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Annals of Biological Research, 2010, 1 (3) : 85-100
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0976-1233
CODEN (USA): ABRNBW

Resetting of adult hormonal axes and male germ cell kinetics: neonatal melatonin excess and developmental plasticity

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ABSTRACT

Neonatal hypermelatonemia and adult plasticity in terms of testis growth and functions and endocrine axes have been studied in rats. Melatonin was administered for 21 days post partum and adult rats at 90 days were evaluated for hormonal profile and germ cell dynamics by histometric enumeration. The results clearly suggest that, neonatal hypermelatonemia induces adult plasticity in body weight gain but not of testes and, permanently lowers the set point of the neuroendocrine reproductive axis and elevates the set point of the thyroid axis. Functionally, testicular germ cell number is increased by decreased apoptotic loss but there is a paradoxically increased loss of spermatids and spermatozoa by way of altered Sertoli-germ cell adhesive properties. Plasticity changes in the form of decreased tubular length, basement area and Sertoli cell number are also recorded. It can be concluded from the observations made herein that, neonatal melatonin perturbation can shape later phenotypic responses of male gonads and their controlling hormonal axes.

Key words: neonatal, melatonin, plasticity, hormonal axis, germ cell dynamics.

INTRODUCTION

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 [1,2]. The pineal

gland as the primary source of melatonin is now recognised as the preceptor of seasonal changes in day length, and thus for the proper timing of reproduction in seasonally breeding species [3]. The duration of the nocturnal melatonin elevation is modulated by photoperiod and this hormone effects reproductive activity by regulating the activity of hypothalamic neuroendocrine circuitry [1].

Adult rats are reported to be insensitive to exogenous melatonin in altering reproductive functions [4, 5, 6]. However, immature rats have been shown to be responsive to melatonin. Melatonin administration to immature rats has been reported to retard testes and accessory sex organ development [7,8,9]. 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 [10]. 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 [11]. 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 [11]. Since the influence of melatonin on reproductive development has been known to commence during the pre-natal period and extend into postnatal life [12], 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 [13]. Apparently, melatonin administration in the early neonatal periods has definite influence on body and organ growth, reproductive axis, as well as on metabolic functions. However, long term influences of neonatal melatonin excess have not received any attention. Pineal maturation and establishment of rhythmic secretion of melatonin commences only by 15 days of age in rats [14]. In the absence of light induced synchronization of melatonin synthesis in the new born rats due to their being blind, lactational transfer of maternal melatonin seems as the principal synchronizer [15]. Lactational transfer of maternal cues is a conduit of phenotypic information transfer that could impact on developmental plasticity resulting in long term consequences in terms of adult physiology and metabolism [16]. An organism`s ability to change its physiology or developmental events within an inherent plasticity range in relation to endogenous or exogenous cues is known as phenotypic plasticity. A major regulator of phenotypic plasticity is expectedly the environmentally induced differences in the endocrine system [17]. Hormones can bring about epigenetic alterations and, in fact, can function as essential link between the environment and the genome. Perturbation in endocrine signals in developmentally critical periods like the foetal and neonatal stages can bring about physiological alteration in organ systems within a range of phenotypic plasticity from the same genotype. Such perturbations in endocrine signals in developmentally critical periods are potentially capable of establishing altered adult structural and functional status of organs and, even, impact health positively or negatively by inducing changes in gene transcription, modification of metabolic rates in target cells, changes in cell number or even by bringing about subtle changes in receptor type and number [18].

The present study in this context has attempted to evaluate the long term plasticity change in male gonadal functions and hormonal axes due to induced neonatal melatonin excess.

MATERIALS AND METHODS

Animals and maintenance

Healthy male albino rats (Charles Foster strain) used for the present study were maintained in the animal house under a constant temperature range of 21-23°C and a lighting regimen of LD 10:14 throughout the period of study as per the CPCSEA guidelines and clearance by the ethical committee (Approval No. 827/ac/04/CPCSEA). The animals were fed with standard pelleted food (Lipton Rat Feed) and provided with 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 (Weaning).

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 (ip) 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 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. Fifteen 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. The testes were then 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 an ocular meter. The total Sertoli and germ cell number was estimated using morphometric methods based on the count of round objects in sections of known thickness [18] as modified from the reports of Wing and Christensen [19] and Russell *et al*.[20]. Inherent error was corrected using Floderus equation [21].

Hormone assay:
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 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 (Cort) and Testosterone (T) were assayed by RIA using kits obtained from Amersham International PIc.

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

Postnatal growth:
The body and testes weight 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). The growth rate of body and testes paralleled the changes in body and testes weights. Though the weights of testes expressed relative to body weight was in general lesser in melatonin treated rats, was nevertheless statistically insignificant.

