CHAPTER - VI

Neonatal corticosterone deprivation decreases germ cell number and sperm density in the adult testis

Foetal impressions in terms of environmental and endocrine conditions have long-term effects. Profound effects on birth weight and adult organ system functions have been reported as consequences (Smith and Waddell, 2000). Among endocrine conditions, variations in foetal glucocorticoid exposure have been found to be of greater significance. Though foetal glucocorticoids have been implicated a role in the maturation of organ systems (Baxter and Rousseau, 1979), excess glucocorticoids have been shown to retard foetal growth and have been linked with the development of adult diseases (Smith and Waddell, 2000). Foetal exposure to glucocorticoids has also been considered significant in programming normal postnatal development and physiological maturation of various systems. One system that has been known to be affected is the postnatal reproductive development, as puberty onset is delayed in the offsprings of mothers subjected to stress (Politch and Herrenkohl, 1984) or treated with adreno-corticotrophic hormone (ACTH) during pregnancy (Harvey and Chevins, 1987). In a recent study, reduced offspring birth weight and delayed onset

of puberty in male offsprings of mothers subjected to glucocorticoid excess from gestation day 13 to termination has been reported (Smith and Waddell, 2000). Since there was no studies on neonatal exposure to glucocorticoids, we had previously tested the influence of glucocorticoid administration to rat neonates during the preweanling period on the course of development and adult histoarchitecture and functions of the testis (Chapters 2 & 3). These studies suggested a definite influence of glucocorticoid status during neonatal period on qualitative and quantitative aspects of spermatogenesis and steroidogenesis as, long-term effects of glucocorticoids. Apart from long-term effects on the various neuroendocrine axes, increase in germ cell population in the testes was a significant feature, which was accredited to a probable decrease in apoptosis.

The above findings raised a natural and logical question of the effect of glucocorticoid deprivation or insufficiency during neonatal period on the observed functions. Hence, present study was planned as a corollary to the above studies to answer these questions and test the assumption of the reduced germ cell loss due to glucocorticoid excess. To this end, rat neonates have been treated with metyrapone (Glucocorticoid synthesis inhibitor) during the preweanling period for 21 days and the ensuing effects on adult testis structure and functions have been assessed to have an idea about the influence of neonatal glucocorticoid insufficiency on various

neuroendocrine axes. To this end, circulating levels of TSH, T_4, T_3, LH, T and

CORT have been measured.

MATERIALS AND METHODS:

Animals and Maintenance:

As in chapter one.

Preparation of Metyrapone:

Metyrapone (2,methyl 1, 2 di-3-pyridyl-1-propanone) 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, some of them

consisting of subgroups as mentioned below.

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 *i.p.* with vehicle (0.9% saline) in morning (0800 hrs)

Group II Metyrapone treated; (MET):

Rat neonates were injected with metyrapone (2,methyl 1, 2 di-3-pyridyl-1-propanone) in the following dose,

(i)   0.5 mg metyrapone/animal/day from day 0 to day 10 and 1.0 mg metyrapone/animal/day from day 11 to day 21.

Parameters and Methods of Evaluation:

As in chapter one.

Histology and Histometry:

As in chapter one.

Hormone Assays:

As in chapter one.

Statistical Analysis:

As in chapter one.

RESULTS:

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

Postnatal Growth:

Body and testes weights of experimental animals tended to be slightly lower during the treatment period, significantly increased during post-treatment period upto 60 days and, noticeable lower weight at 90 days (Table 1; Figures 1a & 1b). These changes in body and testes weights are clearly evidenced in the recorded growth rates, with higher growth rates between 15-60 days in MET animals. The decline in growth rate in both body and testes weights between 60-90 days in MET animals are perceptible and significant (Table 2; Figures 3a & 3b). The relative weight of testes is not significantly different in any of the stages studied except between 60 and 90 days whence there was a decrement (Table 1; Figure 2)

Histology and Histometry:

The sections of testis of control animals showed full spermatogenic activity and presence of sperms in the tubules at 60 days. The experimental animals in contrast showed presence of sperms by 45 days. There was no significant difference in tubule diameter or germinal epithelial thickness between controls and experimentals at any age. Overall, the germ cell number appears to be decreased in experimental animals with many empty spaces; such loss of cells appear to be increased during postpubertal (45, 60 and 90 days) as compared to prepubertal stage (35 days). Quantitative alterations, marked by low density of sperms was the feature at 60 and 90

Table 1: Chronological alterations in body weight (g) and absolute (g) and relative weight (g/100 g) in Control and Metyrapone treated rats

Treatment	Body Weight					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	34.75 ±1.942	82.0 ±2.804	122.5 ±3.677	197.33 ±6.396	360.9 ±6.280	0.215 ±0.019	0.86 ±0.050	1.346 ±0.058	2.603 ±0.085	3.586 ±0.104	0.67 ±0.034	1.056 ±0.060	1.108 ±0.062	1.399 ±0.074	0.986 ±0.054
MET	31.0 ±1.864	100.0[a] ±6.164	175.0[c] ±3.106	258.3[c] ±6.540	354.16 ±5.665	0.179 ±0.013	0.86 ±0.034	1.913[c] ±0.048	3.173[c] ±0.052	3.480 ±0.052	0.562 ±0.057	0.562 ±0.054	1.091 ±0.056	1.228[a] ±0.068	0.982 ±0.041

