Neonatal thyroid hormone deficiency programs for reduced body and ovarian weights and induces plasticity changes in adult ovarian functions

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ABSTRACT

The present investigation evaluates the long-term effects of programming by neonatal hypothyroidism (HPOT) on adult ovarian functions, ovarian histoarchitechture and, serum hormone profiles in the Charles foster strain of rats. Hypothyroidism was induced in neonates through lactating mothers by feeding 0.1% 6-propyl 2-thiouracil in drinking water from day 0 to day 21. The animals were screened at 22, 45 and 90 days of age in terms of body and ovary weights, ovarian histometry and serum titres of T3, T4, estradiol and progesterone. There was significant decrease in adult body and ovary weights in HPOT animals and significant decrease in ovarian volume and number of follicles. The number of antral follicles showed a significant decrement and the number of atretic follicles were high. From these findings, it can be concluded that neonatal HPOT programming has a long-term deleterious effect on adult ovarian functions in terms of growth, maturation and folliculogenesis and, alters serum titres of estradiol and progesterone as developmental plasticity changes.

Keywords: Hypothyroid, rats, ovary, follicular dynamics.
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INTRODUCTION

Thyroid hormones are required for normal body functions in mammals to control a variety of physiological processes. Hypothyroidism is a common disorder associated with a range of reproductive abnormalities including menstrual disorders, amenorrhea, infertility and frequent abortions in many female animals, including humans [1-6]. Hypothyroidism affects normal follicular maturation in the ovary of adult rats [7-11] and gonadotropin secretion [7], resulting in irregular estrous cycles [12, 13]. Experimentally induced inadequate thyroid hormone supply reportedly disturbs folliculogenesis [14]. Thyroidectomy before puberty increases the number of follicles [15, 16] and decreases pre-ovulatory luteinizing hormone (LH) surge [16]. Importance of thyroid hormones in embryonic or foetal development of vertebrates stands well established [17]. Hypothyroidism in rats results in fewer pregnancies, delays parturition and reduces litter

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size [18, 19]. Dijkstra *et al.* [14] in their study on pre-pubertal hypothyroidism induced by PTU feeding from birth to day 40 post-partum, recorded decreased body and ovarian weights. They noted more secondary and less number of antral follicles, smaller non-atretic antral follicles and more atretic follicles. Even postnatal mice subjected to hypothyroidism show reduced number of Graafian follicles and multi laminar follicles in the immediate post-treatment periods [6]. There are also many other studies on the effect of PTU treatment during postnatal period, which have recorded retarded growth and physical development, delayed eye opening and teeth eruption [20-25]. However, there is no information on long-term plasticity consequences of neonatal programming by hypothyroidism in terms of adult ovarian functions and hormonal profile. It is in this context the programming effect of neonatal hypothyroidism on adult ovarian functions and hormonal profile has been undertaken.

MATERIALS AND METHODS

Animals and Maintenance
Healthy female laboratory rat neonates (*Charles foster* strain) were used for the present study. The animals were maintained at the Sarabhai Research Center, with a constant temperature range of 21 ±2°C and under a lighting regimen of LD 8:16 throughout the experimental period of study. The animals were fed with standard diet (Amrut Rat Feed) and water *ad libitum*. The treatment was initiated on day '0' (day of birth) a nd terminated on day 21 postpartum. The studies were conducted as per the CPCSEA guidelines and clearance by the ethical committee (Approval No. 827/ac/04/CPCSEA).

Experimental Protocol
The experimental setup consisted of two groups.

Group I Control (C):
Female rat neonates maintained in the laboratory till day 90 served as controls. This consisted of 2 subgroups (as follows) of 10 animals each:
(i) Control rats (Maintained as such)
(ii) Injected *i.p.* with vehicle (0.9% saline) in the evening at 16.30 hrs.

Group II Hypothyroid (HPOT):
Female rat neonates consisting of 10 animals were subjected to transient hypothyroidism (HPOT) by feeding lactating mothers with 0.1% 6-propyl 2-thiouracil (PTU) in drinking water from day 0 to day 21 post-partum.