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 sperm could be seen (Plates I and II). At 60 days, sperm 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 sections of MT rats showed significantly higher number of germ cells starting with 45 days though, at 35 days the number and size were significantly lesser. The degree of loss of advanced germ cells (by degeneration/apoptosis) was found to be greater in experimental rats marked by the formation of empty areas within 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 was higher by 8 % in the experimental rats (Table 2).

Serum hormone profile:
Corticosterone (Cort):
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 MT treated rats, serum Cort 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 (Fig 1).

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 levels at 60 days. At 90 days, the levels were found to be slightly decreased (Fig 2, 3). The T_3 level after an initial increase between 15 and 35 days was slightly decreased at 45 days, wherafter it increased at 60 and 90 days (Fig 4). In MT treated rats, the levels of TSH, T_4 and T_3 showed significant temporal increase between 15 and 35 days. Whereas the TSH level showed significant decrease at 45 and 60 days compared to 35 days, the T_4 level decreased to 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. Though the TSH level showed a decline after 45 days, the levels were still higher than the the controls at 60 and 90 days.

LH and Testosterone:

The levels of both LH and T in control rats showed a continuous and gradual increase from 15 day onwards 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) (Fig 5, 6).

Table I: Chronological alterations in body weight (g) and relative testis weight (g/100g) of testes in control and melatonin treated rats

GROUPS	BODY WEIGHT					RELATIVE TESTIS WEIGHT				
	Age in days					Age in days				
	15	35	45	60	90	15	35	45	60	90
C	37.33 ±1.91	85.51 ±1.90	117.16 ±0.30	193.66 ±5.49	322.49 ±4.07	0.755 ±0.07	0.944 ±0.02	1.09 ±0.04	1.178 ±0.07	0.933 ±0.03
MT	31.45[a] ±1.47	110.3[c] ±2.49	152.3[c] ±2.49	233.7[c] ±3.24	398.7[c] ±9.89	0.677 ±0.08	0.854 ±0.08	1.167 ±0.04	1.16 ±0.06	0.847 ±0.04

C- Control, MT- Melatonin treated ; Values are expressed as Mean±SEM of six animals, [a] $p<0.05$, [b] $p<0.005$, [c] $p<0.0005$

Table 2: Histometric enumeration of seminiferous tubules of control and melatonin treated rats at 90 days

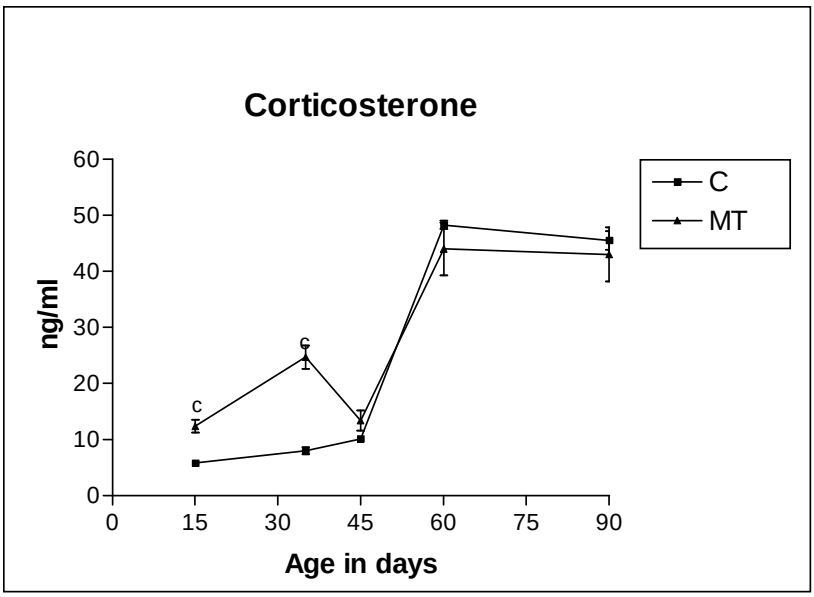
GROUPS	T_v In cc	S_D In cm	GE In cm	S_V In cm	S_L In cm	bm in cm^2	SC_N x 10^6	TGC_T x 10^6	AGC_T x 10^6	TGC_M x 10^6	AGC_M x 10^6	% LOSS
C	1.503 ±0.03	0.027 ±0.0006	0.0074 ±0.0003	1.427 ±0.05	2321.03 ±94.2	204.04 ±5.23	32.49 ±1.8	311.00 ±6.3	280.84 ±5.6	13.39 ±0.26	12.1 ±0.15	10.00 ±0.0002
MT	1.541 ±0.070	0.033[c]± 0.001	0.0098 ±0.002	1.448 ±0.06	1725.16 [c]±45.30	177.205 [c]±4.69	24.15[b] ±1.6	340.00[c] ±5.6	279.45 ±2.8	19.70[c] ±0.35	16.20[c] ±0.21	19.00[c] ±0.2

C- Control, MT- Melatonin treated, Values are expressed as Mean±SEM of fifteen observations.