C – Control, **MET** – Metyrapone treated

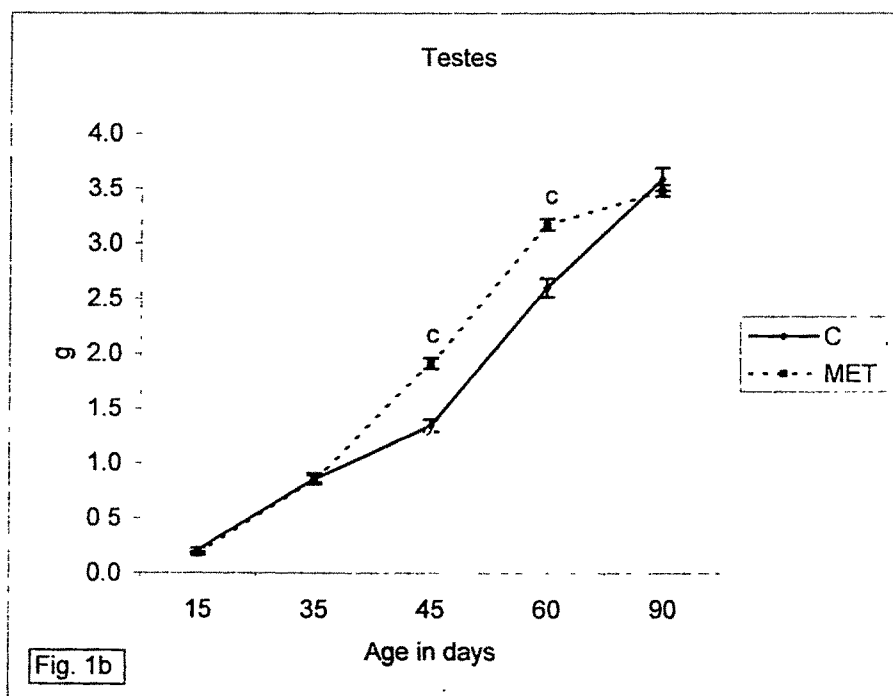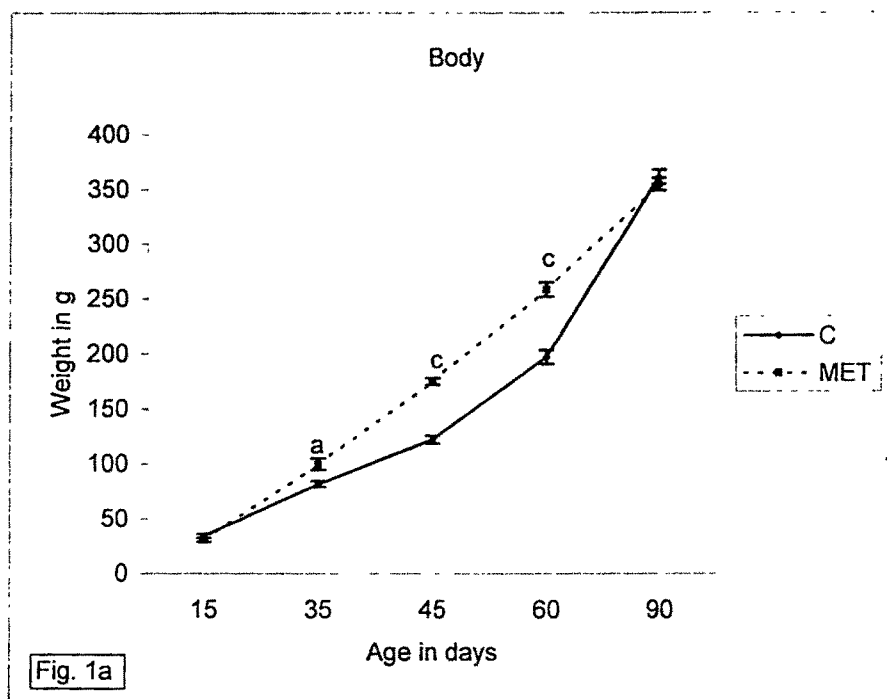Values expressed as Mean ± SEM of six animals.   [a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

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

C - Control, MET - Metyrapone, Values expressed as Mean ± SEM of six animals, [a] p< 0.05, [b] p< 0.005, [c] p< 0 0005
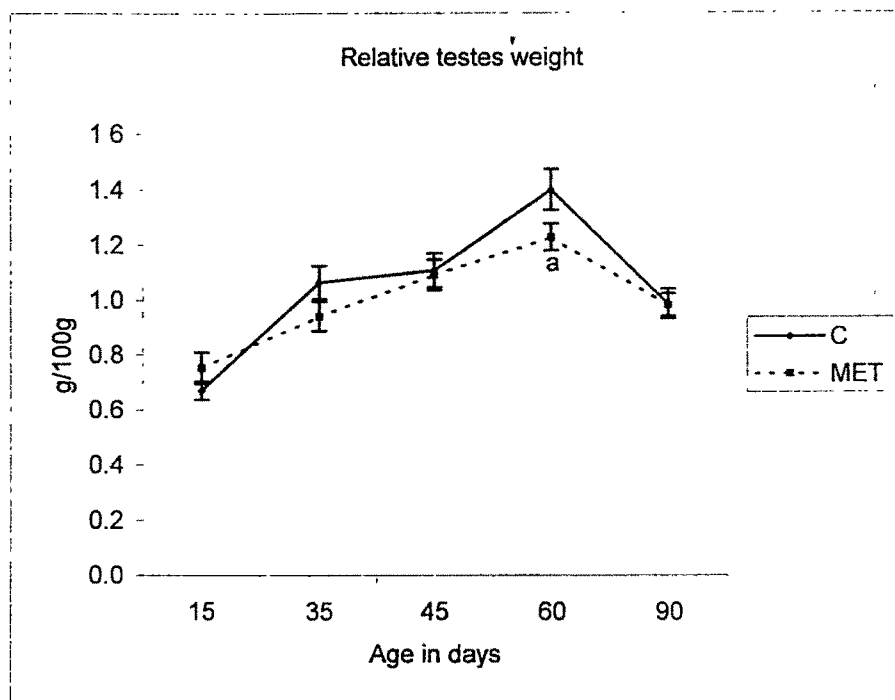
Fig.2: Chronological alterations in relative weight (g/100 g) of testes in
Control and Metyrapone treated rats
C - Control, MET - Metyrapone, Values expressed as Mean ± SEM
of six animals, [a] p< 0.05, [b] p< 0.005, [c] p< 0.0005

Table 2: Per day Body and Testes Growth Rate (g/day) in Control and Metyrapone 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	1.945	2.36	4.050	4.989	5.452	0.014	0.032	0.049	0.084	0.033
MET	1.633	3.45	7.5	5.55	3.195	0.012	0.034	0.105	0.084	0.010

