CHAPTER - I

Neonatal nocturnal hypermelatonemia increases adult germ cell number and degeneration: Paradoxical effects

The two most widely recognised physiological roles of melatonin are the control of seasonal reproduction and the regulation of circadian rhythms. The major physiological role of melatonin in the regulation of seasonal reproduction in mammals has been well recorded (Karsch *et al.,* 1984; Underwood and Goldman 1987; Foster *et al.,* 1989; Bartness *et al.,* 1993; Weaver, 2000). The pineal gland is now recognised as the perceptor of seasonal changes in day length, and thus for the proper timing of reproduction in seasonally breeding species, as it is the primary source of circulating melatonin (Lewy *et al.,* 1980; Weaver, 2000) whose level is elevated at night. The duration of the nocturnal melatonin elevation is modulated by the photoperiod and this hormone affects reproductive activity by regulating the activity of hypothalamic neuroendocrine circuitry. Seasonal pattern of reproduction is more clearly marked in temperate zone species of mammals (Bronson, 1989). Depending on the time of the year when they are sexually active, species are characterised as either short day breeders (Sheep) or long day breeders (Hamster, Horse). The nocturnal secretion of

melatonin has been considered to be the primary transducer of photoperiodic information to the neuroendocrine reproductive axis (Bartness *et al.,* 1993; Arendt, 1995). The relationship between the pineal gland and photoperiodic control of seasonal reproduction has been clearly shown by the significantly altered functions in pinealectomized animals. Pinealectomy (Px) in Syrian hamster prevents the seasonal reduction in gonadotrophin secretion and gonadal regression normally brought about by a short photoperiod (Reiter, 1980), an effect that can be reversed by melatonin replacement. Such early works led to the concept of an anti-gonadotrophic action of melatonin, but further studies particularly in short day breeders have led to the understanding of a more generalised role of melatonin in the control of seasonal rhythms (Herbert, 1981; Hoffman, 1981; Karsch *et al.,* 1984). The concept that has evolved out of such studies is that Px can suppress response to both short and long photoperiod, and that melatonin depending on its specific pattern can reinstate both these responses (Malpaux *et al.,* 1999). The role of melatonin is to provide an endocrine code for day length, which is well illustrated by replacement studies in Px animals.

The reproductive system of adult rats is reportedly insensitive to exogenous melatonin (Reiter, 1980; Goldman *et al.,* 1981). Though there are reports of varying effects of either Px or melatonin administration in adult male rats, the general consensus has veered to a concept of no major role of pineal (Reiter, 1980; Goldman *et al.,* 1981; Binkley, 1983). However,

immature rats have been shown to be responsive to melatonin. Melatonin administration to immature rats has been reported to diminish ovarian and uterine weights (Wurtman *et al.,* 1963; Motta *et al.,* 1967) and to retard testes and accessory sex organ development (Debeljuk, 1969; Kinson and Robinson, 1970; Kinson and Peat, 1971). Exogenous melatonin has been shown to have a dose dependent inhibitory action on sexual maturation, when given daily in the afternoon from 20 to 40 days of age (Lang *et al.,* 1983). But no influence of melatonin was seen when injected during pre-pubertal period from 5 to 20 days or adulthood from 70 to 90 days (Lang *et al.,* 1984). Administration of melatonin between 20 and 45 days of age showed a delay in sexual maturation of male rats, but no inhibition as development was found to be normal in 80-day-old rats that received melatonin between 20 and 40 days (Lang *et al.,* 1984). Since the influence of melatonin on reproductive development has been known to commence during the pre-natal period and extend into the postnatal life (Weaver, 2000), melatonin administration either in the morning or in the evening in the infantile to prepubertal period (10 to 25 days) has been tested in our laboratory. This study showed decreased body weight and testes weights in the period immediately after melatonin treatment, more pronouncedly in the evening treatment (Patel and Ramachandran, 1992). Apparently, melatonin administration in the early neonatal periods has definite influence on the body and organ growth, reproductive axis, as well as on metabolic functions.

However, long-term influence of neonatal melatonin administration has not been investigated. Hence in the present study the influence of evening injections of melatonin in the preweanling period (0 to 21 days) in terms of body and testes weights, testis histoarchitecture and serum hormone profiles have been evaluated.

MATERIAL AND METHODS:

Animals and Maintenance:

Healthy albino rats (Charles Foster strain) were used in the present study. The animals were maintained in the animal house of Sarabhai Research Centre with a constant temperature range of 19 - 22°C and under a lighting regimen of LD 8:16 throughout the period of study. The animals were fed with standard food (Lipton Rat feed) and water *ad libitum*. When the mated females delivered pups, males and females were separated and equal number of males were assigned to lactating mothers. The treatment was started on day 0 post-partum and continued till 21 days.

Preparation of Melatonin:

Melatonin (N-acetyl 5-methoxytryptamine) procured from Sigma Co. USA was weighed and the requisite amount was dissolved in 0.9 % saline.

Experimental Protocol:

The experimental set-up was divided into two major groups of study.

Group I (control) **(C)**:

Newborn rat pups maintained till 90 days served as controls. This consisted of 2 subgroups (as follows) of 30 animals each:

(i) Control rats **(N)**.

(ii) Injected intraperitoneally (*i.p.*) with vehicle (0.9% saline) in the evening (1600 hrs).

Group II (melatonin treated) **(MT)**:

30 newborn rat pups were injected *i.p.* with Melatonin, at 1600 hrs (40μg melatonin/animal/day) from day 0 to day 21 post partum.

Parameters and Methods of Evaluation:

The treatment was discontinued from day 22 and the animals were sacrificed at 35, 45, 60 and 90 days of age and, various morphometric, gravimetric and histocytometric studies were carried out. 15-day-old animals were sacrificed for serum collection during the treatment period. The animals were killed 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 testes were excised, blotted free of tissue fluids and weighed accurately in a Mettler

balance. The absolute weights so obtained were converted to relative weights and expressed as percentage of body weight. These testes were fixed in Bouin's fluid and processed for paraffin wax histology.

Histology and Histometry:

Testis was fixed immediately in Bouin's fluid and processed for histological studies. Paraffin sections of 5 μ thickness were cut on a microtome and stained with Haematoxylin-Eosin (HE). For morphometry and enumeration of seminiferous tubules, homologous cross-sections from the middle part of testis showing the largest cross-sectional diameters were chosen. The diameter of seminiferous tubules and germinal epithelial thickness were measured using ocular micrometer. An approximate count of Sertoli cells was done in 20 tubular cross sections. The total Sertoli and germ cell number was estimated using morphometric methods based on the count of round objects in section of known thickness, modified from the reports of Wing and Christensen (1982) and Russell *et al.* (1990). Inherent error was corrected using Floderus equation (Floderus, 1944). The following parameters were determined:

- Testicular volume (T_V) in cubic cm.

$$T_V = \frac{\text{Absolute weight of single testis}}{\text{Sp. gravity of water (1.04)} \times \text{Fixation shrinkage (1.053)}}$$

Testis sectional area in cm^2 (T_A)

$T_A = \pi r^2$ (r = radius of the cross section of testis)

- Seminiferous Tubule diameter in cm (S_D)

$S_D = 2 r_s$ (r_s = mean radius of seminiferous tubules)

Seminiferous Tubule area in cm^2 (S_A)

$S_A = \pi r_s^2$

Effective Tubular area in a testis cross section (E_A)

$E_A = T_A - I$

(I = interstitial area) (I is 5% of T_A)

Number of tubules per section (N_T)

$$N_T = \frac{E_A}{S_A}$$

Relative area of tubule in cm^2 (R_T)

$$R_T = \frac{E_A}{T_A}$$

❒ Seminiferous tubule volume in cm^3 (S_V)

$$S_V = R_T \times T_V \qquad \text{(Hess } et\ al.\text{, 1993)}$$

❒ Seminiferous tubular length in cm (S_L)

$$S_L = \frac{S_V}{S_A}$$

❒ Total basement membrane area in cm^2 (bm)

$$bm = 2\ \pi\ r_s\ S_L$$

❒ Total number of Sertoli cells in testis (SC_N)

$$SC_N = \left[\frac{SC_T}{\text{thickness of section}} \right] \times S_L$$

(SC_T = number of Sertoli cells per seminiferous tubule)
(thickness of section in cm, 0.0005)

Total number of Sertoli cells per unit basement membrane area in (cm^2) (SC_{bm})

$$SC_{bm} = \frac{SC_T}{bm}$$

Theoretical Germ cell number (GC_N) in a tubule section

$$GC_N = \frac{GC_a}{GC_{a1}}$$

(GC_a = effective area – area occupied by Sertoli cells)