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

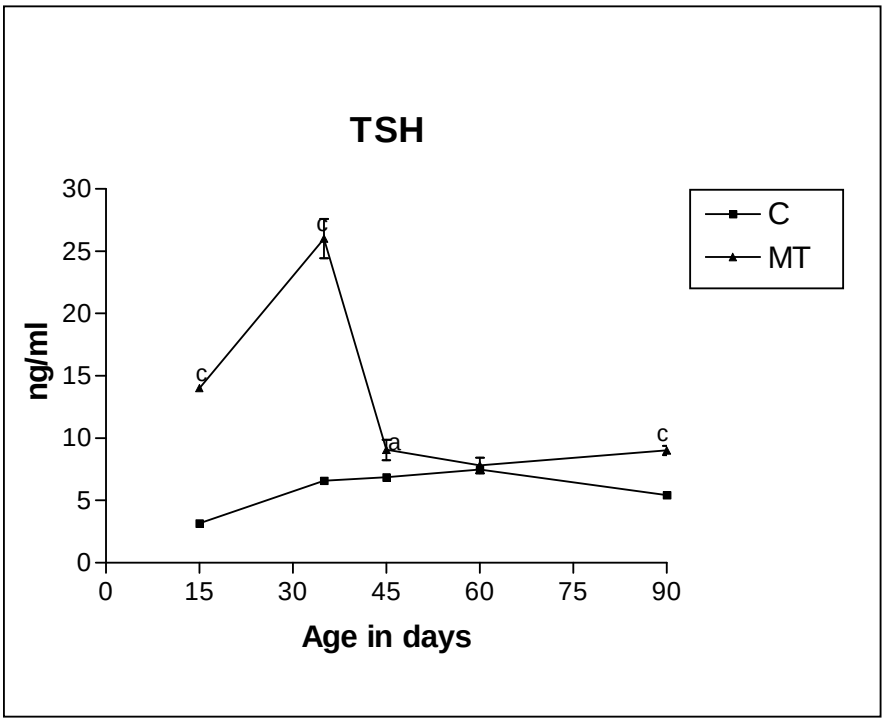
T_v – Volume of testis, S_D- Seminiferous tubule diameter, GE- Germinal epithelium thickness, S_v- Volume of seminiferous tubule, S_L- Length of seminiferous tubule, bm- basement membrane area of the seminiferous tubule, SC_N- Total Sertoli cell number in testis, TGC_T- Theoretical germ cell number per 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.

Fig 1: Serum corticosterone level (ng/ml) in control and melatonin treated rats



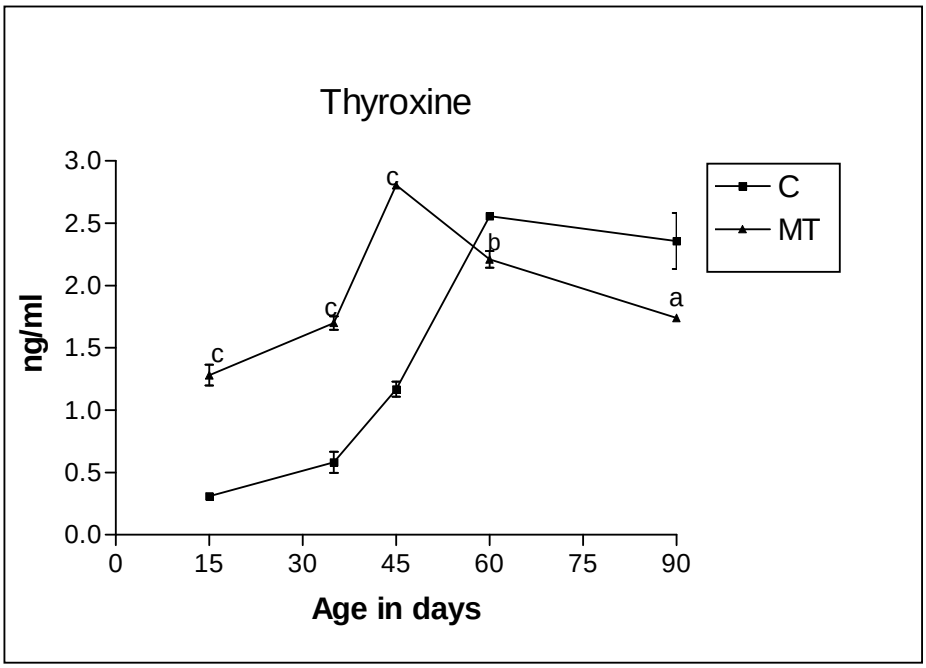
C- Control, MT- Melatonin treated ; Values are expressed as Mean±SEM of four samples.
[c] p<0.0005