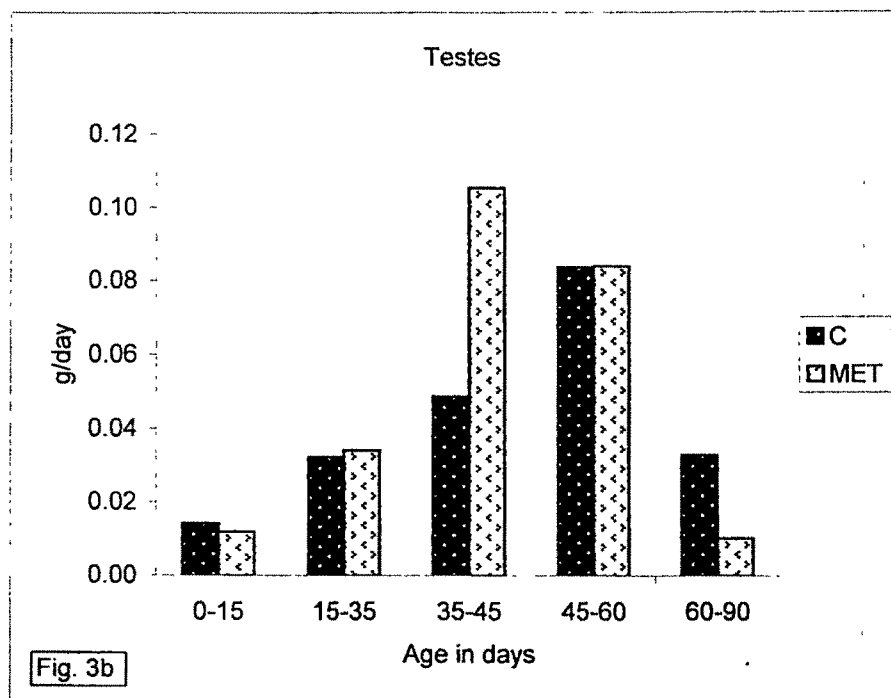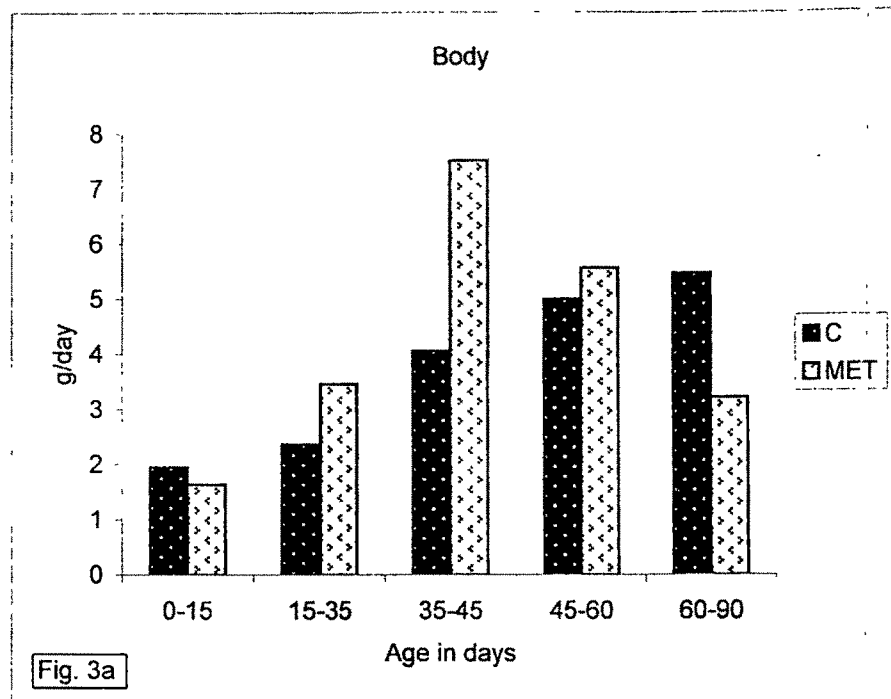C – Control, **MET** – Metyrapone treated

Fig 3a and 3b. Per day Body and Testes Growth rate (g/day) in Control and Metyrapone treated rats

C - Control, MET - Metyrapone

Table 3: Histometric enumeration of seminiferous tubules of Control and Metyrapone 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 x10^6	TGC_T x10^6	AGC_T x10^6	TGC_M x10^6	AGC_M x10^6	% 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 ±6.300	280.84 ±5.600	13.39 ±0.260	12.1 ±0.150	10.00 ±0.0002
MET	1.589 ±0.090	0.0290 ±0.0010	0.0088 ±0.0010	1.525 ±0.070	2323.66 ±56.900	211.040 ±4.500	32.531 ±1.900	361.00[c] ±4.600	232.27[c] ±3.800	14.430 ±0.659	9.990 ±1.260	30.760[c] ±0.150

C – Control, MET – Metyrapone treated rats

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.

days. Leydig cells in general appear to be small at 90 days. Various histometry parameters such as seminiferous tubular volume, tubular length and total basement area did not show any significant difference, compared to control testis at 90 days. The Sertoli cell number per testis or number per meter length of tubule was identical to those of controls. However, the total germ cell number per testis or per meter length of the tubule was slightly more in MET as compared to controls (14.43×10^6 v/s 13.39×10^6). The total number of cells per meter length of tubule, obtained by actual count was significantly lower in experimental animals (9.97×10^6) than controls (12.1×10^6). The germ cell loss was as high as 31% (Table 3) (Plates I, IXa and IXb).

Serum Hormone Profile:

Corticosterone:

Serum corticosterone level was significantly lower during the treatment period but then increased to a higher level at 35 and 45 days. During 60 and 90 days the hormone level increased gradually to reach near normal level at 90 days (Table 4; Figure 4).

TSH, T_4 and T_3:

Except for treatment period (15 days), all other periods of study showed increasing higher levels of TSH in MET animals with a significantly higher level at 90 days (Table 5; Figure 6). The serum T_4 level was

Table 4: Serum Corticosterone, LH and T levels (ng/ml) in Control and Metyrapone 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	45.55 ±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 ±0.620
MET	2.7 ±0.015	23.0 ±0.654	20.0 ±0.850	24.8 ±0.746	39.0 ±0.156	11.4[a] 0.967	28.56[c] ±1.24	71.25[c] ±2.876	86.35[c] ±2.678	56.32 ±2.794	0.485[c] ±0.028	1.954[c] ±0.042	2.150 ±0.016	2.569 ±0.026	1.48[b] ±0.034

C – Control, MET – Metyrapone treated

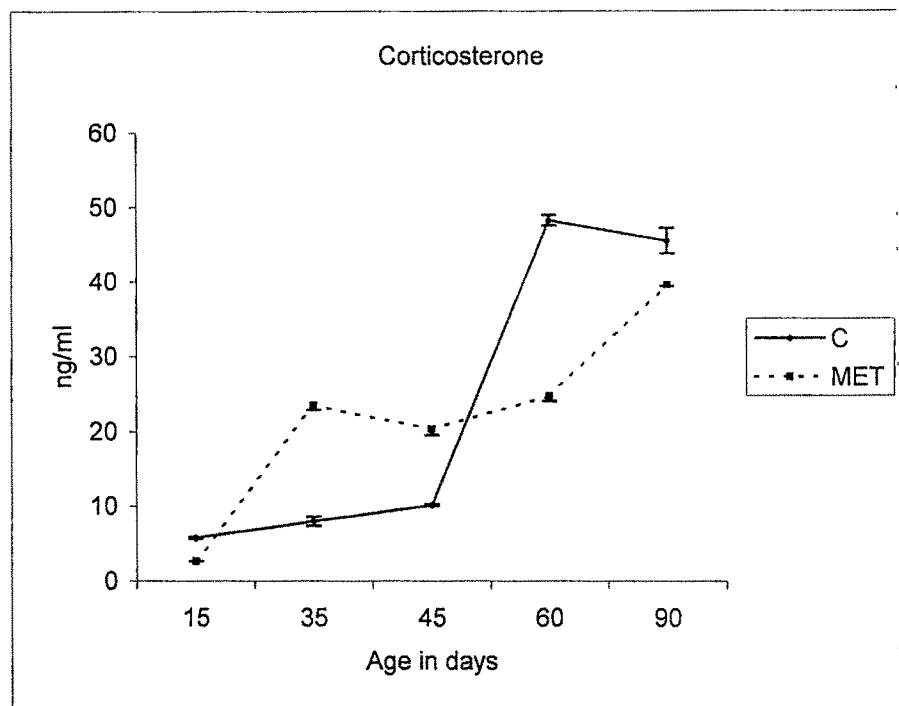Values expressed as Mean ± SEM of four Samples. [a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