1. effective area of tubule section containing cells – ea
2. Sertoli cell area – Sca
3. Area occupied by one germ cell – GC_{a1}

ea = Area of tubule – Area of lumen

Note: Area of tubule is calculated by subtracting the area of lumen from area of tubule section (the diameter of lumen is calculated by subtracting the germinal epithelial height **(GE)** × 2, from the diameter of tubule)

GC_a = GC_N with Correction of area occupied by Sertoli cells

($GC_a = ea - SC_a$)

SC_a = Sertoli cell area in tubule section

$SC_a = SC_N \times$ area of one SC
(SC - $2\pi rh$)
* r = 0.000075 cm radius of Sertoli cell
* h = 0.009 cm is the Sertoli cell height
= 0.00004239 cm^2 is average Sertoli cell area based on standard Sertoli cell dimension known

GC_{a1} = Area of one germ cell

($GC_{a1} = \pi r^2$)

(r- radius of germ cell i.e. 0.000675)

$\therefore$ Average $GC_{a1} = 0.000014313882\ cm^2$

$= 1.4314 \times 10^{-5}\ cm^2$

- Theoretical Germ cell number per testis (TGC_T)

$$TGC_T = \frac{GC_N \times S_L}{\text{thickness of section}}$$

(section thickness = 0.0005 cm)

TGC_T = Final theoretical count with correction of inherent error using Floderus equation

$$TGC_T = \frac{\text{Total germ cell count}}{D + T - 2h}$$

D – Diameter (2.5×10^{-7})
T – Section thickness (0.0005 cm)
h – height of smallest recognisable nucleus, which is assumed to be 0.00001 cm

i.e. $D + T - 2h = 5.002$

- Theoretical Germ cell number per meter length of seminiferous tubule (TGC_M)

$$TGC_M = \frac{TGC_T}{S_L}$$

True germ cell count per section (TGCs) (with correction of inherent error using Floderus equation)

$$TGCs = \frac{\text{Raw count}}{D + T - 2h}$$

Raw count is the number of germ cells counted in terms of nuclei at stages V and VI under the microscope and D + T – 2h = 5.002, as calculated above

- Actual Germ cell number per Testis (AGC_T)

$$AGC_T = TGCs \times S_L$$

- Actual Germ cell number per meter length of seminiferous tubule (AGC_M)

$$AGC_M = \frac{AGC_T}{S_L}$$

- Degeneration of germ cells

Theoretical germ cell count – True germ cell count

Hormone Assays:

The blood for hormone assays was collected from the brachial vein under mild anaesthesia 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 in ng/ml of serum.

TSH, LH, corticosterone and testosterone were assayed by RIA. Rat TSH (NIDDK-rTSH-I-9) and LH (NIDDK-rLH-I-7), were iodinated by the chloramines T method with carrier free ^{125}I obtained from Amersham International Plc as described by Greenwood *et al.* (1963).

Pure rat hormones (2.5 μg) were incubated with specific concentrations of chloramine T (5 μg for TSH; 7 μg for FSH; 4 μg for LH) for 45 s. The reaction was stopped with 20 μl sodium metabisulphite (80 μg), and purified in a PD-10 column presaturated with barbitone buffer (0.07 M) and precoated with BSA (1%) (Pharmacia LKB Biotechnology, Bromma, Sweden), eluted in barbitone buffer and tubes with peak specific activity of 60-80 μCi/μg were used for RIA.

- RIA of Peptide hormones:

Peptide hormones were measured by liquid-phase RIA using specific antibodies and reference preparations from NIDDK. Antisera were anti-rTSH-S-5, anti-rFSH-S-11, anti-rLH-S-10, rFSH-RP2 and rLH-RP-3.

Hormones and antisera were dissolved in peptide assay buffer (pH 7.4)

containing sodium dihydrogen phosphate (3.05 g/l), sodium chloride (8.8 g/l), sodium azide (100 mg/l) and BSA (5 g/l). EDTA (0.025 M) was also added. The tracer was diluted in the peptide assay buffer containing 0.5 % normal rabbit serum to have approximately 20,000 – 30,000 c.p.m./100 µl tracer. Anti-rabbit γ-globulin (ARGG) in peptide assay buffer (1:26) and 8 % polyethylene glycol (PEG) in saline were used for separation of bound and free hormones.

Two hundred micro litres each of antiserum, sample and tracer in 600 µl assay buffer were incubated for 24 h at 4°C, followed by the addition of 100 µl ARGG and the incubation was continued for another 24 h. At the end of the second incubation, 1 ml PEG was added and centrifuged at 1500 g for 45 min. The radioactivity in the pellet was counted for 1 min in a microprocessor-based LKB gamma counter. The sensitivity of the assays was as follows: TSH 0.01 ng/ml, FSH 0.2 ng/ml, LH 0.14 ng/ml. Inter-assay variations were TSH 8.2 %, FSH 12.2 %, and LH 9.9 %. Intra-assay variations were TSH 4.7-6.9 %, FSH 5.7-8.9 % and LH 4.9-8.4 %. The antibodies were highly specific to the respective rat antigens with very minimal cross-reactivity with other peptides as per NIDDK specifications. The maximum binding of the antibodies recorded by us was TSH 39 %, FSH 30 %, and LH 32 %.

RIA of Testosterone:

Testosterone was assayed by liquid-phase RIA using the protocol and reagents from World Health Organisation. Maximum binding of the testosterone antiserum was 40 % and its cross-reactivity to cortisol, dehydrotestosterone, androstenedione and «^{5}-androstenediol was 0.001 %, 14 %, 0.8 % and 6 % respectively. The inter-assay and intra-assay variations were 6 % and 4 % respectively. The sensitivity of the assay was 0.3 pg/ml.

RIA of Corticosterone:

The RIA for corticosterone was carried out by RIA kits, procured from Amersham International Plc. and were expressed as ng/ml.

Statistical Analysis:

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

RESULTS:

Since no significant difference was observed between vehicle and non-vehicle controls, the data represented is of vehicle control **(C)** only.

Postnatal Growth:

The body and testes weights of melatonin treated animals (MT) were significantly less during the treatment period (15 days) by 17% and 24% respectively. However, the weights at 35, 45, 60 and 90 days were significantly greater in the melatonin treated rats (Table 1; Figures 1a & 1b). The growth rate of body and testes paralleled the changes in body and testes weights (Table 2; Figures 3a & 3b). The weight of testes expressed relative to body weight was in general lesser in melatonin treated rats at all periods except at 45 and 60 days when it was identical to the controls (Table 1; Figure 2).

Histology and Histometry:

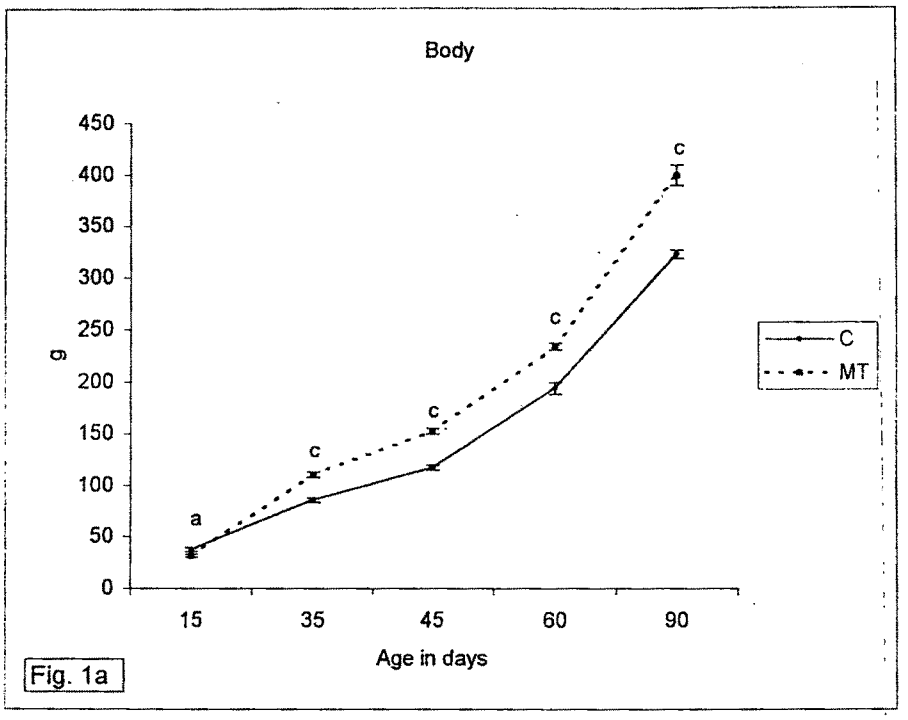
The testis sections of melatonin treated rats showed reduced tubular diameter at 35 days with relatively more germ cell loss. Sperm could be seen at 45 days with increased tubular diameter and hyperplastic interstitium in MT treated rats, compared to controls where no sperms could be seen (Plates I & II). At 60 days, sperms were seen in the testis of control animals. At both 60 and 90 days, there was increased tubular diameter, germinal epithelial thickness and prominent interstitium in MT treated rats compared to controls. The tissue sections of MT showed significantly increased higher germ cell number starting with 45 days and, at 35 days the number and size were significantly lesser. The degree of germ cell loss by

Table 1: Chronological alterations in body weight (g) and absolute (g) and relative weight (g/100g) of testes in Control and Melatonin treated rats.