Fig 2: Serum TSH (ng/ml) in control and melatonin treated rats



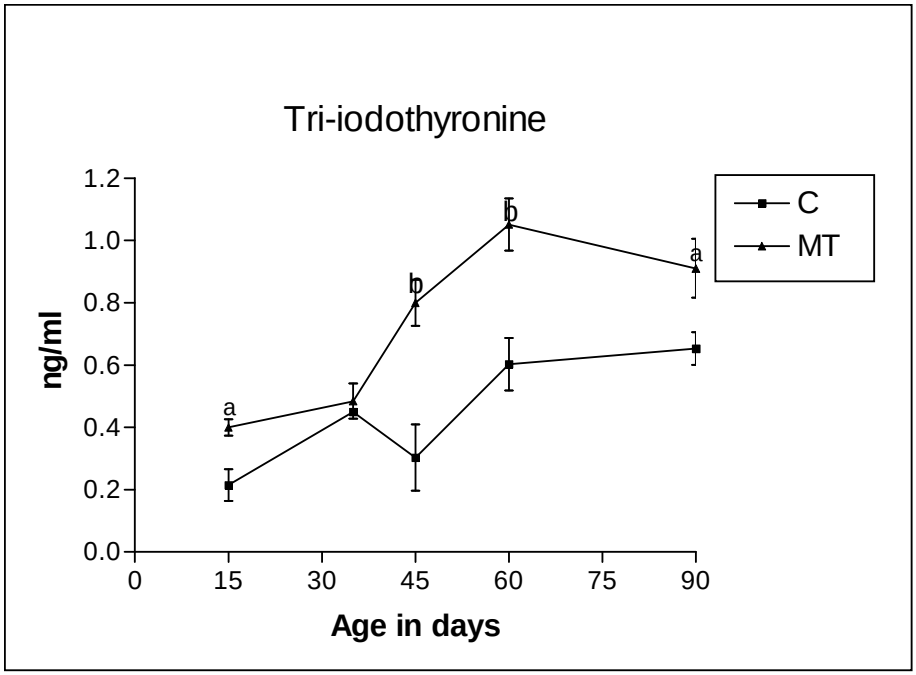
C- Control, MT- Melatonin treated ; Values are expressed as Mean±SEM of four samples.
[a] p<0.05, [c] p<0.0005

Fig 3: Serum T_4 level (ng/ml) in control and melatonin treated rats



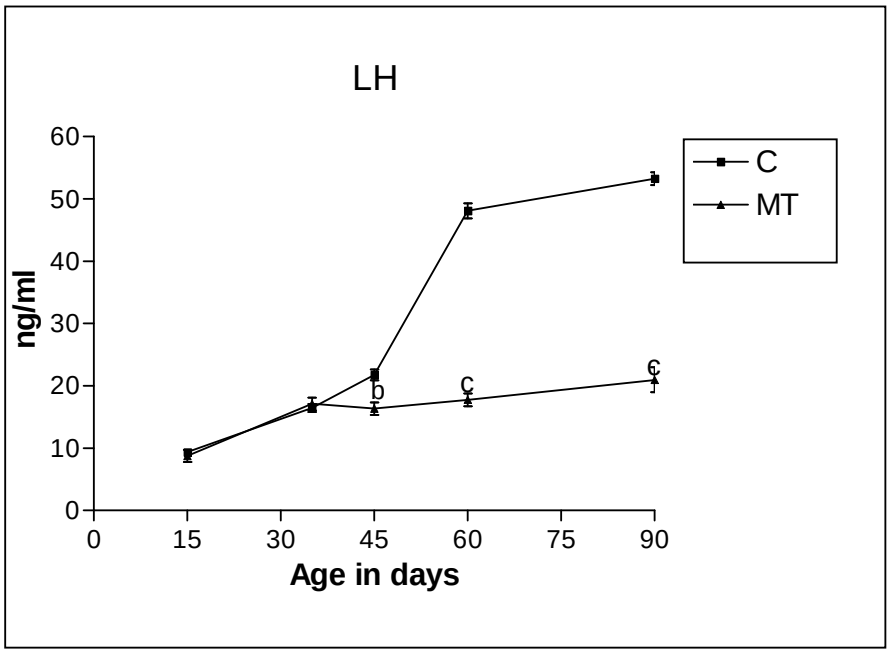
C- *Control, MT- Melatonin treated ; Values are expressed as Mean±SEM of four samples.*
[a] p<0.05, [b] p<0.005, [c] p<0.0005