Fig.4: Serum Corticosterone level (ng/ml) in Control and
Metyrapone treated rats
C - Control, MET - Metyrapone, Values expressed as Mean ± SEM
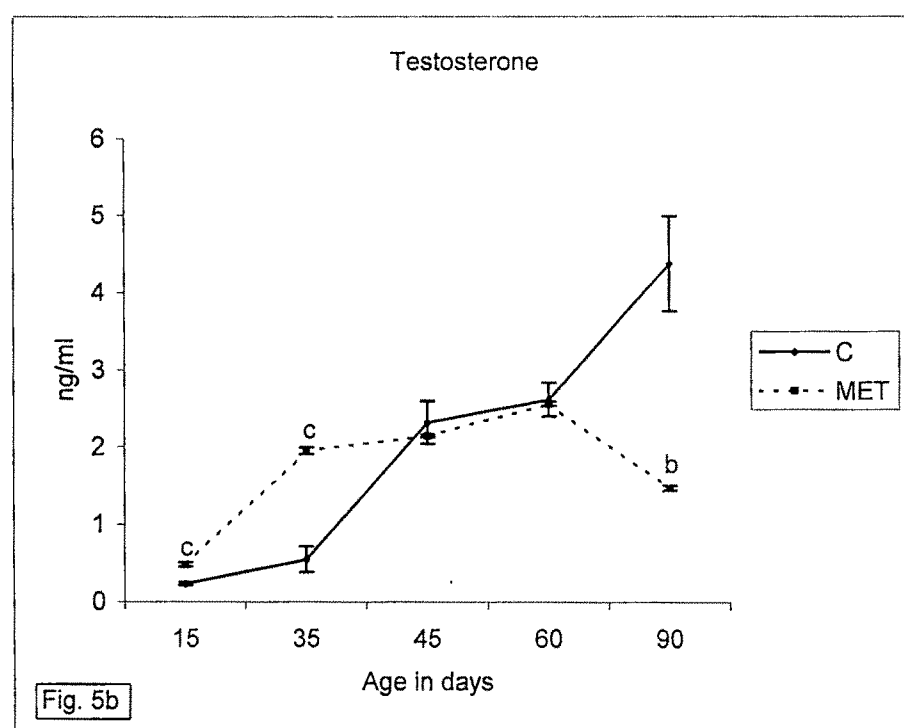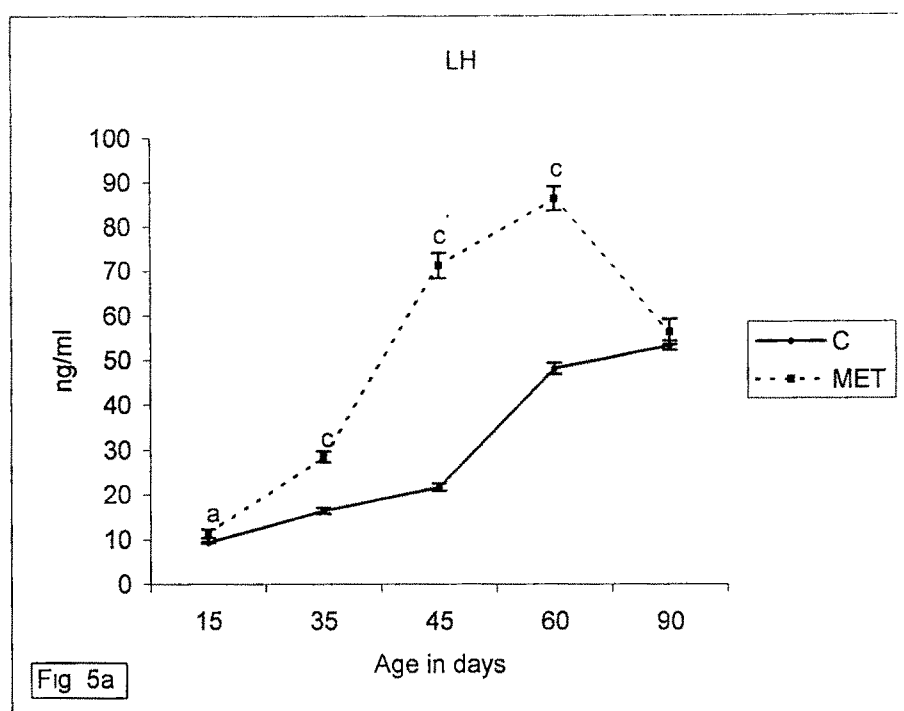of four samples, [a] p< 0 05, [b] p< 0 005, [c] p< 0 0005

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

C - Control, **MET** - Metyrapone, Values expressed as Mean ± SEM
of four samples, [a] p< 0 05, [b] p< 0 005, [c] p< 0 0005

Table 5: Serum TSH, T_4 and T_3 levels (ng/ml) in Control and Metyrapone 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
MET	2.254[b] ±0.134	6.980 ±0.243	7.250 ±0.269	7.840 ±0.341	9.640[c] ±0.398	0.46[b] ±0.024	1.94[c] ±0.024	3.0[c] ±0.032	1.73[c] ±0.032	1.74[b] ±0.021	0.125 ±0.025	0.205[c] ±0.021	0.285 ±0.012	0.264[b] ±0.016	0.367[b] ±0.019

