Observations on the Untreated Progeny of Hypothyroid Male Rats

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The untreated progeny (F_1) of hypothyroid male rats that were either radiothyroidectomized (Tx) or had the neo-T_4 syndrome (an endocrine disorder produced by large doses of thyroxine (T_4) injected during the neonatal period) were studied. The mother rats were all normal. The fathers never had any contact with their progeny.

Unexpectedly, the progeny usually showed delayed eye opening, decreased weaning weights, and increased final body weight. The thyroid glands from F_1 offspring of both Tx and neo-T_4 fathers were enlarged significantly in all but F_1 males of Tx fathers. The F_1 of Tx fathers had significantly smaller uteri, both absolutely and relatively. The ovaries were significantly larger, whereas the testes were significantly smaller. Pituitary TSH, stalk-median eminence (SME) TSH, and serum TSH were all normal with the exception of an increase in SME TSH in F_1 males born of neo-T_4 fathers. The response to thyrotropin releasing hormone (TRH) stimulation of the F_1 adult progeny of neo-T_4 fathers was significantly blunted in the males, whereas the response was normal in the offspring of Tx fathers. The mechanisms by which hypothyroid fathers caused changes in their progeny is not known.

THE INJECTION of large doses of thyroxine (T_4) into rats during the first 5 days of life has been shown to produce a variety of endocrine alterations that persist throughout adult life.[1] The treated animals exhibited an acceleration of eye opening and a delay in puberty[2] and became mildly hypothyroid. Their pituitary and thyroid growth was impaired, sometimes disproportionately greater than the impairment in body growth.[1] Protein bound iodine and free T_4 plasma concentrations were reduced,[2] the pituitary thyrotropin (TSH) content was diminished and the stalk-median eminence (SME) TSH content was usually elevated.[3] These rats also had a subnormal response to thyrotropin-releasing hormone (TRH) stimulation and upon assay of their hypothalamic tissue were shown to have an increased content of TRH.[4] Their serum TRH was significantly reduced.[5] We have called this group of abnormalities the "neo-T_4 syndrome." It was demonstrated that the implantation or injection of T_4 in systemically ineffective doses into the arcuate area of the hypothalamus of neonatal rats produced the neo-T_4 syndrome.[6] Implants in nearby areas were ineffective. We also demonstrated[5] that neo-T_4 animals have an abnormal sensitivity to T_4 feed-back similar to that observed after bilateral lesions in the "thyrotropic area" of the hypothalamus. From these changes we concluded that T_4 given during a critical neonatal period acted directly on certain develop-

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ing hypothalamic centers regulating pituitary function, resulting in mild hypothyroidism.

The fertility of female rats with the neo-T_4 syndrome was studied by mating them with normal males. Because of an interest in the normal neonatal pituitary and hypothalamic TSH content, the 304 pups of controls and 264 pups of neo-T_4 mothers were killed at five days of age and the tissues pooled for assay. Unexpectedly, it was found that the untreated offspring of neo-T_4 mothers had a significantly depressed pituitary TSH content and a significantly elevated hypothalamic TSH content.[7] Following these findings, experiments were repeated allowing the progeny to mature. When endocrine abnormalities were found in these offspring,[8,9] two experiments were carried out mating neo-T_4 males with normal females. The progeny of these matings were also found to have abnormalities as compared with controls. Since these neo-T_4 males are only mildly hypothyroid, two additional experiments were performed using severely hypothyroid (thyroidectomized) males. Their progeny also showed abnormalities. These unexpected findings are the substance of this report.

MATERIALS AND METHODS

Timed pregnant Sprague-Dawley rats were obtained by limiting sexual exposure to a selected 24-hr period. Rats born in a given 24-hr period were sorted to make up litters of equal size (usually ten) and of one sex. Neo-T_4 fathers were treated as neonates by subcutaneous injection of a total of 135–150 ug of l-T_4 in saline during the first 7–10 days of life. Control males were injected on the same schedule with diluent alone. At maturity, control and neo-T_4 males were bred with normal females for 2 wk and then the males were removed. They never had further contact with the pregnant dams or with the offspring.