Treatment	Absolute Body Weight					Absolute Testes Weight					Relative Testes Weight				
	Age in days					Age in days					Age in days				
	15	35	45	60	90	15	35	45	60	90	15	35	45	60	90
C	37.333 ±1.919	85.513 ±1.902	117.16 ±0.307	193.66 ±5.493	322.49 ±4.078	0.281 ±0.081	0.803 ±0.064	1.280 ±0.043	2.28 ±0.050	3.067 ±0.063	0.755 ±.072	0.944 ±0.025	1.09 ±0.040	1.178 ±0.073	0.933 ±0.039
MT	31.45[a] ±1.477	110.3[c] ±2.490	152.3[c] ±2.490	233.7[c] ±3.242	398.7[c] ±9.898	0.231[a] ±0.061	0.938 ±0.073	1.779[c] ±0.053	2.714[a] ±0.102	3.375[c] ±0.078	0.677 ±0.081	0.854 ±0.084	1.167 ±0.042	1.16 ±0.063	0.847 ±0.043

C – Control, **MT** – Melatonin treated

Values expressed as Mean ± SEM of six animals. [a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$



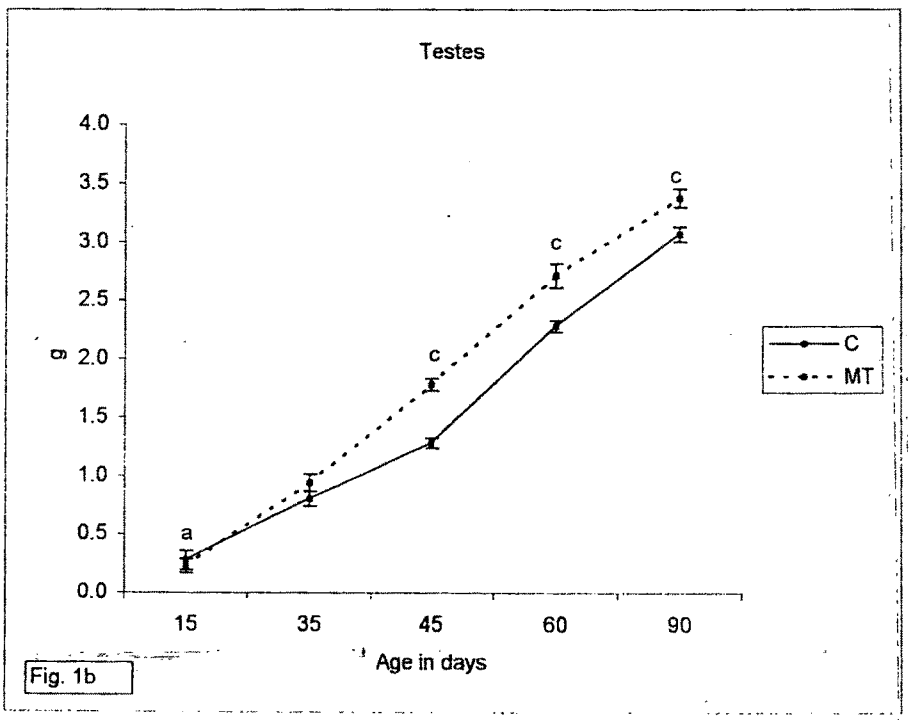


Fig. 1a and 1b: Chronological alterations in body weight (g) and absolute weight (g) of testes in Control and Melatonin treated rats.

C – Control, MT – Melatonin treated

Values expressed as Mean ± SEM of six animals. [a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

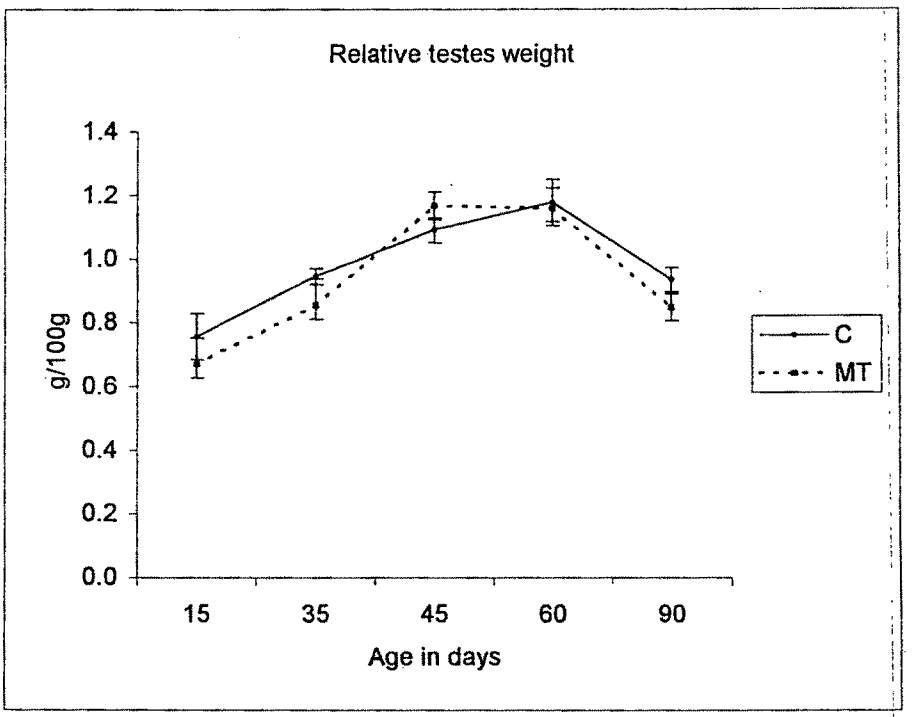


Fig. 2: Relative testes weight (g/100g) in Control and Melatonin treated rats

C – Control, MT – Melatonin treated

Values expressed as Mean ± SEM of six animals. [a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

Table 2: Per day Body and Testes Growth Rate (g/day) in Control and Melatonin treated rats.

Treatment	Per Day Body Growth Rate					Per Day Testes Growth Rate				
	Age in days					Age in days				
	0-15	15-35	35-45	45-60	60-90	0-15	15-35	35-45	45-60	60-90
C	2.117	2.409	3.165	5.100	4.294	0.019	0.0261	0.048	0.067	0.026
MT	1.663	3.942	4.200	5.422	5.500	0.015	0.035	0.084	0.062	0.022