C – Control, **MET** – Metyrapone treated

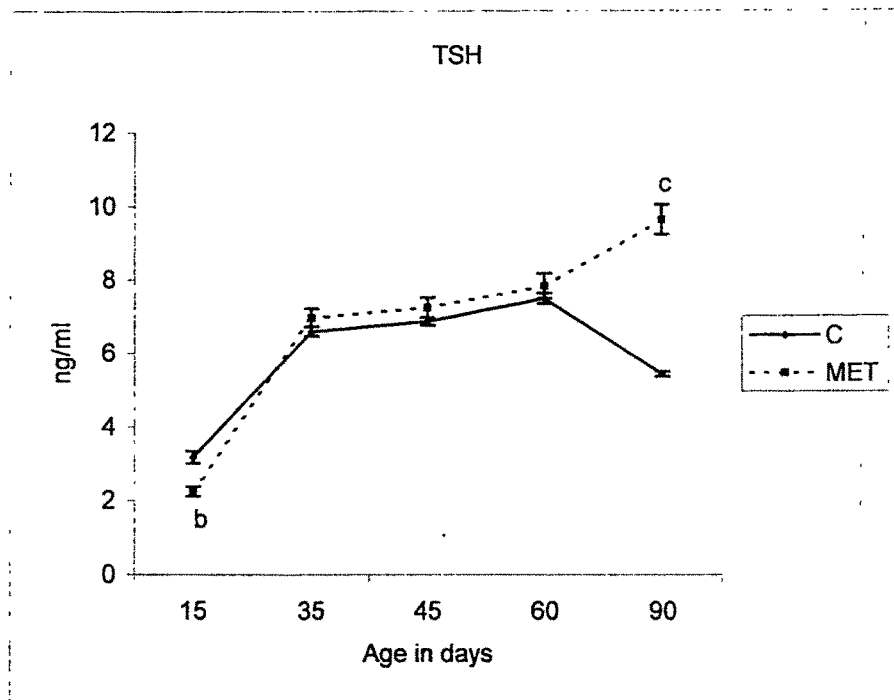Values expressed as Mean ± SEM of four Samples. [a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

Fig.6: Serum TSH level (ng/ml) in Control and Metyrapone treated rats
C - Control, MET - Metyrapone, Values expressed as Mean ± SEM
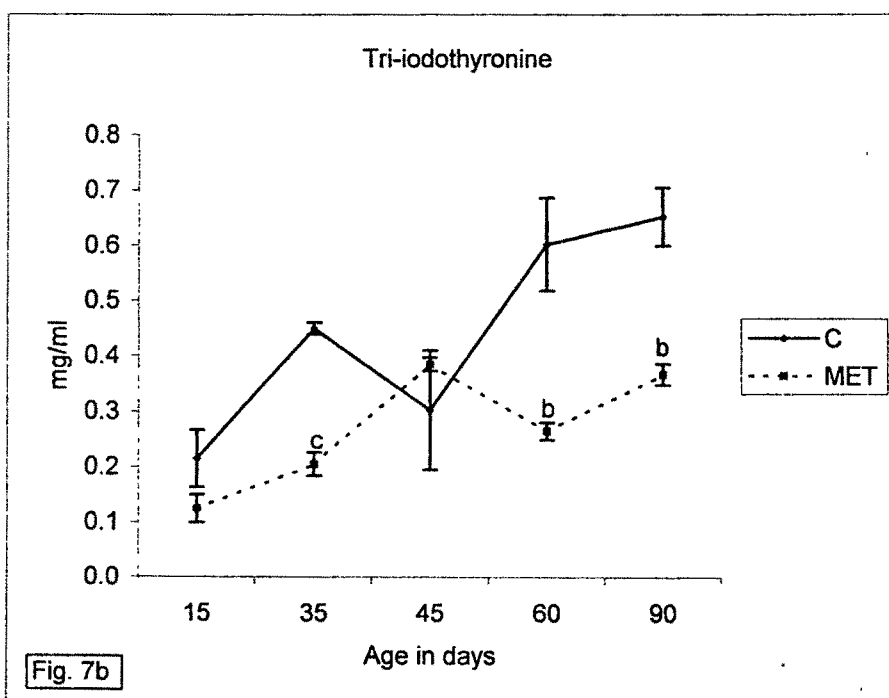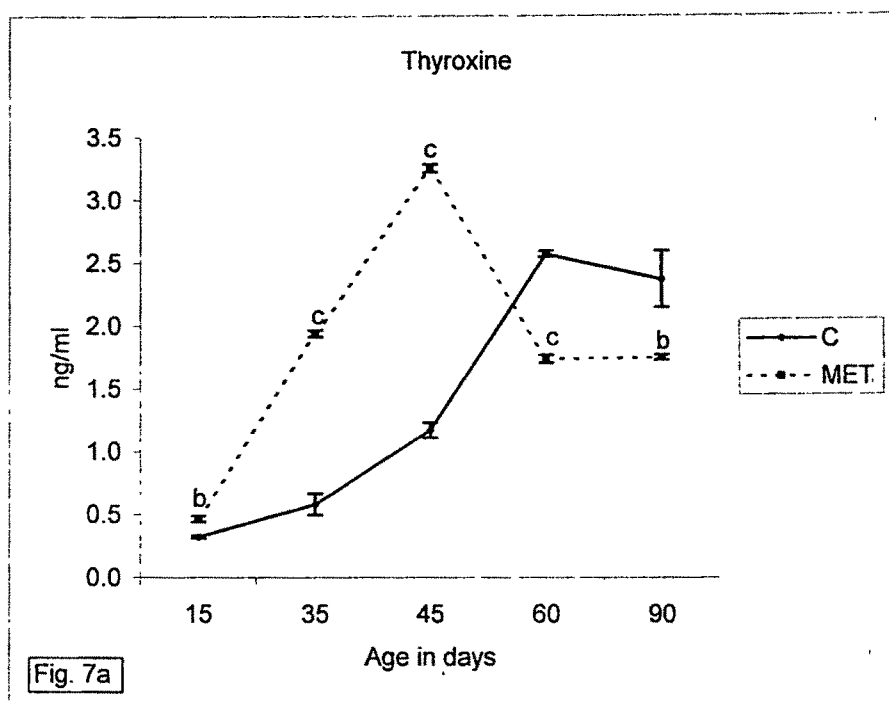of four samples, [a] p< 0.05, [b] p< 0.005, [c] p< 0.0005

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

C - Control, **MET** - Metyrapone, Values expressed as Mean ± SEM of four samples, [a] p< 0.05, [b] p< 0.005, [c] p< 0 0005

significantly higher in the experimental animals at 15, 35 and 45 days but the levels at 60 and 90 days were significantly lower compared to controls (Table 5; Figure 7a). In contrast, serum T_3 showed consistently lower levels with significantly lower level at 90 days in MET animals (Table 5; Figure 7b).

LH and Testosterone:

Serum LH level was significantly higher at all ages of study as compared to controls (Table 4; Figure 5a). However, serum testosterone level was significantly higher at 15 days (treatment period) and at 35 days (post-treatment period), but then decreased significantly to subnormal level at 90 days (Table 4; Figure 5b).

