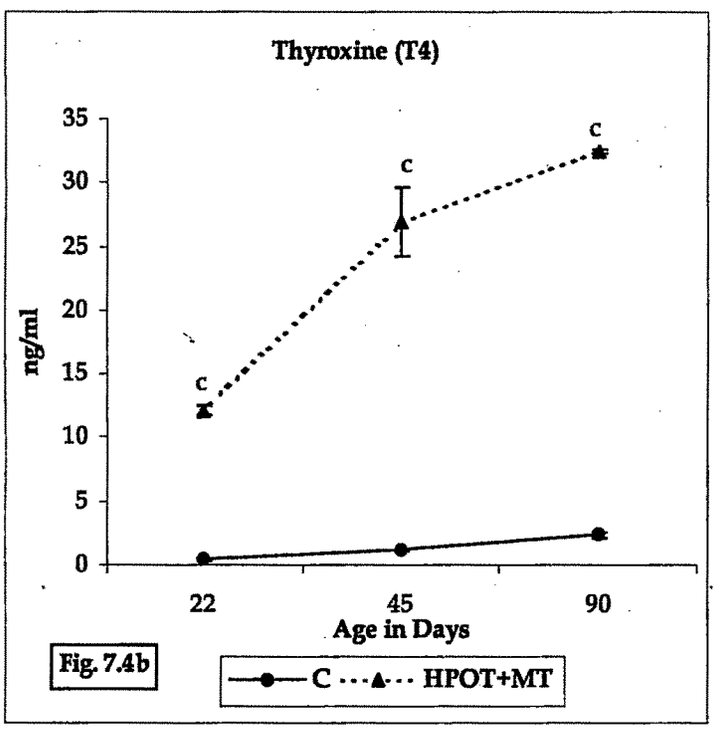
Treatment	Estradiol			Progesterone		
	Age in Days			Age in Days		
	22	45	90	22	45	90
C	4.02 ±1.30	8.73 ±2.55	10.39 ±3.10	2.75 ±0.88	8.55 ±2.41	15.19 ±4.36
HPOT+MT	5.01 ±1.92	6.85 ±2.37	8.77 ±3.64	7.809[c] ±0.356	12.96 ±1.391	10.27 ±4.473