C – Control, **MT** – Melatonin treated

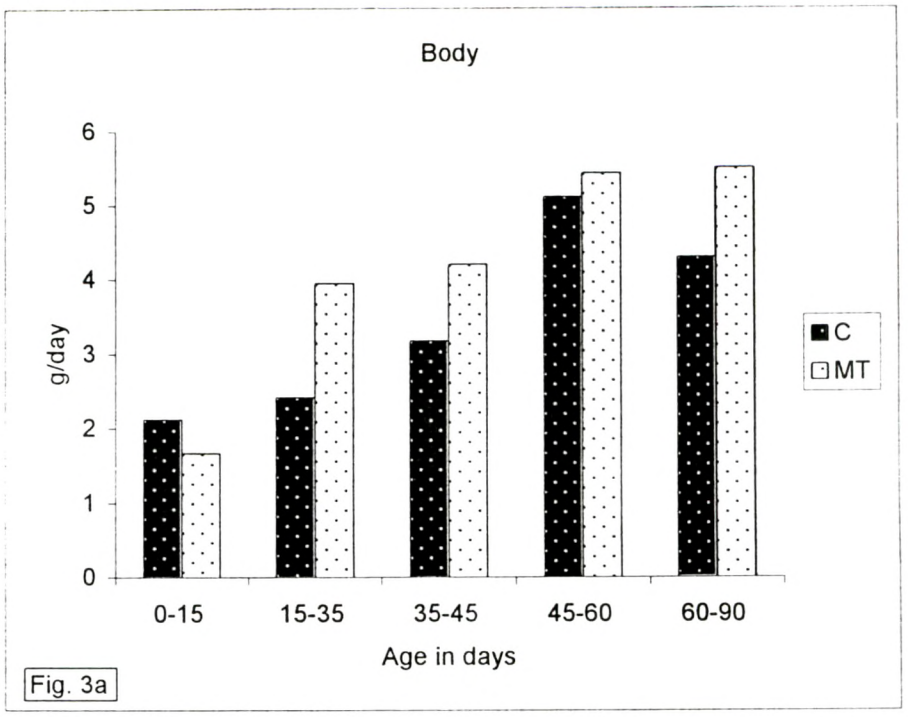


Fig. 3a

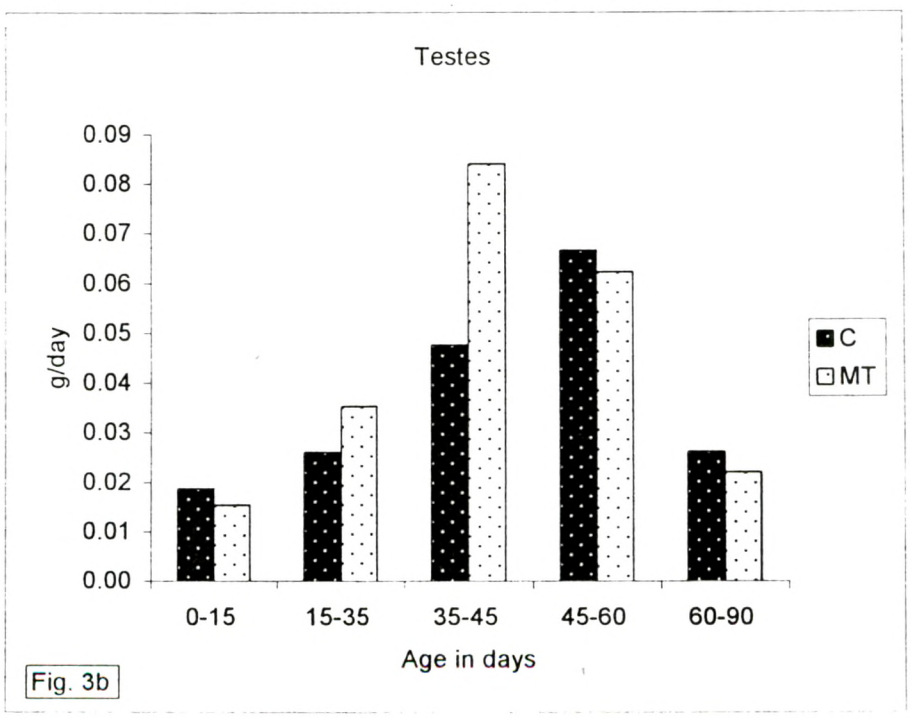


Fig. 3b

Fig. 3a and 3b: Per day Body and Testes growht rate (g/day) in Control and Melatonin treated rats

Table 3: Histometric enumeration of seminiferous tubules of Control and Melatonin treated rats at 90 days

Treatment	T_V in cc	S_D in cm	GE in cm	S_V in cc	S_L in cm	bm in cm^2	$SC_N \times 10^6$	$TGC_T \times 10^6$	$AGC_T \times 10^6$	$TGC_M \times 10^6$	$AGC_M \times 10^6$	% Loss
C	1.503 ±0.030	0.0279 ±0.0006	0.0074 ±0.0003	1.427 ±0.050	2321.03 ±94.200	204.045 ±5.230	32.49 ±1.800	311.00 ±6.300	280.84 ±5.600	13.39 ±0.260	12.1 ±0.150	10.00 ±0.0002
MT	1.541 ±0.070	0.033^c ±0.001	0.0098 ±0.002	1.448 ±0.060	1725.16^c ±45.30	177.205^c ±4.690	24.15^b ±1.600	340.00^b ±5.600	279.45 ±2.800	19.70^c ±0.350	16.20^c ±0.210	19.00^c ±0.200

C – Control, **MT** – Melatonin treated

Values expressed as Mean ± SEM of minimum fifteen observations. $^a p < 0.05$, $^b p < 0.005$, $^c p < 0.0005$

T_V - Volume of Testis, **S_D** - Seminiferous tubule diameter, **GE** - Germinal epithelial thickness, **S_V** - Volume of Seminiferous tubule, **S_L** - Length of seminiferous tubule, **bm** - basement membrane area of the seminiferous tubule, **SC_N** - Total Sertoli cell number in testis, **TGC_T** - Theoretical germ cell number per testis, **AGC_T** - Actual germ cell number per testis, **TGC_M** - Theoretical germ cell number per meter of seminiferous tubule, **AGC_M** - Actual germ cell number per meter of seminiferous tubule.

degeneration was found to be greater in experimental rats marked by the formation of empty areas within the germinal epithelium. The advanced stages of germ cells i.e. spermatids and spermatozoa seemed to be showing poor adhesive properties as these cells were found frequently being sloughed off from the epithelium. The overall sperm mass was also found to be less. Whereas there was no difference in testis and tubule volume, total tubular length, total basement membrane area and Sertoli cell number were all decreased in MT rats at 90 days. Most of the growth in tubular length occurred by 35 days and the growth further till 90 days was only 28% as against 190% in the controls. Total germ cell count per meter length of the tubule was increased significantly, but total percentage of germ cell loss by degeneration was higher by 8% in the experimental rats (Table 3).

Serum Hormone Profile:

Corticosterone:

The control animals showed a consistent increase in corticosterone level from 15 day onwards with a significant increase to the adult level between 45 and 60 days. In melatonin treated rats, serum corticosterone level was significantly higher at 35 days, which then decreased significantly at 45 days (still higher than the control) and again increased to control levels by 60 days. This level persisted even at 90 days as in the controls (Table 4; Figure 4).

Table 4: Serum Corticosterone, LH and T levels (ng/ml) in Control and Melatonin treated rats.

Treatment	Corticosterone					LH					T				
	Age in days					Age in days					Age in days				
	15	35	45	60	90	15	35	45	60	90	15	35	45	60	90
C	5 825 ±0.085	8.000 ±0.618	10.150 ±0.155	48.300 ±0.705	45.50 ±1.699	9.390 ±0.132	16.450 ±0.634	21.750 ±0.854	48.125 ±1.235	53.250 ±1.031	0.235 ±0.019	0.550 ±0.166	2.235 ±0.278	2.625 ±0.217	4.375 ±0.265
MT	12.40[c] ±1.106	24.70[c] ±2.075	13.40 ±1.80	44.01 ±4.732	43.01 ±4.856	8.761 ±1.009	17 12 ±1.024	16.39[b] ±1.008	17.77[c] ±1.025	20.99[c] ±2.025	0.221 ±0.016	1.501[c] ±0.048	1.503[a] ±0.054	1.300[c] ±0.058	2.002[b] ±0.088

C – Control, **MT** – Melatonin treated

Values expressed as Mean ± SEM of four samples. [a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