DISCUSSION:

Previous studies on reduced exposure of the foetus to endogenous glucocorticoid by maternal metyrapone treatment had shown higher birth rate and early onset of puberty in male rats. Metyrapone is known to inhibit glucocorticoid synthesis by inhibiting 11-β hydroxylase (Milkovic et al., 1975; Smith and Waddell, 2000). The 11-β hydroxylase inhibition induced minimal foetal glucocorticoid exposure is reported to account for the enhanced foetal growth and advancement of puberty in males (Milkovic et al., 1975; Smith and Waddell, 2000). This is the first study, which evaluates the effect of reduced endogenous glucocorticoid exposure in the neonatal period on adult testis structure and functions and neuroendocrine hormonal status. Though

there are no significant effects on testicular volume, testicular weight, tubular volume, tubular basement membrane area or Sertoli cell number, there are nevertheless marked influences on the time of appearance of sperms, germ cell number per unit area as well as the degree of germ cell loss by apoptosis and/or degeneration. The appearance of sperms in the tubules by 45 days, which is slightly earlier than in the controls, is similar to the observations made in neonatal corticosterone exposed animals (Chapters 2 & 3). This similarity of early onset of spermatogenesis in diametrically opposite setups (hypercorticalism and hypocorticalism) is rather paradoxical. However, a critical review of the experimental observations in the two set up shows, an identical increased serum corticosterone titre in the prepubertal period (35 days) subsequent to the period of experimental treatment. This increase in the endogenous glucocorticoid level seen at 35 days (about 14 days after treatment) could be inferred to be due to two unrelated consequences. Whereas, the increased corticosterone level in animals exposed to neonatal glucocorticoid excess can be related to the probable decreased metabolic clearance due to inhibition of 11β-HSDH as inferred earlier (Chapter 2), the increase obtained in MET treated animals could be accredited to a rebound effect mediated by the higher adreno-corticotrophic hormone (ACTH) levels prevailing in the experimental period (upto 21 days) due to the reduced negative feedback effect as a consequence of lowered corticosterone synthesis. In this context, the prevailing glucocorticoid levels during the

prepubertal periods appear to be of crucial significance in controlling postnatal growth and maturation. This assumption gains justification from the recorded higher growth rates of body and testes between 35-60 days in animals subjected to either glucocorticoid excess or deficit (Chapters 2 & 3). The early completion of spermatogenesis and appearance of sperms in the tubules by 45 days seen under both neonatal hyper and hypocorticalism may also suggest a potentiated testosterone action in a background of higher prepubertal corticosterone level (Chapters 2 & 3).

Our previous studies involving neonatal corticosterone exposure had suggested a possible long-term quantitative influence on spermatogenesis by way of increased germ cell number. This was related with the possibility of neonatal corticosterone excess in modifying the genetic programming of Sertoli cells resulting in reduced apoptosis. Looked in this context, neonatal corticosterone insufficiency could be expected to have a reverse effect and thereby favour increased apoptosis. The histoarchitectural features seen in testis section of MET animals suggest greater loss of germ cells as seen by the empty spaces within the germinal epithelium. Such sites might represent areas previously occupied by germ cells and subsequently lost by apoptosis. As can be seen from table 3, the total number of germ cells that can be theoretically present per meter length of seminiferous tubule based on the area available is 14.43×10^6. Calculations based on actual counts provide a figure of 9.9×10^6 cells. This amounts to a 31% difference compared to age

matched controls, much more than that seen under neonatal exposure to melatonin or corticosterone (Chapters 1, 2 & 3). This observation of increased germ cell loss by apoptosis seen with metyrapone treatment strengthens and provides validity to our earlier contention of decreased apoptotic loss of germ cells in the adult, due to neonatal corticosterone exposure.

Unlike neonatal corticosterone exposure, which resulted in a permanently lowered set-point of the hypothalamo-hypophyseal-adrenal (HHA) axis, MET treatment does not seem to alter the set point of the axis significantly as the circulating serum corticosterone level tended to increase gradually to control levels. The set point of the hypothalamo-hypophyseal-gonad (HHG) axis seems to be permanently altered under MET treatment as marked by consistently higher levels of LH at all ages. However, there seems to be a lowered responsiveness of the post pubertal Leydig cells to LH as seen by the reducing testosterone levels. Nevertheless, there is either an increased number and/or increased sensitivity of prepubertal Leydig cells as denoted by the higher testosterone levels at 15 and 35 days, which can be favorably related with early appearance of sperms as discussed above. The hypothalamo-hypophyseal-thyroid (HHT) axis shows a differential effect under neonatal corticosterone deficit. The increased TSH levels right from 35 to 90 days (except for a slight decrease at 15 days; treatment period) indicates an upregulation of hypothalamo-hypophyseal set point or an

increased sensitivity of pituitary thyrotropes to thyrotrophin releasing hormone (TRH). In contrast, the sensitivity of thyroid to TSH seems to be decreased as revealed by consistently lower T_3 levels at all ages and of T_4 levels at 60 and 90 days. The levels of T_3 at all ages suggest either a decreased thyroid output and/or decreased peripheral conversion. Apparently, the HHT axis responds differentially to neonatal corticosterone deficit.

Overall, the present study on neonatal corticosterone deficiency has not only shown an altered response of HHT and HHG axes with no significant effect on HHA axis, but has also demonstrated increased germ cell loss and low sperm density, strengthening our earlier contention of neonatal corticosterone excess in physiological range providing a long-term anti-apoptotic effect.