Parameters and Methods of Evaluation
The treatment was discontinued from day 22 and the female animals were sacrificed at 22, 45 and 90 days of age, and various morphometric, gravimetric and histocytometric studies were carried out. The animals were sacrificed under mild anesthesia 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 s amples were utilized for assay of various hormones. The viscera was cut open and both testes and ovary 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. The ovaries were fixed in Bouin's fluid and processed for paraffin wax histology.

Histology and Histometry

Ovaries were fixed immediately in Bouin's fluid and processed for histological studies. Paraffin sections of 3µm thickness were cut on a microtome and stained with Haematoxylin-Eosin (HE). For morphometry and enumeration of ovarian follicles, homologous cross sections of entire ovary showing better area of vision were chosen. The section area was calculated by integrating the area inside the traced perimeter and volume was calculated by multiplying by the section thickness. The section volume was multiplied by 10 (to account for the number of sections skipped) to give the "10—section" volume; sum of al l 10-section volumes gave an estimate of the total ovarian volume (in mm^3) [26-27]. A total count of different types of follicles was made.

Hormone Assays

The blood for hormone assays was collected from the brachial vein under mild anesthesia before sacrificing the animals. T_3 and T_4 were assayed by ELISA using kit purchased from Glaxo (product code H -T_3H-0010 and H-T_4H-0010) and expressed in ng/ml of serum.

Statistical analysis

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

RESULTS

Body and Ovary weight

The body weight of hypothyroid rats was significantly lower at all ages from 22 days to 90 days. The final weight at 90 days was 36% lesser than the controls (144.5 ±2.55 gm v/s 226.16±9.57 gm) (Table 1). The absolute and relative weights of paired ovaries were also significantly less in hypothyroid rats. The absolute weight was almost 50% lesser than the controls.

Table1 Chronological alteration showing body weight, absolute and relative ovary weight of control and hypothyroid female rats

| Treatment	Body weight			Absolute Ovary weight			Relative Ovary weight		
	Age in Days			Age in Days			Age in Days		
	22	45	90	22	45	90	22	45	90
C	51.33 ±1.873	136.667 ±4.176	226.167 ±9.575	0.024 ±0.0003	0.062 ±0.0069	0.076 ±0.0074	0.046 ±0.0006	0.045 ±0.0015	0.034 ±0.0025
HPOT	27.00 ±0.447 [c]	70.667 ±2.512 [c]	144.5 ±2.552 [c]	0.014 ±0.0004 [c]	0.017 ±0.0016 [c]	0.039 ±0.0066 [b]	0.051 ±0.0016 [c]	0.025 ±0.0021 [c]	0.027 ±0.0044 [b]

C – Control; HPOT – Hypothyroid
Values expressed as Mean ± SEM of ten animals; [a] p<0.01; [b] p<0.005, [c]p<0.0005

Table 2 Differential total follicular count in ovary of control and hypothyroid female rats

Treatment	Age in Days	Follicle Type						Ovarian Volume (mm^3)
		Primordial	Primary	Secondary	Antral	CL	Atretic	
C	22	821±48	531±25	350±20	181±04	-	10±0.8	0.58
	45	426±17	162±11	245±10	142±08	-	29±02	0.68
	90	300±13	168±08	96±04	72±05	48±05	36±03	1.31
HPOT	22	523 ±39 [c]	354 ±31 [c]	296±21	38 ±07 [c]	-	247 ±23 [c]	0.23 [c]
	45	339±31	146±13	192±19	56 ±05 [c]	-	321 ±16 [c]	0.36 [c]
	90	251±22	83 ±06 [c]	72±10	18 ±02 [c]	33±03	336 ±27 [c]	0.85 [c]

C – Control; HPOT – Hypothyroid
Values expressed as Mean ± SEM of ten animals; [a] p<0.01; [b] p<0.005, [c]p<0.0005