Fig 4: Serum T_3 level (ng/ml) in control and melatonin treated rats



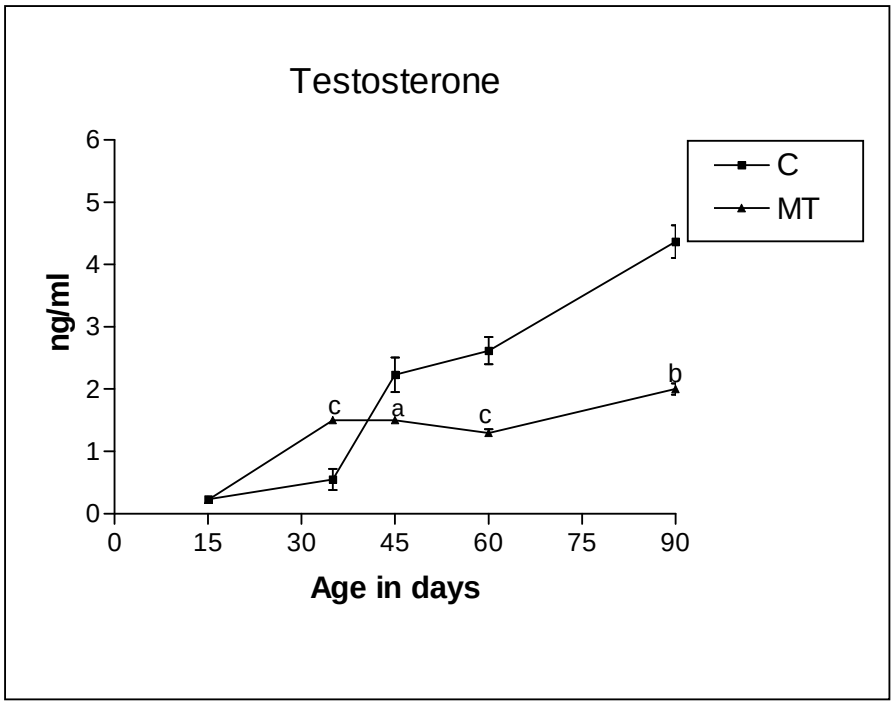
C- *Control, MT- Melatonin treated ; Values are expressed as Mean±SEM of four samples.*
[a] p<0.05, [b] p<0.005

Fig 5: Serum LH level (ng/ml) in control and melatonin treated rats



C- Control, MT- Melatonin treated ; Values are expressed as Mean±SEM of four samples.
[b] p<0.005, [c] p<0.0005

Fig 6: Serum T level (ng/ml) in control and melatonin treated rats



C- Control, MT- Melatonin treated ; Values are expressed as Mean±SEM of four samples.
[a] p<0.05, [b] p<0.005, [c] p<0.0005

Plate I: Figures 1-8: Photomicrographs of sections of testis of control rats

Figures 1 and 2: Sections of testis of 35 day old rats showing Seminiferous tubules and interstitium, Figures 3 and 4: Sections of testis of 45 day old rats showing advanced stages of spermatogenesis and appearance of sperms in few tubules, Figures 5 and 6: Sections of testis of 60 day old rats showing well-established spermatogenesis with sperm in lumen, Figures 7 and 8: Sections of testis of 90 day old rats showing prominent interstitium and fully established spermatogenesis.

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

Abbreviations : I-Interstitium, L-Lumen, st-spermatids, S-sperm, D-Degeneration, rs- round spermatids.