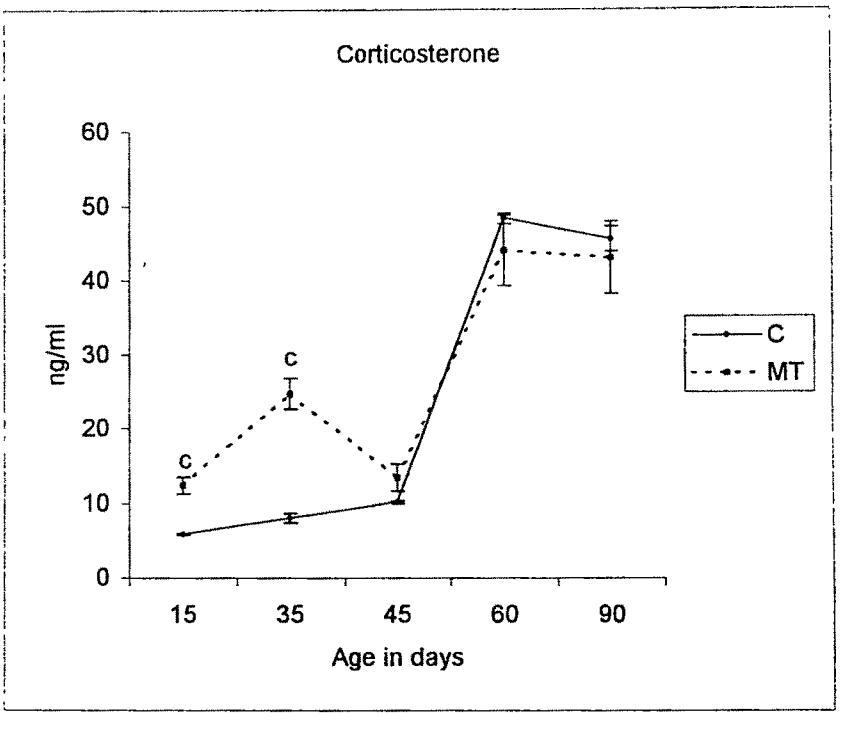
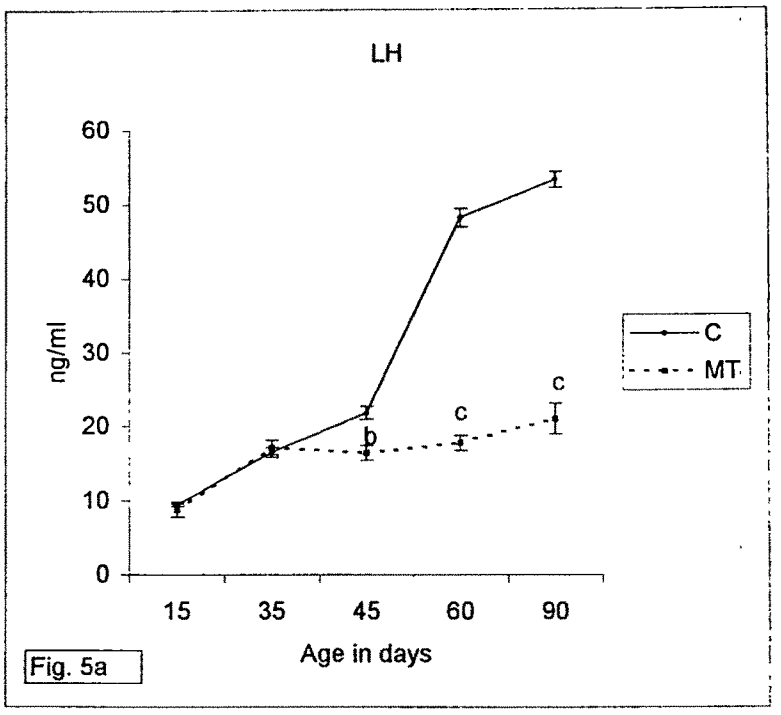


Fig.4: Serum Corticosterone level (ng/ml) in Control and Melatonin treated rats
C – Control, **MT** – Melatonin treated
Values expressed as **Mean ± SEM** of four samples
a $p < 0.05$, b $p < 0.005$, c $p < 0.0005$



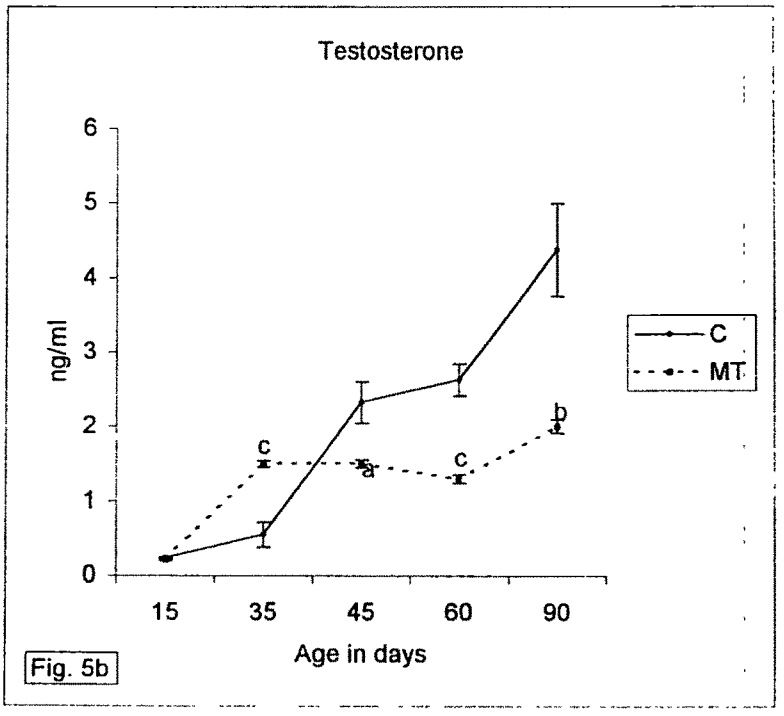


Fig.5a and 5b. Serum LH and T levels (ng/ml) in Control and Melatonin treated rats

C – Control, **MT** – Melatonin treated

Values expressed as Mean ± SEM of four samples

[a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

TSH, T_4 and T_3:

In control rats, the levels of TSH and T_4 showed gradual and continuous increase from 15 to 60 days to attain maximum value at 60 days. At 90 days the levels were found to be slightly decreased. The T_3 level after an initial increase between 15 and 35 days, was slightly decreased at 45 days, whereafter it increased at 60 and 90 days (Table 5; Figure 7b). In melatonin treated rats, the levels of TSH, T_4 and T_3 were significantly higher at 15 and 35 days and in the ascending order. Whereas the TSH levels showed significant decrease at 45 and 60 days compared to 35 day, the T_4 level increased to a maximal level at 45 days and then decreased at 60 and 90 days to be below the control levels and, T_3 increased to a maximal level at 60 and 90 days to be above the control levels (Table 5; Figures 7a & 7b). Though the TSH level showed a decline after 45 days, the levels were still higher than the control at 60 and 90 days (Table 5; Figure 6).

LH and Testosterone:

The levels of both LH and Testosterone in control rats showed a continuous and gradual increase from 15 days to reach maximal levels at 90 days. In melatonin treated rats, the levels of the hormones were subnormal during the treatment period and above normal in the post-treatment period at 35 days. However, the levels were consistently subnormal thereafter at all ages (45, 60, 90 days) (Table 4; Figures 5a & 5b).

Table 5: Serum TSH, T_4 and T_3 levels (ng/ml) in Control and Melatonin treated rats.

Treatment	TSH					T_4					T_3				
	Age in days					Age in days					Age in days				
	15	35	45	60	90	15	35	45	60	90	15	35	45	60	90
C	3.175 ±0.165	6.600 ±0.129	6.873 ±0.111	7.495 ±0.143	5.440 ±0.066	0.31 ±0.013	0.583 ±0.085	1.170 ±0.061	2.568 ±0.024	2.368 ±0.225	0.215 ±0.051	0.450 ±0.011	0.303 ±0.107	0.603 ±0.084	0.653 ±0.053
MT	14.01^{c} ±0.104	26.01^{c} ±1.577	9.050^{a} ±0.804	7.804 ±0.652	9.001^{c} ±0.365	1.282^{c} ±0.084	1.700^{c} ±0.053	2.805^{c} ±0.015	2.212^{b} ±0.068	1.742^{a} ±0.018	0.400 ±0.026	0.484 ±0.056	0.800^{c} ±0.074	1.051^{c} ±0.084	0.910^{c} ±0.095

C – Control, **M** T– Melatonin treated

Values expressed as Mean ± SEM of four samples. ^{a} $p < 0.05$, ^{b} $p < 0.005$, ^{c} $p < 0.0005$

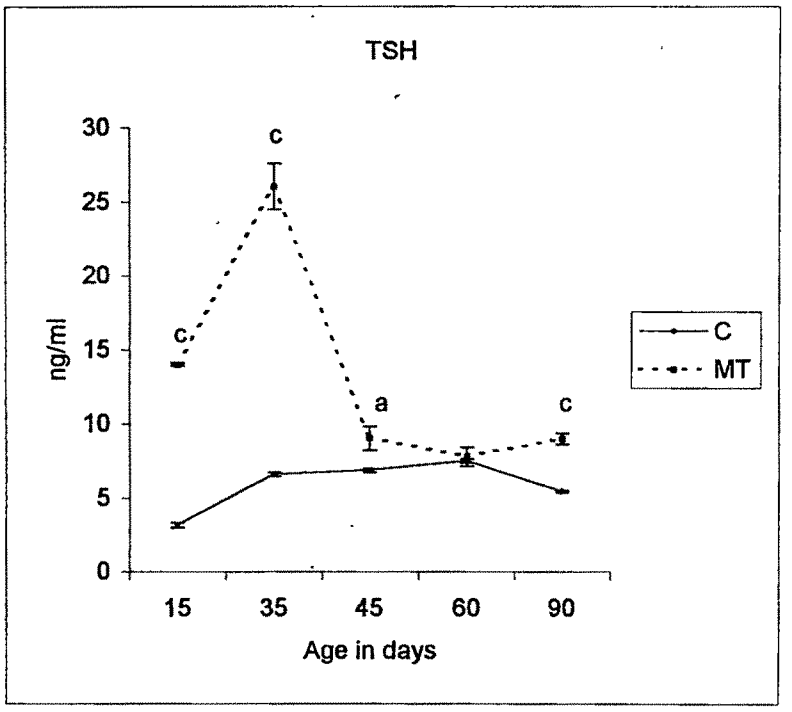


Fig.6: Serum TSH level (ng/ml) in Control and Melatonin treated ra
C – Control, **MT** – Melatonin treated
Values expressed as Mean ± SEM of four samples
[a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