Figure 1 Photomicrograph of sections of ovary of control and hypothyroids rats.
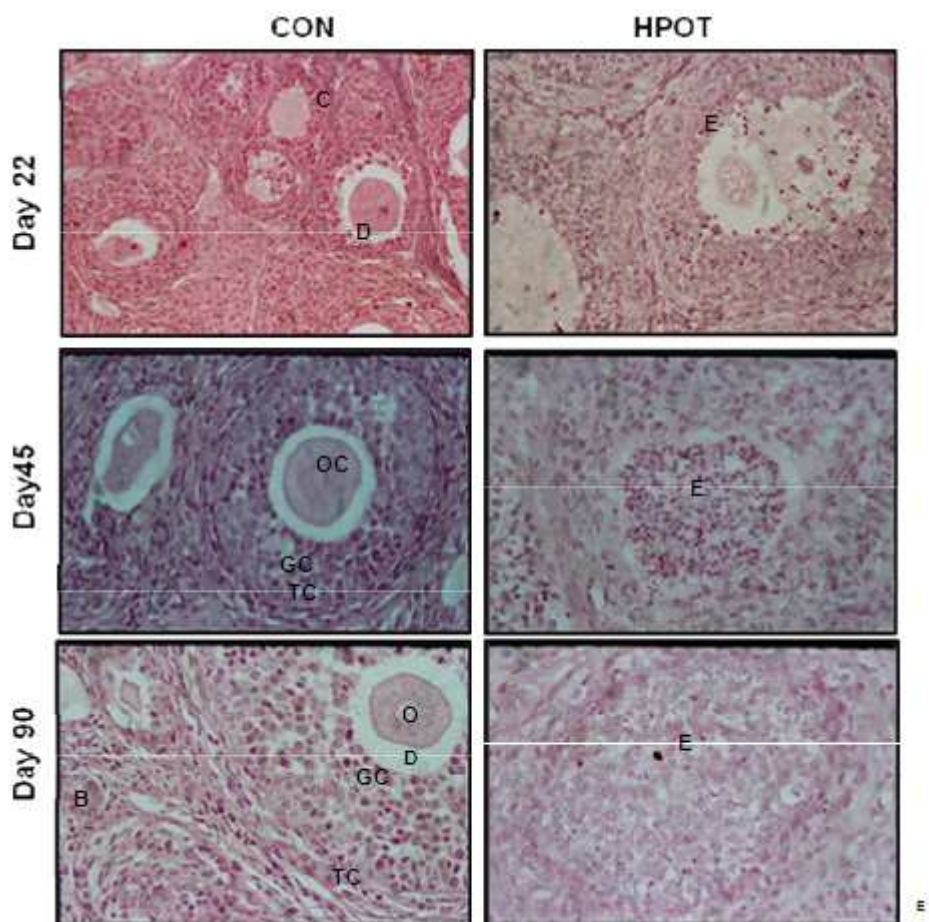
Sections of ovaries of 22, 45 and 90 days old control rats showing less number of atretic follicles compared to age-matched hypothyroid rats that shows more number of atretic follicels and poorly developed follicles.

C- Secondary follicle, D- Antral follicle, E- Atretic follicle, GC- Granulosa cells, TC- Thecal cells, O-Oocyte.