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.
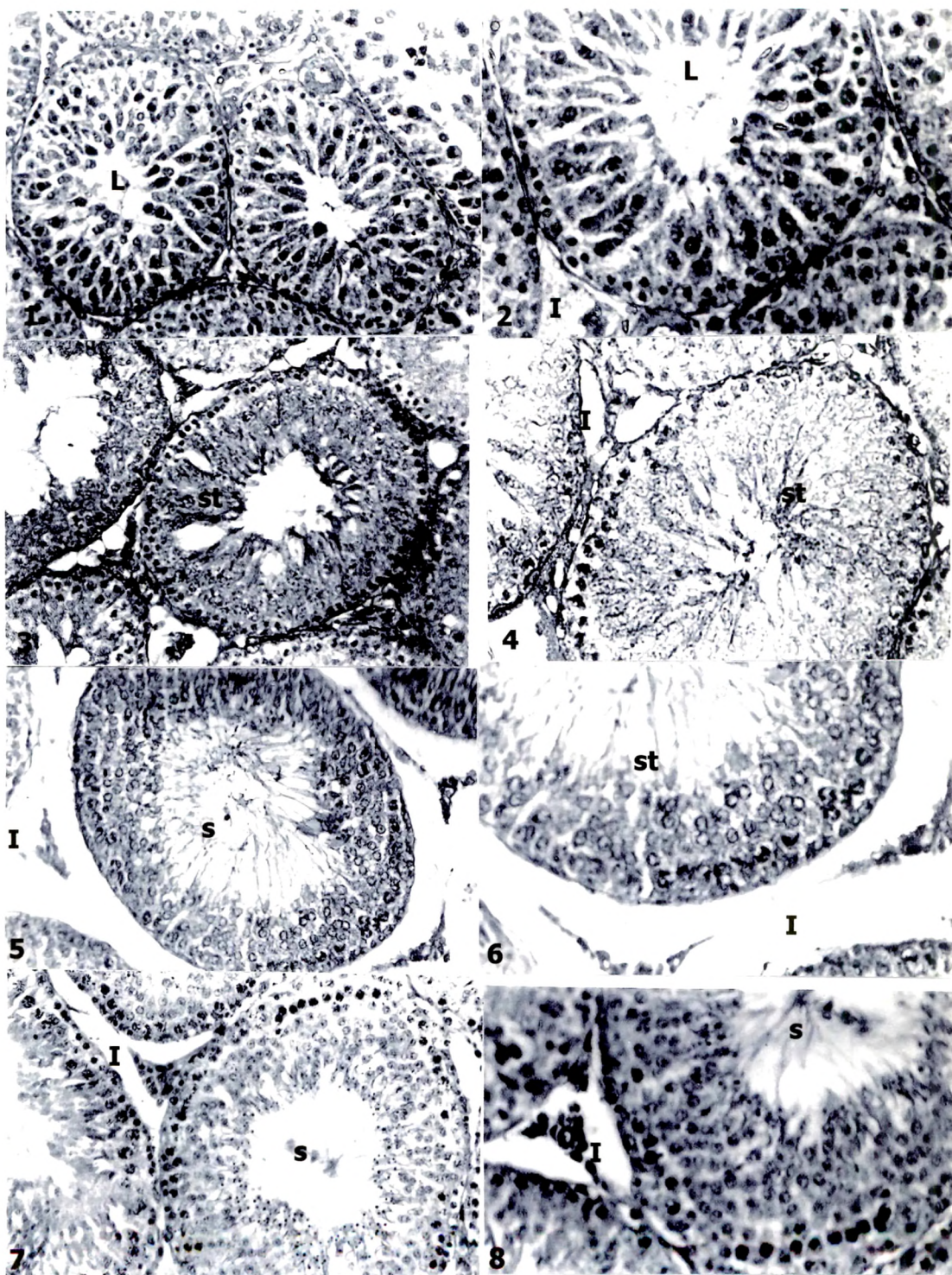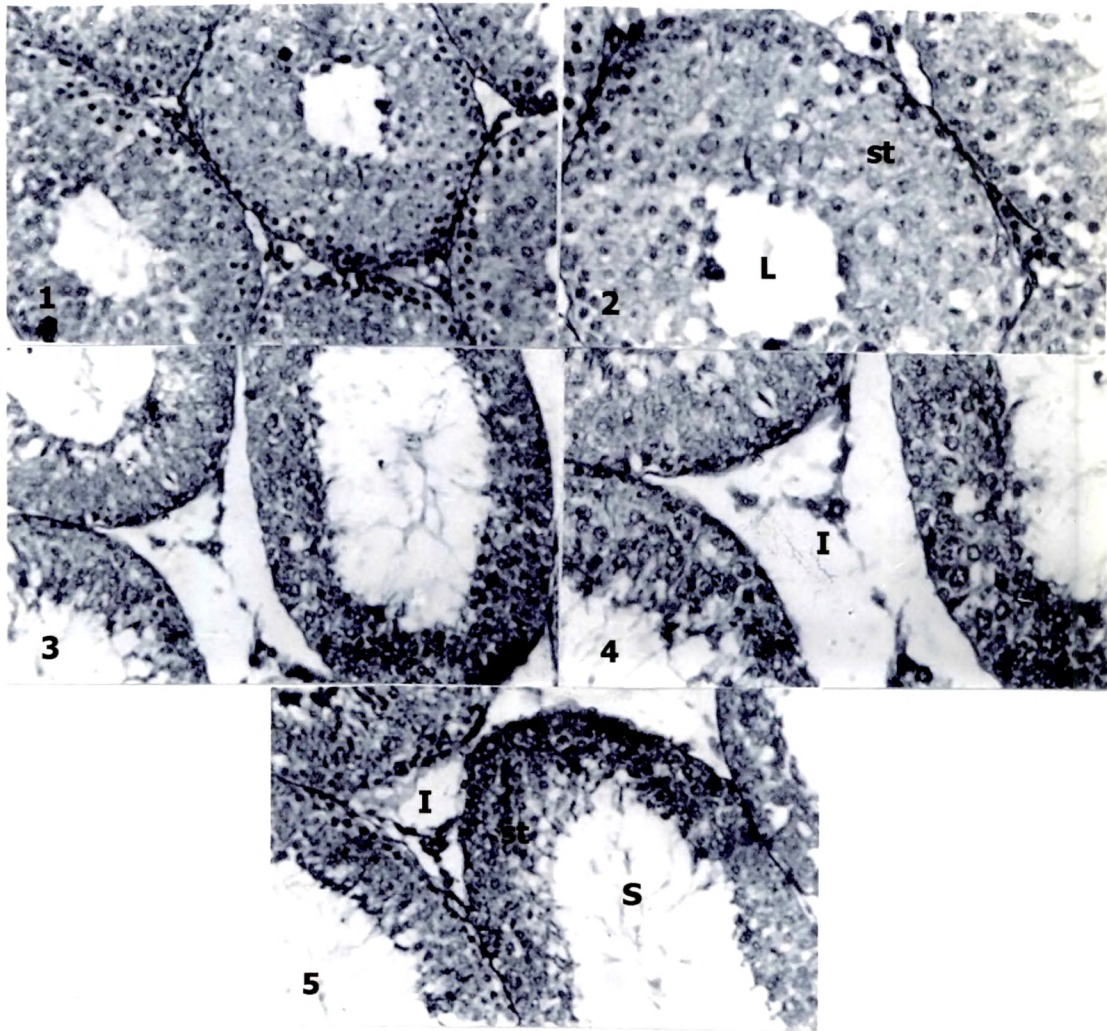
PLATE – IX a

Figures 1 – 5: Photomicrographs of sections of testis in rats treated with
Metyrapone.

Figures 1 and 2 : Sections of testis of 35 day old MET treated rats showing,
showing elongated spermatids and degenerating germ cells.

Figures 3 to 5 : Section of 45 day old testis of MET treated rats
showing, high degree of degeneration and mixed population
of interstitial cells, also sperms are seen.

MET – Metyrapone treated rats

Figures: 1 and 3 – 250 x
Figures: 2 to 5 – 400 x

PLATE – IX b

Figures 1 – 7: Photomicrographs of sections of testis in rats treated with metyrapone.