Radiothyroidectomized (Tx) fathers were prepared by putting 55 day-old Sprague-Dawley males on a low iodine diet (ICN Pharmaceuticals) for 3 days prior to a 500 μCi dose of ^{131}I, s.c., for radiothyroidectomy. Two months later, when all radioactivity was gone, blood samples were taken from seven animals selected at random. Their serum TSH levels were approximately 10 times normal, confirming their severe hypothyroidism. Two weeks later control and Tx males were bred with normal females.

When pups (F_1) were born, they were sorted to make up litters of ten of one sex. The nursing pups were examined daily for eye opening, which was designated as the day both eyes were open. At 21 days of age, all rats were weaned and housed two to a cage. They were maintained as were the parents, on Purina Laboratory Chow and tap water ad lib. and kept in quiet quarters with a controlled temperature (24 ± 0.5°C) and diurnal lighting with 14 hr of light.

At maturity, groups of both males and females were anesthetized with sodium pentobarbital and injected via the femoral vein with 100 ng synthetic TRH kindly provided by Abbott Laboratories. Blood samples were obtained 15 min later for TSH assay.

When killed, the rats were lightly anesthetized with sodium pentobarbital intraperitoneally and decapitated as soon as they were drowsy. All rats were killed between 1 p.m. and 4 p.m. to avoid possible circadian variations in TSH secretion.[10] The sera or plasma samples were frozen individually for TSH radioimmunoassay. The thyroids, ovaries, uteri, testes, ventral prostates, and pituitaries were removed and individually weighed on the appropriate size Roller-Smith torsion balances. Stalk-median eminence fragments were homogenized in Krebs-Ringer phosphate buffer. All organ weights were recorded as wet weights and presented as means with standard errors. Relative organ weights were also calculated and expressed per 100 g body weight.

Pituitary glands were homogenized individually in Kontes glass homogenizers at 4°C in isotonic Krebs-Ringer phosphate buffer at pH 7.4, one pituitary per 3 ml, and further diluted in 1% bovine serum albumin buffer and frozen for TSH assay. All assays used the radioimmunoassay reagents supplied by the NIH Rat Pituitary Hormone Distribution Program. The TSH standard was stated to contain 0.22 USP (Bovine) U/mg. All results are expressed as means ± S.E. Student's t test was used to determine statistical significance.

Table 1. F_1 Offspring of Treated Fathers

Experiment	Father	Sex	N	Eye Opening Age (d)	Weaning Weight (g)
I	Co	M	40	15.2 ± 0.1	49.1 ± 1.1
	neo-T_4	M	40	15.7 ± 0.1†	40.7 ± 0.8†
	Co	F	40	15.3 ± 0.2	42.4 ± 0.7
	neo-T_4	F	40	15.8 ± 0.1†	42.9 ± 0.7
II	Co	M	25	15.0 ± 0.1	46.4 ± 0.8
	neo-T_4	M	31	15.2 ± 0.2	46.1 ± 0.6
	Co	F	25	14.6 ± 0.2	43.1 ± 0.8
	neo-T_4	F	29	14.9 ± 0.2	43.5 ± 0.8
III	Co	M	19	15.5 ± 0.2	44.2 ± 0.9
	Tx	M	20	16.1 ± 0.2*	44.7 ± 0.9
	Co	F	20	15.1 ± 0.2	44.6 ± 0.6
	Tx	F	20	15.3 ± 0.2	39.1 ± 0.5†
IV	Co	M	25	15.0 ± 0.2	42.4 ± 0.9
	Tx	M	25	16.0 ± 0.1†	40.1 ± 0.7*
	Co	F	25	14.6 ± 0.2	42.5 ± 1.0
	Tx	F	25	15.5 ± 0.1†	37.6 ± 0.6†

*$p < 0.05$.
†$p < 0.01$.

RESULTS

Table 1 shows eye opening and weaning weights in all four experiments in which neo-T_4 or Tx fathers were mated with normal mothers. Eye opening was significantly delayed in five of the eight comparisons and weaning weight was significantly reduced in four of the eight comparisons.