Ovarian Histology and Histometry
The ovary of HPOT rats showed significantly lower volume at all ages with reduced number of follicles of all developmental grades. Though follicles of all developmental stages showed significant reduction in HPOT rats, the most remarkable changes were with reference to antral follicles, which were very low and atretic follicles, which were very high (Table 2, Figure 1).
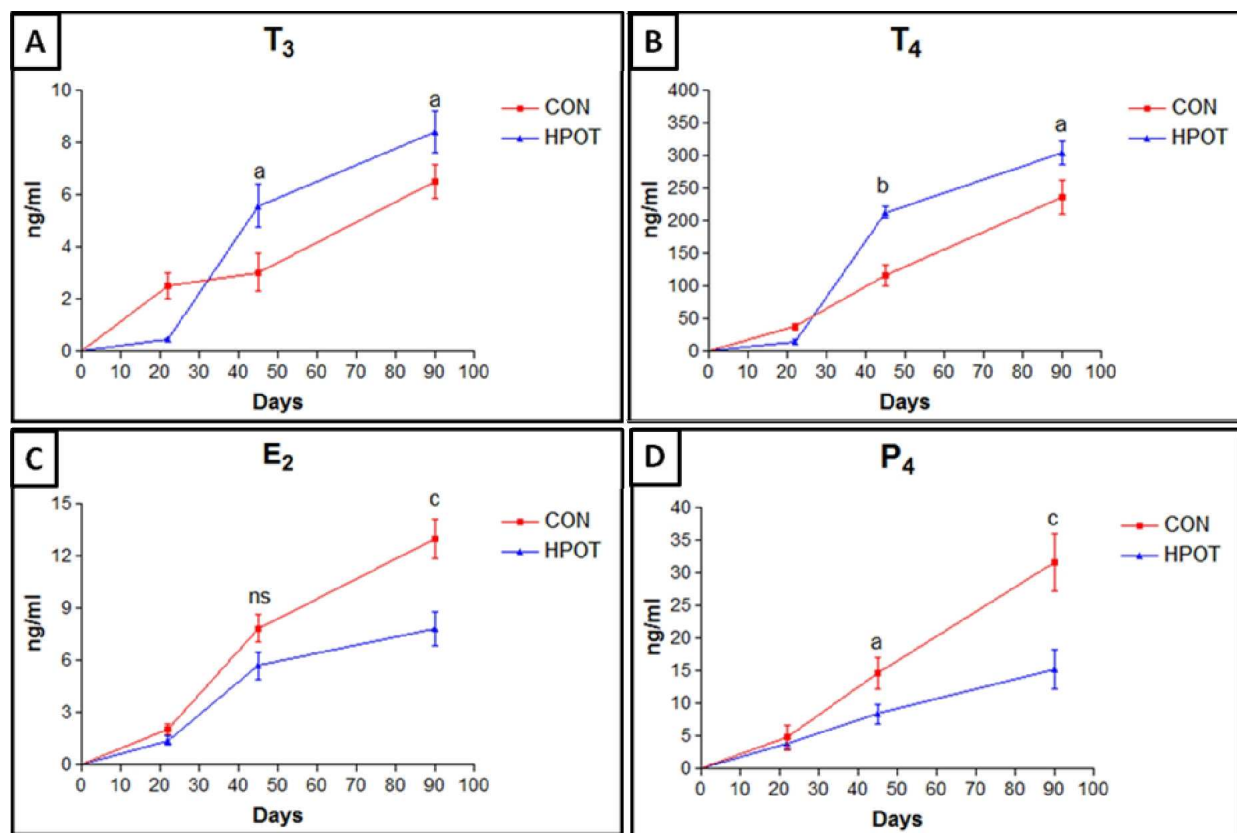
Serum Hormone profiles
The circulating titres of both T_3 and T_4 were significantly low on 22nd day, a day after cessation of PTU treatment. However, by7 45th day, both T_3 and T_4 registered significant increase and the adult levels at 90 days were significantly below control levels (Fig.2A and 2B). On the other

hand, estrogen and progesterone levels showed significant reduction at all ages post-treatment with the difference becoming more pronounced from weaning to puberty to adulthood. In order to avoid the contradiction due to differential levels of oestrogen and progesterone during the oestrus cycle, the serum levels assayed were of late diestrous of the last cycle before sacrifice,

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representing an average of 3-6 animals (Fig. 2C and 2D).

Figure 2 Serum hormone profile in control and hypothyroid rats.

DISCUSSION

The results of the present study on PTU induced hypothyroidism from PND 0 to 21 days reveal compromised body weight gain, ovary growth and, ovarian function marked by follicular dynamics and serum hormone profiles. Though there are many reports on prenatal or postnatal hypothyroidism induced reproductive disturbances, the present study is a singular one, which shows a clear long-term negative influence of hypothyroidism on folliculogenesis, body weight gain and adult hormonal profiles. The presently recorded poor growth rate and significantly low adult body weight are well corroborated by a previous observation of reduced body and ovarian weight in rats rendered hypothyroidic from day 0 to day 25 [28]. A clear strain difference in the degree of hypothyroidism induced decrement in body and ovarian weights is evident as, in the present study with Charles foster strain of rats, a decrement in body weight by 36% and in ovarian weight by 49% has been recorded as against 19.8% and 26.1% respectively with Sprague-Dawley strain of rats by Zertashia *et al* [28].