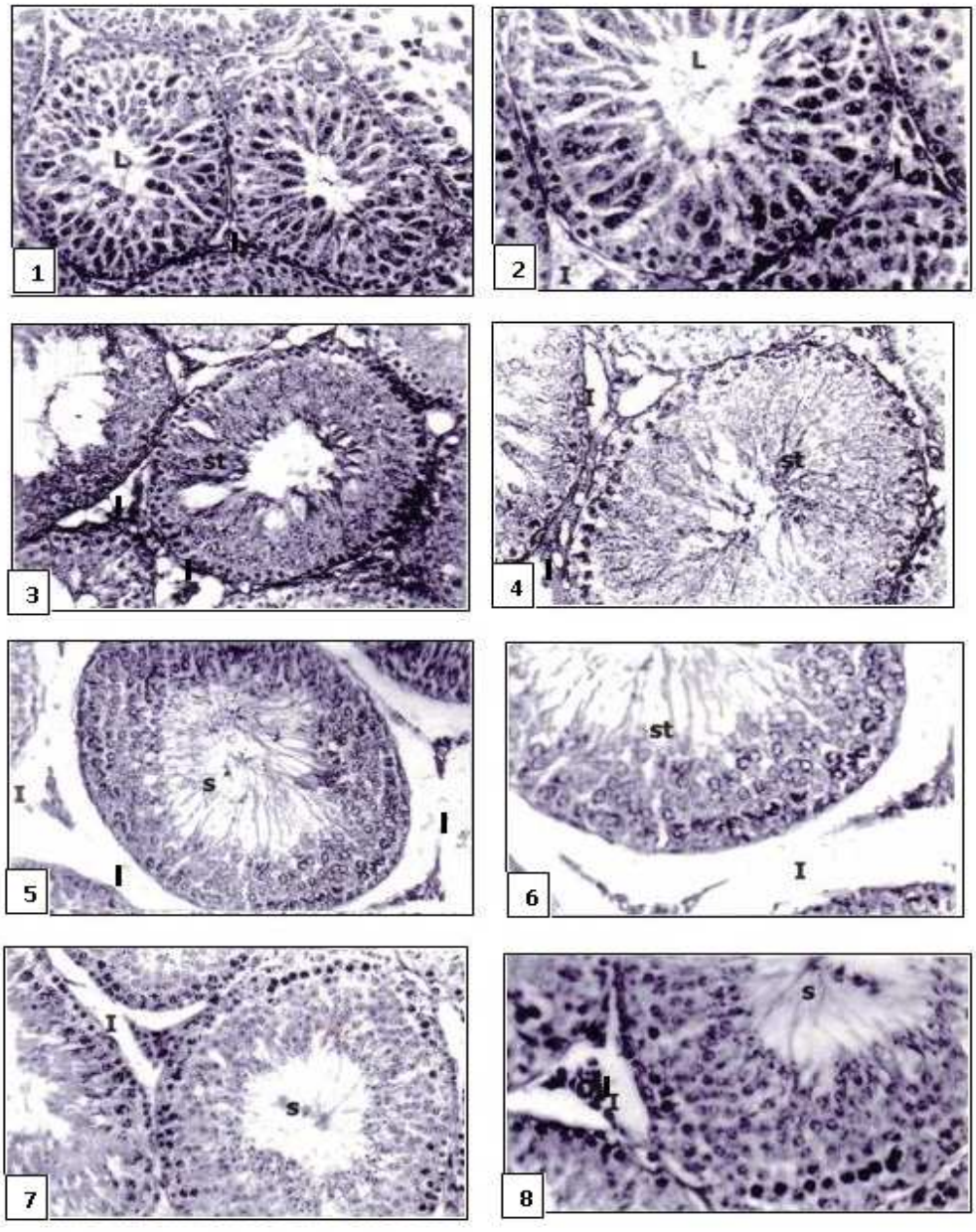
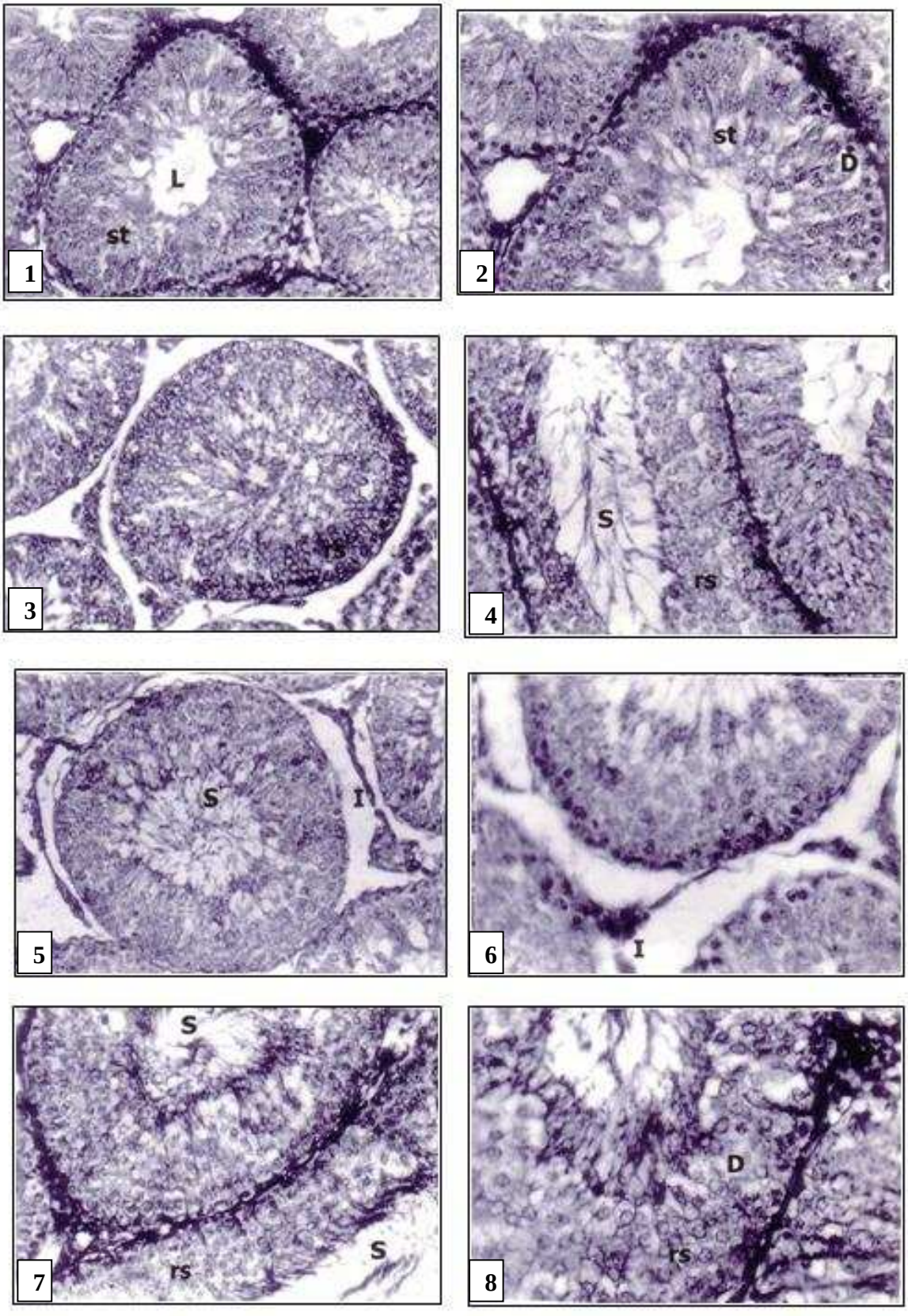