Table 2 shows the second experiment in which the untreated offspring (F_1) of neo-T_4 fathers and normal mothers were allowed to mature. The ages at vaginal opening and first estrus were normal. Final body weight was significantly

Table 2. The Untreated F_1 of Controls Versus Untreated F_1 of Neo-T_4 Fathers and Normal Mothers*

	F_1 Males		F_1 Females	
	Control	Neo-T_4	Control	Neo-T_4
Final number	8	10	7	10
Final Weight (g)	468 ± 8	479 ± 12	248 ± 7	266 ± 4†
Vaginal opening age (days)			35.3 ± 0.9	35.9 ± 0.6
First estrus age (days)			35.7 ± 0.9	36.0 ± 0.5
Pituitary Weight (mg)	11.38 ± 0.36	12.80 ± 0.31	15.15 ± 0.59	13.61 ± 0.47^{RR}
Thyroid Weight (mg)	15.2 ± 0.4	19.9 ± 1.1‡^{R}	13.0 ± 0.4	16.1 ± 0.9†^{R}
Gonad Weight (g, mg)	3.69 ± 0.08	3.89 ± 0.08	81.8 ± 4.4	89.5 ± 4.4
V prostate/uterus (mg)	572 ± 36	478 ± 42	538 ± 29	593 ± 31
Pituitary TSH (mU/gland)	246 ± 27	312 ± 31	223 ± 48	244 ± 26
SME TSH (μU/gland)	166 ± 16	270 ± 30‡	157 ± 11	223 ± 29
Serum TSH (μU/ml)	50.1 ± 10.4	82.0 ± 15.2	41.7 ± 6.8	50.8 ± 5.1

*These rats, from experiment II, were killed when 134 days of age.
Mean ± SE. †indicates significance of $p < 0.05$ and ‡$p < 0.01$.
^{R} and ^{RR} indicate significance of relative weight differences (per 100 g body weight).

Table 3. TRH Stimulation of the Untreated Offspring (F_1) of Neo-T_4 Fathers

		F_1 Control		F_1 Neo-T_4	
		Uninjected	TRH	Uninjected	TRH
Number		7–8	6–7	10	9–10
Serum TSH (μU/ml)	Male	50.1 ± 10.4	1111 ± 103	82.0 ± 15.2	721 ± 76.3†
	Female	41.7 ± 6.80	587 ± 113	50.8 ± 5.10	593 ± 53.0
Pituitary TSH (mU/gland)	Male	246 ± 27.4	323 ± 36.9	312 ± 30.8	258 ± 18.1
	Female	223 ± 47.9	270 ± 23.7	244 ± 25.5	223 ± 23.2
SME TSH (μU/gland)	Male	166 ± 15.8	191 ± 48.6	270 ± 29.8	125 ± 21.4†
	Female	157 ± 10.6	209 ± 26.4	223 ± 28.8	161 ± 26.1

The rats from experiment II were 134 days old.
Same experiment as in Table 2. The TRH, 100 ng/100 g body weight, was injected i.v. 15 min before kill.
†as in Table 1. All comparisons are between F_1, neo-T_4, and controls.

greater in females but not in males. Relative pituitary weights were normal in males, but significantly reduced in females. Thyroid weights were significantly greater in males and females both absolutely and relatively. Gonads, uterine and ventral prostate weights were not significantly affected. Pituitary TSH and serum TSH were not significantly affected, but in male offspring the SME TSH was significantly increased.

Table 3 shows the results of TRH stimulation of the untreated adult offspring of neo-T_4 fathers. The response in males was significantly blunted and the SME TSH content was significantly reduced. Females did not show these changes.

Table 4 shows the effects of radiothyroidectomy on the fathers of the progeny presented in Table 5. Body weight was significantly reduced. Pituitary, adrenal, and ventral prostate weights were significantly less than in controls both absolutely and relative to body size. Relative testes weights were significantly increased. Pituitary and SME TSH contents were significantly reduced while serum TSH was elevated to more than ten times the normal level in these hypothyroid rats. TRH stimulation showed the typical hyper-response.