Figures 1 to 3   : Sections of testis of 60 day old MET treated rats showing, sloughing off of spermatids, less no. of sperms and less germ cell number.
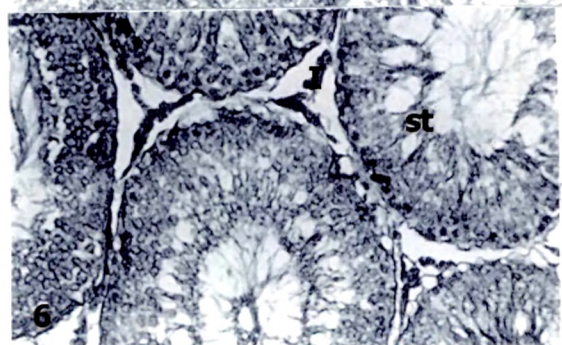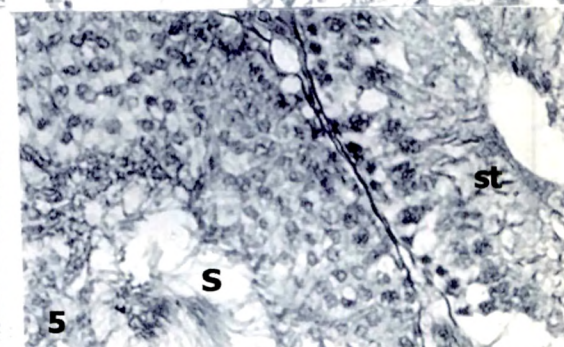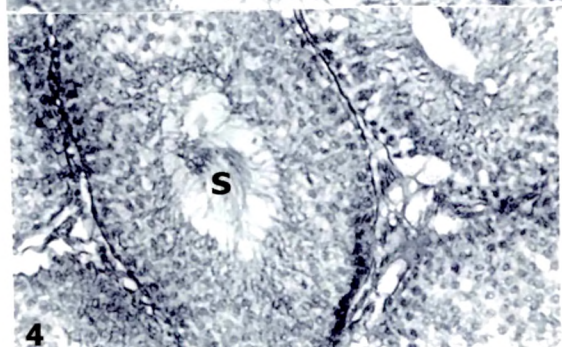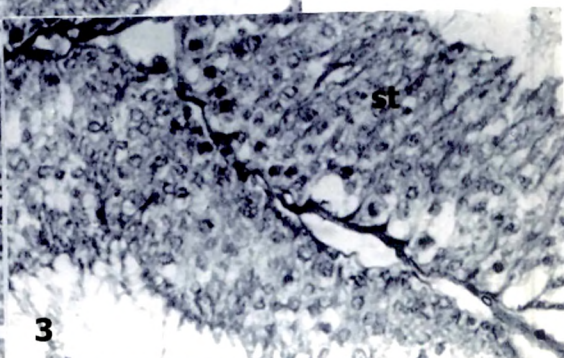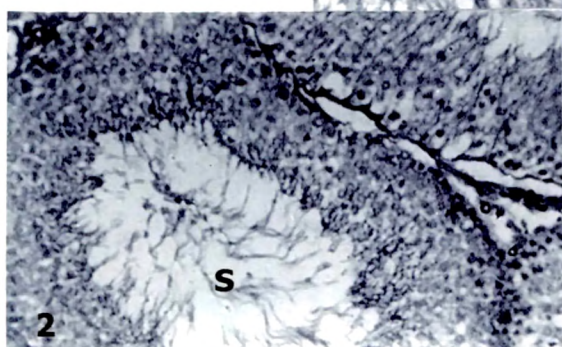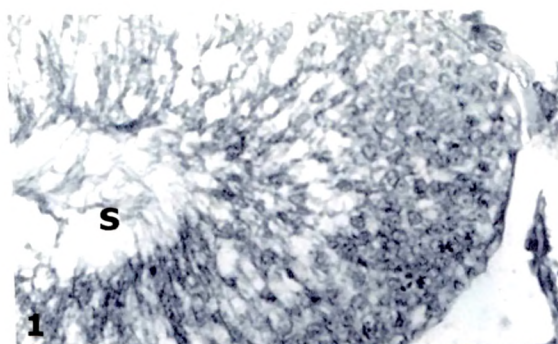

Figures 4 to 7   : Section of 90 day old testis of MET treated rats showing, degeneration and less sperm density.

MET – Metyrapone treated rats

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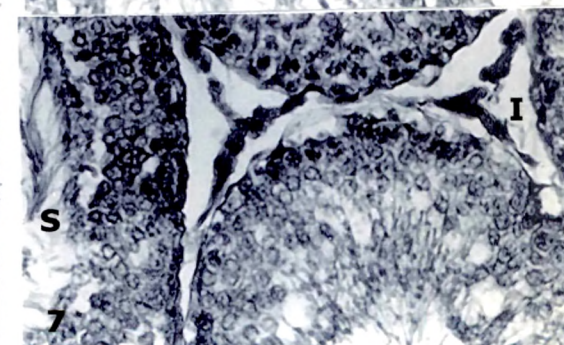
Summary

The long-term influence of neonatal hypocorticalism on adult testis structure and functions and serum hormonal profiles has been assessed in Charles Foster strain of rats. The neonates were subjected to corticosterone deprivation by treatment with metyrapone (2-methyl, 1,2 di-3-pyridyl-1-propanone) from day 0 to day 21 at a dosage of 0.5 mg/day from day 0 to day 10 and 1 mg/day from day 11 to day 21. The present study conducted, as a corollary to previous studies on neonatal corticosterone excess, has shown no significant difference in either body or testes weights. There was an increase in germinal epithelial thickness, total basement membrane area and theoretical germ cell count. However, the actual germ cell count was significantly lower and the percentage of germ cell loss was very high (10% in control v/s 31% in MET). In general, serum LH level was high and testosterone (T) level was significantly low. The serum corticosterone and T_3 and T_4 levels were also significantly lower though the TSH levels were high. The early onset of spermatogenesis seen in the present study has been correlated with the increased serum corticosterone level in the prepubertal period. The observation of empty spaces within the germinal epithelium as well as the calculated higher germ cell loss are confirmatory of the earlier inferred protective role of corticosterone in preventing apoptosis by genetic

programming of the Sertoli cell secretory function. The sensitivity of the postpubertal Leydig cells is significantly reduced and so is the case with reference to sensitivity of thyroid to TSH. The findings suggest alterations in the hypothalamo-hypophyseal-thyroid (HHT) and hypothalamo-hypophyseal-gonad (HHG) axes in the form of increased sensitivity of the pituitary for releasing factors and paradoxically lower sensitivity of the target glands.