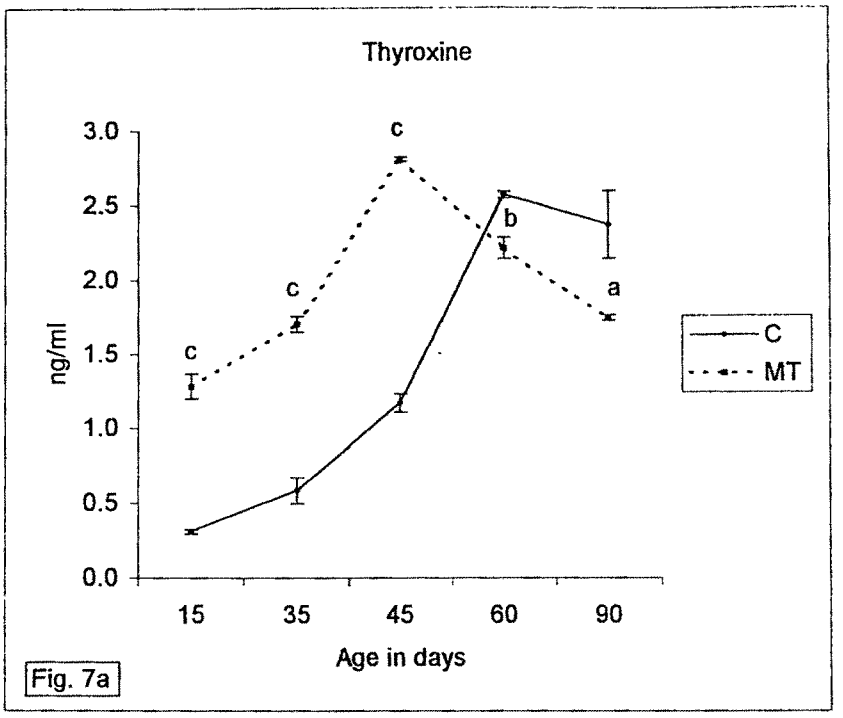


Fig. 7a

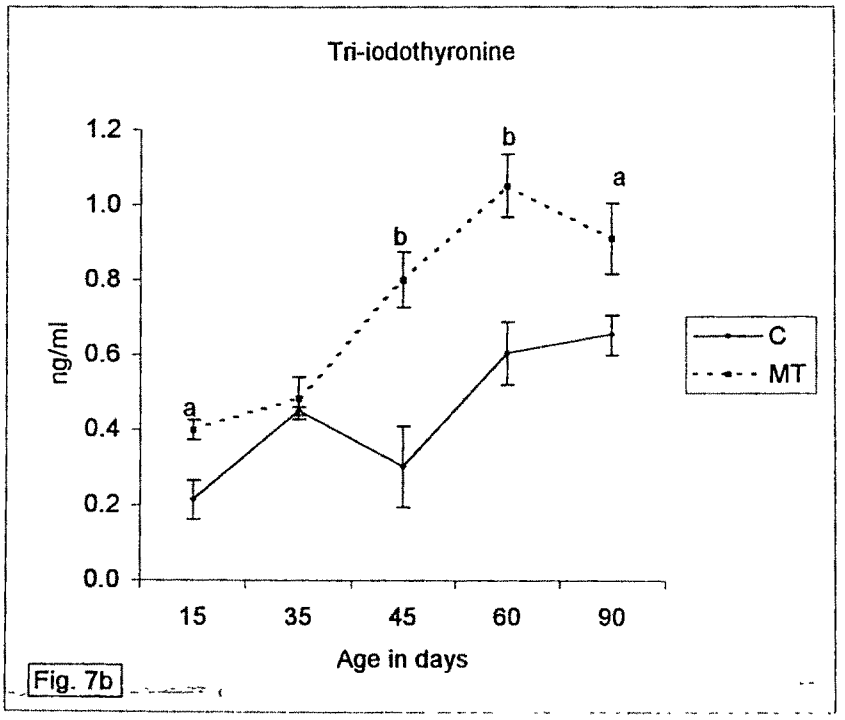


Fig. 7b

Fig.7a and 7b: Serum T_4 and T_3 levels (ng/ml) in Control and Melatonin treated rats.

C – Control, **MT** – Melatonin treated

Values expressed as Mean ± SEM of four samples

[a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

DISCUSSION:

The results of the present study involving hypermelatonemia in the neonatal period by exogenous administration of melatonin from day 0 to day 21 suggest long-term permanent alterations affecting the adult testis functions, the neuroendocrine reproductive (NER) axis and other hormonal axes. A previous study involving neonatal administration of melatonin in Wistar rats had shown an inhibitory influence on the reproductive axis marked by reduced FSH and LH levels and decreased testes and accessory gland weights when melatonin administration was between 20-40 days but not when between 5-20 days, (Lang *et al.,* 1983). But, in our laboratory, melatonin administration between 10 to 25 days in Charles Foster neonates showed significant reduction in testes weight, both in absolute terms as well as relative to body weight (Patel and Ramachandran, 1992). The experiments on Wistar rats had shown the sensitivity to melatonin to be exhibited when administered either 9 hours into the photophase or at the beginning of the scotophase, but with no effect at the beginning of photophase (Lang *et al.,* 1984). Again in contrast, our studies in Charles Foster neonates showed sensitivity to melatonin both at the beginning of photophase as well as scotophase, though the effect was more pronounced in the latter phase (Patel and Ramachandran, 1992). These paradoxical observations find no explanation but, a possible strain difference in laboratory rats that needs to be evaluated thoroughly.

The body weight of MT neonates in the present study remained significantly lower during the treatment period, but in the post-treatment periods the body weight increased significantly, resulting in the MT rats having significantly greater body weight as adults (Table 1; Figure 1a). The post-treatment increase in body weight gain is due to both an increased growth rate as well as, prolongation of the temporal increase in growth rate (Table 2; Figure 3a). Melatonin has been shown to induce hyperphagia by a possible suppressive action on central 5-HT (2A) receptor (Raghvendra and Kulkarni, 2000). However, this mechanism of body weight gain in the present experimental regimen does not seem relevant as the body weight of melatonin treated rats was significantly lower during the preweanling treatment period and showed a reverse trend of enhanced body weight gain in the post-treatment period extending to adulthood. A probable increase in growth hormone secretion and/or sensitivity on a long-term basis due to neonatal excess melatonin exposure could be speculated. In this context, the influence of neonatal melatonin excess on the SCN-pineal axis and/or hypothalamic-pituitary-growth hormone axis on a long-term basis needs to be evaluated, and as such melatonin induced elevation of growth hormone (GH) has in fact been reported (Mckeown *et al.,* 1975; Vriend *et al.,* 1990)

The paired testes weight was 24% lesser during the treatment period; post-treatment period showed compensatory growth increase which persisted till 90 days and the increase was manifested by 35 days itself (17%), which

increased to a maximum of 38% at 45 days and the weight remained still higher at 90 days. These changes in absolute weight of testes are clearly reflected in the growth rate at various ages (Figure 3b). The growth rate between 15-45 days was 1.6 times greater in melatonin rats. However, the relative weight of testes was consistently lesser in melatonin rats than controls, except at 45 and 60 days, when it was like control, thereby suggesting no hyperplastic growth of testes due to neonatal melatonin excess.

Obviously, neonatal melatonin excess seems to be more favourable for body weight gain than for testes weight. Nevertheless, melatonin administration in the evening during the first 21 days (Preweanling period) does show some influence on the neuroendocrine reproductive (NER) axis and cytoarchitecture of the testis. The influence on the NER axis is clearly shown by the consistently decreased LH and testosterone levels during the treatment as well as post-treatment periods extending upto adulthood (45 to 90 days). Such an inhibitory influence of melatonin on GnRH induced FSH and LH has been shown in neonatal rats (Vaneck, 1999). A permanent hyposetting of the central set point of neuroendocrine reproductive axis is deducible and, suggest the possible action of neonatal melatonin excess on the maturation of the reproductive axis either directly or indirectly. The possibility of such an alteration of reproductive neuroendocrine set point is strongly supported by an observation of altered feed back response of

testosterone on LH, FSH and PRL secretion in the pre-pubertal and pubertal periods due to prenatal melatonin administration (Diaz *et al.,* 2000). There was only one period of testosterone excess, at 35 days, in the present study, which could be related with the early appearance of spermatozoa in MT rats. However, this increase in testosterone level at 35 days, which remains more or less so till adulthood, cannot be explained and may only signify an early attainment of adult level characteristic of melatonin treated rats. It may be speculated that the increased serum titre of testosterone at 35 days could be due to the sudden increase in Leydig cell number that occurs between 35-45 days. This could result in augmented release of testosterone contributing to the higher serum testosterone level before the permanent down regulation of the LH sensitivity of these cells.