C – Control; HPOT+MT – Melatonin treated Hypothyroid

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

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

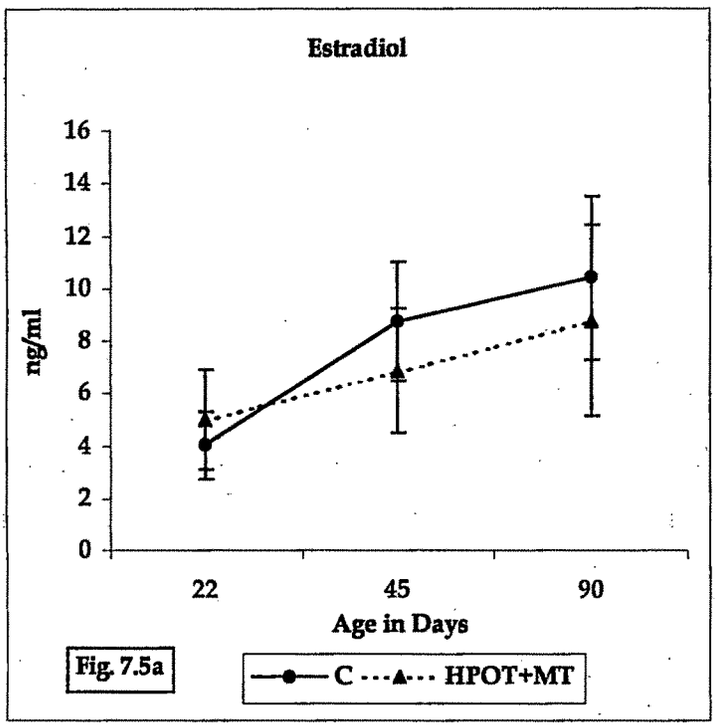


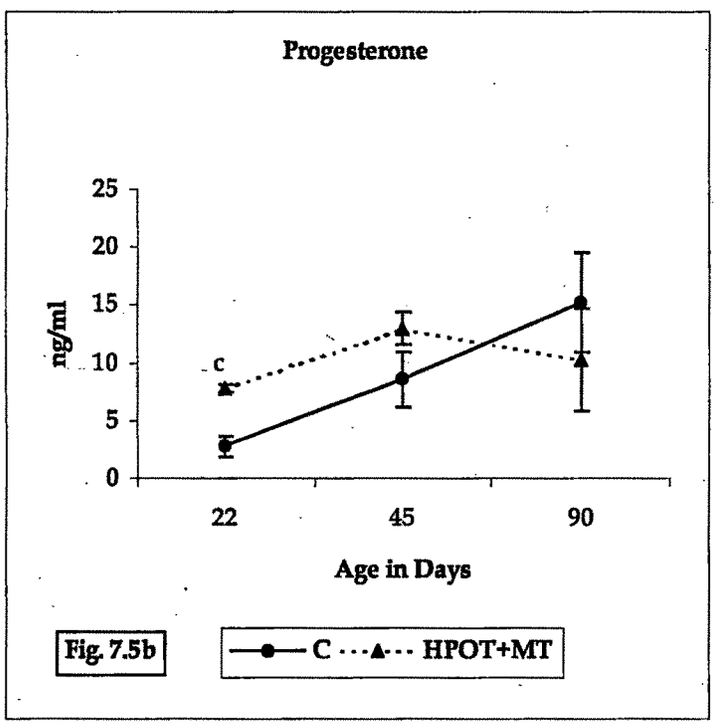


Figures 7.4a&b: Chronological alterations showing serum T3 and T4 (ng/ml) levels in control (C) and melatonin treated hypothyroid (HPOT+MT) rats

Values expressed as Mean ± SEM of ten animals;

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





Figures 7.5a&b: Chronological alterations showing serum Estradiol and Progesterone (ng/ml) levels in control (C) and melatonin treated hypothyroid (HPOT+MT) rats

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

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

DISCUSSION:

The results of the present study clearly reveal differential effects of both melatonin and hypothyroidism. Body weight, and ovarian weight and volume are all significantly less compared to controls, very much comparable with animals rendered hypothyroid neonatally. The HPOT+MT rats showed 26% deficit in body weight and 18% deficit in ovarian weight against 36% and 21% deficient respectively in HPOT alone rats. Clearly, simultaneous melatonin administration is not able to rectify the hypothyroidism-induced retardation in body weight as well as ovarian weight. A distinctive sex difference is inferable as both HPOT and HPOT+MT males showed increased testis size (Unpublished) as against decreased ovarian weight in females. Apparently as the testes respond favourably, the ovaries respond unfavourably to neonatal hypothyroidism in terms of adult gonadal weight. Thyroid hormone seems to be a crucial hormone required for both body and ovary growth in females, while in males the hypothyroidism induced testis enlargement is related with prolonged phase of Sertoli cell proliferation coupled with delayed differentiation (Hess *et al.*, 1993; Simoringker *et al.*, 1995). Further, while melatonin administration had a potentiating influence of hypothyroidism in male in terms of Sertoli cell proliferation (Lagu, 2001), the same in

females could not reverse the ovarian growth retardative influence of hypothyroidism.