Plate II: Figures 1-8: Photomicrographs of sections of testis of melatonin treated rats



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: Sections of testis of 45 day old rats showing well established spermatogenesis with sperm in many tubules, Figures 5 and 6: Section of testis of 60 day old rats showing greater population of germ cells and well established spermatogenesis, Figures 7 and 8: Section of testis of 90 day old rats showing more number of germ cells and sloughing off of spermatids and sperms.

Figures: 1, 3, 5 and 7 – 250x, Figures: 2, 4, 6 and 8 - 400x, Abbreviations: I-Interstitium, L-Lumen, st-spermatids, S-sperms, D-Degeneration, rs- round spermatids.

DISCUSSION

The results of the present study involving hypermelatonemia in the neonatal period suggest long-term permanent alterations affecting adult testis functions and neuroendocrine reproductive and thyroid axes. A previous study involving exogenous 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 in the period immediately after, when melatonin administration was between 20-40 days but, not when between 5-20 days [10]. But, in our laboratory, melatonin administration between 10- 25 days in Charles Foster neonates showed significant reduction in testes weight, both in absolute terms as well as relative to body weight [13]. The experiments on Wistar rats have shown sensitivity to melatonin to be exhibited when administered 9 hours into the photophase or at the beginning of the scotophase, but not at the beginning of photo phase [11]. 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 [13]. These paradoxical observations find no explanation but, a possible strain difference in laboratory rats or, a maintenance temperature difference as our animals in the above experiments were maintained at 30-32°C. In fact, a temperature effect on adult testicular functions by neonatal hypothyroidism has been reported by us [22]. The present study involving transient neonatal hypermelatonemia demonstrates adult phenotypic plasticity in terms of spermatogenesis, hormonal levels and body weight.

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 greater adult body weight. The post-treatment increase in body weight is due to both an increased growth rate as well as, prolongation of the temporal increase in growth rate. Melatonin has been shown to induce hyperphagia by a possible suppressive action on central 5-HT (2A) receptors [23]. 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 [24, 25]. Long term increment in body weight to the tune of an average of 25-26% right from 30 days till 90 days tends to suggest a possible subtle upregulation of growth hormone axis by neonatal hypermelatonemia, a plausible aspect as part of developmental plasticity and programming as has been seen for other hormonal systems [26].

In contrast to body weight, testes weight does not seem to be affected as seen from the similar relative weights of adult testes in both control and experimental rats though there is a statistically insignificant decrease in the latter. Apparently, neonatal melatonin excess seems to favour phenotypic plasticity for body growth rather than for testis growth. The slight decrease (though insignificant) in testis weight noted herein favourably contrasts with the reported increase in adult testis weight in rats exposed to continuous light in the neonatal period [27].

Though there is no increase of testis size in melatonin treated rats, plasticity changes in terms of quantitative enumeration of testicular spermatogenesis and functional features are clearly evident. 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 adult tubular diameter and testis size due to induced neonatal hypothyroidism, accredited primarily to increased Sertoli cell number as a consequence of prolonged duration of proliferation in the hypothyroid state [22, 28]. The presently observed increase in tubular diameter and germinal epithelial thickness of rats subjected to neonatal melatonin excess however is not relatable to increase in Sertoli cell number as, neither the relative weight of testes nor, the Sertoli cell number 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 melatonin treated rats. The increased germ cell number in MT rats seems to be primarily due to significantly reduced germ cell apoptosis despite a paradoxically higher germ cell loss. Obviously, melatonin induced decrease in germ cell apoptosis far outweighs the increased germ cell degeneration, as evidenced by the significantly increased germ cell number per meter length of the tubule and the 8% increase in germ cell loss compared to the control. 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 (pre-meiotic and meiotic) in MT rats, essentially due to increased survival of those cell types. 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-spermatid/spermatozoa adhesive properties. This is very much feasible in the context of reported presence of melatonin receptors in the neonatal testis [29, 30, 31, 32, 33]. Two of the possible effects of excess neonatal melatonin on adult testis histoarchitecture as seen herein are, increased pre-meiotic and meiotic germ cells and modified adhesion properties between Sertoli cells and spermatids/spermatozoa. Both these effects can be related with the reported presence of melatonin receptors in the neonatal and immature rats and the consequence of genetic reprogramming involving anti-apoptotic factors (for pre-meiotic and meiotic germ cell survival) and junctional complexes between Sertoli cells and spermatids/spermatozoa. Increased tubular diameter, higher germ cell number and reduced apoptosis are features observed by us even in rats subjected to neonatal corticosterone excess (Bhavsar *et al*., unpublished). The reduced incidence of apoptosis in that study is accredited to a rare direct action of Cort on Sertoli cell expression of growth/paracrine factors (Bhavsar *et al*., unpublished). The increased Cort level seen in the immediate post-melatonin exposure protocol (15 and 30 days) in this context