Though there is no increase of testis size in melatonin treated rats, the tubular diameter and the thickness of germinal epithelium, which were lesser by 21% and 23% respectively at 35 days, increased significantly by 17% and 32% respectively at 90 days. Previous studies have shown increased tubular diameter and testis size of neonatal hypothyroid rats to be due to increased Sertoli cell number by prolongation of the period of proliferation in the hypothyroid state (Cooke, 1984; Lagu, 2001). The presently observed increase in tubular diameter and germinal epithelial thickness of rats subjected to neonatal melatonin excess cannot be accredited to increase in Sertoli cell number as, neither the relative weight of testes nor, the Sertoli

cell numbers are higher in the experimental rats. But a justification for the increased tubular diameter and germinal epithelial thickness can be sought in the increased germ cell numbers seen in MT rats (Table 3). The increased germ cell number in MT rats seems to be primarily due to significantly reduced germ cell apoptosis despite the paradoxical germ cell degeneration. Obviously, melatonin induced decreased germ cell apoptosis far outways the increased germ cell degeneration as is evidenced by the significantly increased germ cell number per meter length of the tubule and the 8% increase in germ cell degeneration compared to the controls. A remarkable feature is that the total tubular length at 90 days is decreased by 26% and most of the growth (78%) had occurred by 35 days itself, as against 38% of total length in the controls. Obviously, neonatal melatonin, either directly or indirectly, hastens linear tubular growth but then has an inhibitory influence thereafter.

Apparently, there is significant increase in germ cell number in MT rats, essentially due to increased germ cell survival. The observation of germ cell loss by way of premature sloughing off or detachment of advanced stages of germ cells (spermatids and spermatozoa) in the present study is suggestive of a long-term effect of excess neonatal melatonin on Sertoli cell-germ cell adhesive properties. This is feasible in the context of reported presence of melatonin receptors in the neonatal testes (Valladres *et al.*, 1992;

Vera *et al.,* 1993, 1997; Valenti *et al.,* 1995, 1997). Two of the possible effects of excess neonatal melatonin on adult testis histoarchitecture are:

1. Increased Leydig cells due to increased proliferation and
2. Modified adhesion properties between Sertoli cells and spermatids and spermatozoa. Both these effects can be related with the reported presence of melatonin receptors in the neonatal and immature rats. Whereas, the reduced adhesional properties result in low sperm mass in the tubules, the increased Leydig cell numbers paradoxically seems to have reduced LH sensitivity. However, reduced testosterone production and decreased 17-20 desmolase activity have been reported on Leydig cell activity in MT rats (Valladres *et al.,* 1992; Valenti *et al.,* 1995; Tijmes *et al.,* 1996; Valenti *et al.,* 1997). These aspects need detailed investigations for ascertainment.

The increased tubular diameter, high germ cell number and reduced apoptosis are features, which were also realised in rats subjected to neonatal corticosterone excess (Chapters 2 and 3). The reduced apoptosis was accredited to a rare direct action of corticosterone on Sertoli cell expression of growth/paracrine factors (Bhaysar, 2001) (Chapters 2 and 3).

An earlier study involving melatonin in adult at 50 and 70 days had also shown decreased tubular diameter and serum FSH and LH levels with reduced frequency of spermatids in the maturation phase of spermatogenesis. This strengthens our present inference on decrease sperm population, which apart from the adverse action of melatonin, could also be

due to a synergistic action along with decreased FSH and testosterone levels. So, whereas the observed degeneration of germ cells could be related with melatonin alone or in conjunction with FSH and testosterone, the decreased apoptosis seems to be an indirect action of melatonin mediated by increased corticosterone secretion observed in the treatment and immediate post-treatment periods (Chapters 2 and 3). In this context, melatonin has been reported to increase apoptosis especially affecting spermatocytes and spermatids (Young *et al.,* 1999), which may be a contributory factor for the observed decrease in sperm content. With regard to hypothalamo-hypophyseal-thyroid (HHT) axis, the MT treated rats showed a permanently elevated set point for HHT axis in the adult stage as marked by higher TSH and T_3 levels but lower T_4 level. Though the elevated set point of HHT axis was also noted in rats subjected to neonatal corticosterone excess, the reduced T_4 level, noted herein is a novel feature. This might suggest a direct action of melatonin in either altering the T_4:T_3 secretory ratio or increased peripheral conversion of T_4 to T_3. Support to this suggestion is available from the reported decreased T_4 level and increased T_3/T_4 ratio in melatonin treated pigeons (John *et al.,* 1990), which was again speculated to be possibly due to active peripheral mono-de-iodination (George, 1999). This is in contrast to the reports of melatonin decreasing TSH, T_3 and T_4 levels in adult rats (Ozturk *et al.,* 2000). Apparently, melatonin has differential action in the immature and mature stages and, melatonin excess in the neonatal period,

when the HHT axis is in the process of maturation, has potential effects on its later functioning. The increased thyroid hormone level seen in the melatonin treatment period seems to be also related with the early maturation of testes with appearance of sperm, contributed to mainly by the augmented Sertoli cell differentiation, a function accredited to thyroid hormone (Palmero *et al.,* 1989; Hess and Cooke, 1992; Van Haaster *et al.,* 1992, Hess *et al.,* 1993; Cooke *et al.,* 1994; Panno *et al.,* 1994; Franka *et al.,* 1995).

Overall, it can be concluded that neonatal melatonin administration in the preweanling period has many paradoxical effects on testis functions and on various endocrine axes, all of which are either due to indirect modulation by corticosterone or, a direct action of melatonin or even, interactive actions between melatonin, corticosterone and testosterone.

PLATE – I

Figures 1 – 8: Photomicrographs of sections of testis of control rats.

Figures 1 and 2 : Sections of testis of 35 day old control rats showing interstitium.

Figures 3 and 4 : Section of testis of 45 day of showing advanced stages of spermatogenesis and appearance of sperms in few tubules.

Figures 5 and 6 : Section of testis of 60 day old rats showing well-established spermatogenesis and sperms in lumen.

Figures 7 and 8 : Section of testis of 90 day old rats showing prominent interstitium and fully established spermatogenesis.

Figures: 1, 3, 5, & 7 – 250 x
Figures: 2, 4, 6, & 8 – 400 x

Abbreviations:

I-Interstitium, **L**-Lumen, **st**-spermatids, **S**-sperms,
D-Degeneration,**rs**-round spermatids.