This strain difference in the degree of response is also manifest in follicular dynamics as, Zertashia *et al.* [28] did not observe any difference in the mean number of various types of follicles while, the present study clearly shows a reduction in the number of various non-atretic follicles though, the total number of follicles was not very different compared to those of control animals. This is explainable by the variation in the percentage of atretic follicles. Whereas the ovaries of control animals showed only 5% atretic follicles, those of HPOT animals show as

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much as 42.3% at 90 days. It is inferable that there is no difference in folliculogenesis on a quantitative basis; there is nevertheless a decrease in the number of developing follicles essentially due to significantly higher degree of atresia. Apparently, neonatal hypothyroidism programs adult ovarian functions as part of developmental plasticity by down-regulating follicular survival and up-regulating follicular apoptosis. Our previous study in male rats has shown a contrary favorable programming effect of neonatal hypothyroidism that contributed to increased testis size and sperm production due to an increase in sertoli cell population [29]. Apparently the adult plasticity changes induced by thyroid hormone deficiency in the critical neonatal period in terms of gonadal functions and establishment of central set points of neuroendocrine axes are differential in males and females.

This relationship between hypothyroidism and follicular apoptosis is strengthened by the recent report of Singh *et al.* [30] of hypothyroidism during development down regulating the anti-apoptotic genes and maintaining a high level of the pro-apoptotic gene Bax in the cerebellum. Jiang *et al.* [31] documented hampered folliculogenesis due to hypothyroidism and, improvement in folliculogenesis and estradiol secretion by thyroxine administration [31]. Prepubertal hypothyroidism has also been shown to hamper differentiation of granulosa cells with consequent generation of antral follicles of smaller diameter [14]. Arclor, a polychlorinated byphenyl, and ammonium perchlorate have been shown to reduce thyroid hormone levels when neonates are exposed to it. Moreover, the effects seen with Arclor and ammonium perchlorate treatments with regard to ovarian functions are similar to the present observations suggesting their effects to be essentially a consequence of induced thyroid hormone deficiency, as simultaneous T_4 replacement effectively prevented these ovarian dysfunctional changes [32, 33]. The present study also reveals decreased estrogen and progesterone production as a long-term effect at 90 days due to neonatal hypothyroidism. Since conversion of progesterone to estrogen by the follicles requires the expression of aromatase activity under the influence of FSH, the possibility of neonatal hypothyroidism permanently down-regulating either FSH action or aromatase activity need to be evaluated. Some supportive evidence can be drawn towards this by the reported expression of thyroid hormone receptor mRNA in oocytes, granulosa and cumulus cells [4,34] and the reported ability of thyroid hormone to affect oocyte maturation, aromatase activity, estradiol secretion and functional differentiation of granulosa [14, 35-37]. Interestingly, Kobayashi et al. [38] have shown the need for the expression of FSHR for survival and transition of pre-antral follicles to antral follicles. They have further shown the up regulation of FSHR mRNA in pre-antral follicle cells to be mediated through the action of oocyte growth and differentiation factor 9 (GDF-9), whose expression is in turn induced by a synergistic action between T_3 and FSH. Another study by Tohei, 2004 [39] has shown dysfunction in gonadal axis at the hypothalamic-pituitary level in male rat and inhibition of follicular development accompanied by decreased estradiol secretion and increased progesterone in female rats by hypothyroidism. Since this increase in plasma progesterone concentration due to hypothyroidism in adult female rats has been attributed to a hyposecretion of prolactin [40], the possibility of no such effect on prolactin and consequent increase in progesterone can be considered to be occurring due to neonatal hypothyroidism. The herein observed decrease in estrogen and progesterone levels and reduced number of antral follicles together with higher number of atretic follicles in the ovary of rats neonatally programmed by hypothyroidism bespeak of augmented apoptosis of follicles. Another candidate for the developmental programming effect of hypothyroidism on follicular atresia could be up regulation of FOXO3, a member of the FOXO subfamily of forkhead transcription factors that mediates follicular atresia by promoting follicular cell apoptosis as recorded in porcine ovarian follicles [41].

From the present results it can be concluded that neonatal programming by hypothyroidism has serious effects on adult ovarian functions involving folliculogenesis, atresia and ovarian hormone output. These effects may be centrally mediated as well as by altered local regulatory mechanisms and, such plasticity changes are well supported by the understanding that the first 2 to 3 weeks of neonatal development in rats are critical for not only the development of central nervous system but also in the establishment of neuroendocrine set points.

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