Previous studies have shown a marginal increase in the total number of follicles under HPOT and most substantial increase under hypermelatonemia to the extent of 12% and 22% respectively at 45 and 90 days (chapters 1 and 2). However, HPOT+MT in the present study has revealed further increase to the tune of 29% and 38% respectively at 45 and 90 days. Clearly, neonatal hypothyroidism and hypermelatonemic status have a definite quantitative influence on folliculogenesis. Nevertheless, the qualitative aspect of folliculogenesis seems to be poorer when compared to melatonin rats, as, the percentage of atretic follicles is much higher compared to melatonin rats (22% v/s 1.5% at 90 days). This is however significantly less compared to HPOT rats as the percentage of atretic follicles in HPOT rats at 90 days was 42%. Further, the poorer qualitative aspect is also indicated by the lesser number of corpora lutea in HPOT+MT rats (4.2%), which is quite similar to HPOT rats (4.1%) as against melatonin rats (8.1%). Obviously the favourable quantitative effect of HPOT+MT is offset by the increased atresia and decreased antral follicles and corpora lutea. The antagonistic effect of HPOT+MT on follicular atresia is clearly manifested in the percentage of atretic follicles, which is lesser than the HPOT, but more than the MT rats. As the present

study has used only a smaller dose of 40μg of melatonin/animal, it would be interesting to test the effect of higher dose of melatonin in totally offsetting the negative impact of hypothyroidism, and we presume that the higher dose should be successful. Since there are no studies on hypermelatonemia on a background of hypothyroidism, much less with high hypermelatonemia alone, the observations of the present study are novel and interesting needing further evaluations. The presumed favourable effects of melatonin are strengthened by the reported expression of melatonin receptor gene in the granulosa cells of developing female mice (Lee *et al.*, 2001), the role of melatonin in regulating ovarian functions by way of progesterone production, LH receptor expression as well as GnRH and GnRH receptor gene expression (Woo *et al.*, 2001) and the ability of melatonin to prevent intra-follicle lipid peroxidation and exert radioprotective action to ovarian follicles (Kim and Lee, 2000; Takasaki *et al.*, 2003). The above studies have demonstrated the favourable influence of melatonin on simultaneous administration schedules, while, in the present study, the favourable influence is revealed as a consequence of prior neonatal administration. This is a novel observation and the mechanisms of this long lasting protection afforded by neonatal melatonin administration remains 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 (Markstrom *et al.*, 2002). A possible avenue of future investigations could be the understanding of the possible role of neonatal hypermelatonemia on permanent resetting of local genetic programmes resulting in activation of survival factors and/or inhibition of apoptotic factors.

The effect of HPOT+MT on the endocrine axis is marked by reduced titres of both estrogen and progesterone but increased thyroid hormone titres. The progesterone titre is intermediate to that of HPOT and MT rats suggesting the partial nullifying effect of the antagonistic actions of HPOT and MT. Both T3 and T4 levels are significantly elevated probably indicating the potential effect of MT on T3 and HPOT on T4.

The present study has, overall shown, interesting but differential effects on folliculogenesis, follicular survival, ovarian weight and volume and ovarian and thyroid hormone status, which are either synergistic, cumulative or antagonistic in terms of neonatal hypothyroid and hypermelatonemic status. Further investigations are warranted for proper understanding of a hypermelatonemic

status on a hypothyroid background in the neonatal period on long-term ovarian functions.

SUMMARY:

The impact of simultaneous melatonin administration in neonatal hypothyroid rats (HPOT+MT) on adult ovarian functions and serum hormone profiles has been evaluated in Charles foster strain of rats. The female neonates were made hypothyroid (HPOT) by feeding the lactating mothers with 6-propyl 2-thiouracil (PTU) through drinking water and were simultaneously administered with melatonin (MT) (40μg/animal/day) from day 0 to day 21 in the evening at 16.30 hrs just prior to lights off. The experimental animals (HPOT + MT) consistently showed decreased body weight compared to controls and, also the ovary weight was significantly lower at all ages than that of controls. The total number of follicles was significantly lower in HPOT + MT animals but was greater than the HPOT group of animals, and both showed significantly decreased number of follicles compared to control rats. There was a significant reduction in number of antral follicles and corpora lutea and increased atretic follicles in experimental rats. It can be concluded from the above results that simultaneous melatonin treatment is partially able to nullify the negative influence of hypothyroidism on adult ovarian functions and steroidogenesis.