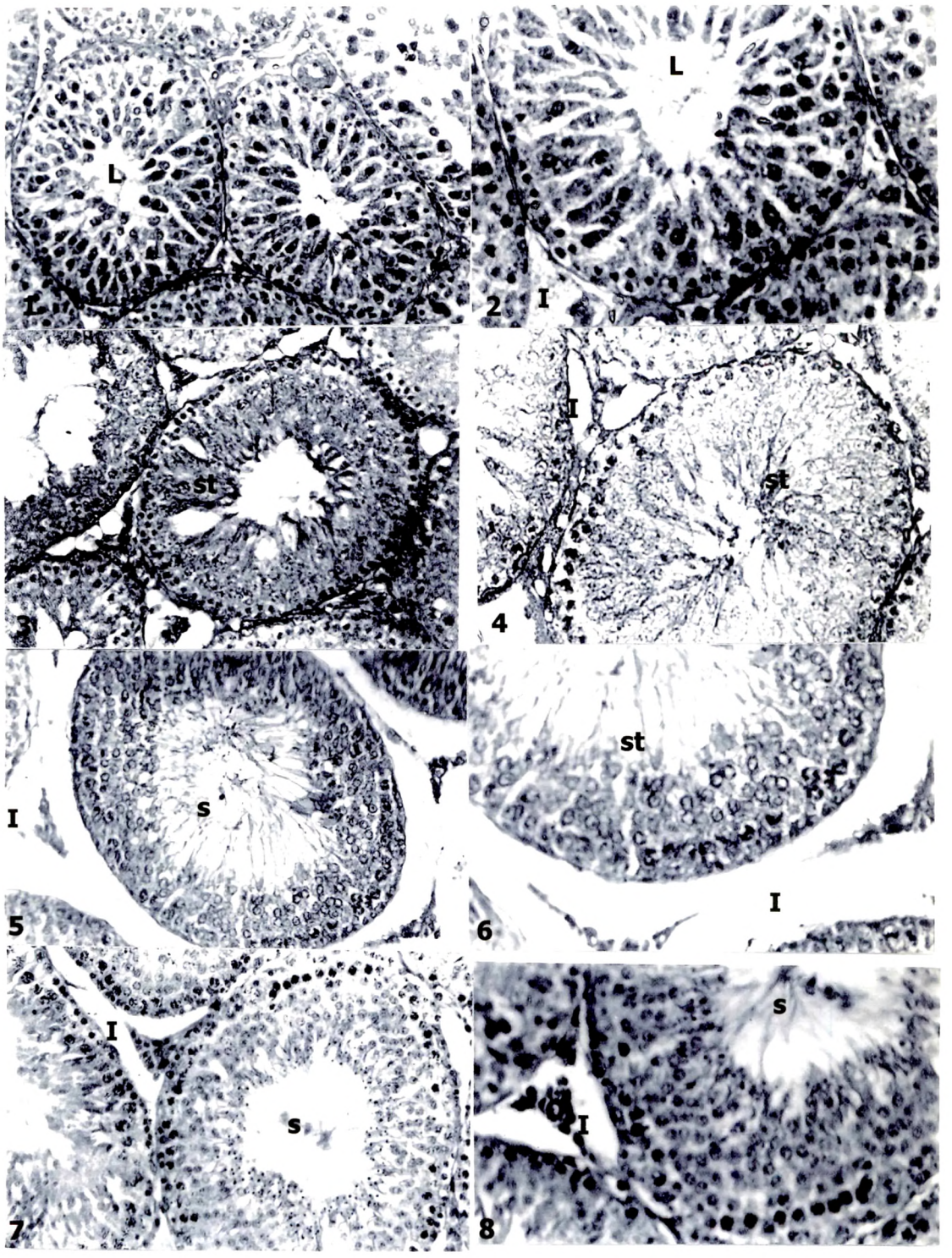

L
1
L
2
I
st
3
I
st
4
I
s
5
st
I
6
I
s
7
s
I
8

PLATE – II

Figures 1 – 8: Photomicrographs of sections of testis of Melatonin treated rats.

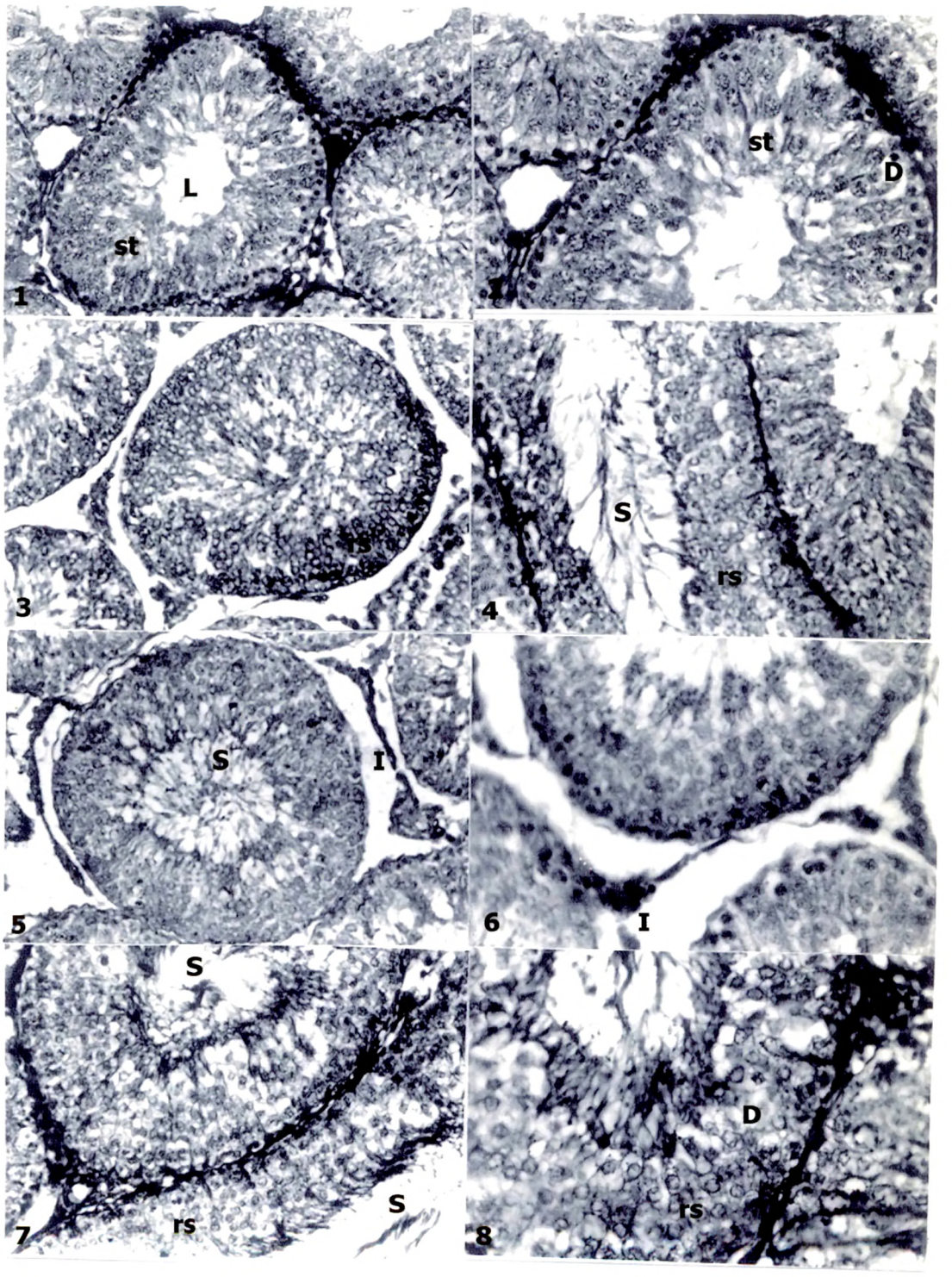
Figures 1 and 2 : Sections of testis of 35 day old rats showing less number of germ cells, inhibited spermatogenesis and more loss due to degeneration.

Figures 3 and 4 : Section of testis of 45 day old rats of showing well established spermatogenesis and sperms in many tubules.

Figures 5 and 6 : Section of testis of 60 day old rats showing greater population of germ cells and well establishment spermatogenesis

Figures 7 and 8 : 90 day old testis section showing more number of germ cells and more degeneration

Figures: 1, 3, 5, & 7 – 250 x
Figures: 2, 4, 6, & 8 – 400 x


L
st
1
st
D
2
S
rs
3
S
rs
4
S
I
5
I
6
S
rs
S
7
D
rs
8

SUMMARY

Long-term effects of neonatal hypermelatonemia have been studied in the Charles Foster strain of rats in terms of testis growth and maturation, histology and histometry and adult (90 days) and serum hormone profiles. Neonates were subjected to melatonin excess by daily *i.p.* administration of 40 μg from day 0 to day 21. The experimental rats showed significantly increased body and absolute testes weight but reduced relative weight. The total seminiferous tubular length, basement membrane area and Sertoli cell number were all significantly decreased. However, theoretical and actual germ cell count were significantly greater together with a higher percentage of germ cell degeneration. Whereas there was no difference in the adult corticosterone level, the serum LH and T levels were significantly elevated in the experimental rats. Serum TSH and T_3 levels were also higher but T_4 levels were lower. The results suggest that neonatal hypermelatonemia is favourable for body weight gain than for testes and that there is a permanent hyposetting of the central set point of the neuroendocrine reproductive axis. The increased germ cell number in experimental rats seems to be primarily due to reduced germ cell apoptosis (a corticosterone effect as inferred in chapters 2 and 3) despite the paradoxical increased germ cell degeneration. It is also deducible that melatonin hastens tubular growth though the ultimate length is lesser. The deleterious effect of melatonin is also manifested in the form of premature sloughing off of spermatids and

spermatozoa. With regard to the hypothalamo-hypophyseal-thyroid (HHT) axis, melatonin treated rats showed a permanently elevated set point. There appears to be a decreased T_4 secretion coupled with increased T_3 secretion and /or increased peripheral conversion of T_4 to T_3. These aspects are explained in detail in the text.