seems to program the testes for reduced early germ cell (pre-meiotic and meiotic) apoptosis. Such a role of Cort is also seen in neutrophils and glomerular endothelial cells [34, 35].

An earlier study involving exogenous melatonin in the peri pubertal period had also shown decreased tubular diameter and serum FSH and LH levels with reduced frequency of spermatids in the maturation phase of spermatogenesis [11]. The present study also reveals a decrease in population, which apart from the adverse action of melatonin, could also be due to a synergistic action along with decreased FSH and testosterone levels. Though the observed degeneration of advanced 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 through increased Cort secretion observed in the treatment and immediate post-treatment periods. In this context, melatonin has been reported to increase apoptosis especially affecting spermatocytes and spermatids [36], which may be a contributory factor for the observed decrease in sperm content.

Neonatal hypermelatonemia seems to exert a permanent resetting effect on the neuro-endocrine-reproductive axis as marked by the decreased adult LH and T levels. Such a lowered setting of the HHG axis due to neonatal exposure to melatonin seems more of a regulatory adjustment in system settings [37] rather than an impairment as, this may insulate the adult neuro-endocrine reproductive axis against any agent which may cause its down regulation including melatonin. Even short term exposure to exogenous melatonin in the neonatal period has revealed an inhibitory effect on GnRH induced FSH and LH secretion. A permanent hyposetting of the control set point of HHG axis is deducible and may also suggest the possible action of neonatal melatonin excess on the maturation of the reproductive axis directly or indirectly. The possibility of such an alteration on set point of HHG axis is strongly supported by an observation of altered feedback response of T on LH, FSH and PRL secretion in the pre-pubertal and pubertal periods due to prenatal melatonin administration [38]. There was only one period of T excess, at 35 days, in the present study, which could be related with the early puberty and appearance of spermatozoa in MT rats. However, this increase in T level at 35 days is not explicable and may only signify the early attainment of an adult level, characteristic of melatonin treated rats. It may be speculated that, the increased serum titre of T 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 T contributing to the higher serum T level before the permanent down regulation of LH sensitivity of these cells. Apparently, neonatal melatonin excess seems to favour Leydig cell proliferation though the sensitivity of these cells towards LH is very much compromised. The lowered levels of both LH and T in melatonin exposed rats are indicative of a centrally (brain) mediated rather than a local (testis) effect [15]. The present tenet of lowered set point of HHG axis is well supported by the ability of melatonin to regulate at all the three levels of the axis as well as the observations of its influence in inhibiting the hypothalamic gonadotropin releasing hormone pulse generator and GnRH gene transcription in GnRH neurons, dampened response of gonadotrops of pituitary to GnRH by way of inhibition of GnRH induced increase in several intracellular messengers and, its negative modulation of Leydig cell response to LH in both adults and neonates [39, 40, 41].

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 (Bhavsar *et al*., unpublished), the reduced T_4 level, noted herein is a novel feature. This might suggest a direct action of melatonin in altering the T_3:T_4 secretory ratio. Inferably, thyroidal type II deiodinase (DIO2) activity is upregulated as a permanent plasticity alteration due to neonatal melatonin excess. Support to this suggestion is available from the reported decrease in T_4 level and increased T_3/T_4 ratio in melatonin treated pigeons [42], which was again speculated to be possibly due to active peripheral mono-de-iodination [43]. Further, both melatonin and TSH are reported to increase the expression of DIO_2 [44, 45, 3,1]. This is in contrast to the reports of melatonin decreasing TSH, T_3 and T_4 levels in adult rats [46]. 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 [22, 47, 48, 49, 50, 51].

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. The observations made herein suggest that, hormonal disturbances early in life can shape later phenotypic responses and apparently, phenotypes remain malleable into the adulthood. More studies on this field are warranted affecting various other organ systems.

Acknowledgments
Thanks are due to Prof.M.M.Aruldhas, Department of Endocrinology, Dr. ALMPGIBMS, Taramani Campus,Chennai, for his kind help in hormone assay.

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