Table 5 shows the untreated offspring of these Tx fathers bred with normal mothers, as compared with controls. The fertility, litter size, and birth mortality

Table 4. The Thyroidectomized Fathers

	Control	T x
N	10	10
Body weight (g)	468 ± 18	281 ± 10‡
Pituitary weight (mg)	13.05 ± 0.60	11.22 ± 0.58†^{RR}
Thyroid weight (mg)	17.5 ± 0.8	absent
Adrenal weight (mg)	53.7 ± 2.7	27.6 ± 1.1‡^{R}
Testes weight (g)	3.22 ± 0.27	3.52 ± 0.08^{RR}
V. prostate weight (mg)	690 ± 48	533 ± 40†^{R}
Pituitary TSH (mU/gland)	257 ± 18	156 ± 29†
SME TSH (μU/gland)	473 ± 118	160 ± 19†
Serum TSH (μU/ml)	96.7 ± 19.7	1058 ± 168‡
15′p TRH (μU/ml)	442 ± 71	13,376 ± 1,796‡

These rats were killed at 155 days of age. Assays were done on groups of five. 50 ng TRH/100g body weight injected into femoral vein.
† and ^{R} as in Table 2.

Table 5. The Untreated F_1 of Controls Versus Untreated F_1 of Tx Fathers and Normal Mothers*

	F_1 Males		F_1 Females	
	Control	Tx Father	Control	Tx Father
Final number	14	15	15	15
Final weight (g)	431 ± 8	471 ± 8‡	261 ± 7	265 ± 4
Pituitary weight (mg)	11.95 ± 0.27	12.84 ± 0.30†	14.68 ± 0.54	15.41 ± 0.52
Thyroid weight (mg)	16.7 ± 0.6	18.0 ± 0.6	13.1 ± 0.5	15.2 ± 0.4‡^{RR}
Gonad weight (g, mg)	3.90 ± 0.10	3.82 ± 0.11^{R}	80.0 ± 2.6	88.5 ± 2.7†
V. prostate/uterus weight (mg)	485 ± 25	536 ± 26	540 ± 31	458 ± 14‡^{RR}
Pituitary TSH (mU/gland)	255 ± 19	281 ± 26	259 ± 21	267 ± 13
SME TSH (μU/gland)	143 ± 8	132 ± 13	103 ± 16	110 ± 12
Serum TSH (μU/ml)	40.4 ± 6.2	35.3 ± 5.2	41.5 ± 7.6	29.5 ± 6.9
TRH Response:§				
Pituitary TSH (mU/gland)	313 ± 31	259 ± 14	232 ± 13	181 ± 22
SME TSH (μU/gland)	143 ± 37	110 ± 25	96 ± 59	113 ± 13
Serum TSH (μU/ml)	770 ± 102	599 ± 41	461 ± 29	489 ± 60

*These rats from Exp III were killed at 124 days of age.
† and ^{R} as in Table 2.
§Fifteen minutes after injection of 100 ng TRH/100 g body weight via femoral vein (n = 5).

of these matings were normal. Final body and pituitary weights were significantly greater in the male offspring of Tx fathers. Thyroid weights in females were greater, both absolutely and relatively. Relative testicular weight was reduced and ovarian weight was significantly increased. Uterine weight was significantly reduced both absolutely and relatively. There were no significant abnormalities in TSH measurements nor in the repsonse to TRH in the small groups tested.

DISCUSSION

That the untreated progeny of hypothyroid fathers had any abnormalities at all was unexpected. From the data presented here it is not possible to formulate an integrated analysis of the significance of these changes. When comparing the progeny of neo-T_4 fathers with Tx fathers, it should be borne in mind that although both are hypothyroid, the two conditions have many differences. The neo-T_4 fathers are only mildly hypothyroid and have other endocrine abnormalities in addition to pituitary-thyroidal defects, while the defect in the Tx fathers is simply one of severe hypothyroidism (and possibly hypoparathyroidism, although no signs of this were manifest). In spite of these differences, their progeny did show some similarities in their abnormalities. The progeny of neo-T_4 fathers in experiment I showed a significant delay in eye opening and the weaning weight was significantly diminished in the males. It should be noted that in the two largest experiments (I and IV) the delay in eye opening was highly significant ($p < 0.01$) in all groups. Also, it may be noted that even when differences were not significant, the means were never deviant from the significant trends. Why the pups in experiment II failed to show changes is not known but we have noted a variability in the completeness or severity of the neo-T_4 syndrome from time to time, although environmental conditions throughout all experiments remained the same. Both serum TSH and SME TSH response to TRH stimulation were significantly blunted in the male

progeny of neo-T_4 fathers (Table 3). The serum TSH after TRH stimulation of the progeny of Tx fathers was 599 ± 41 μU/ml as compared with 770 ± 102 μU/ml in the controls. This difference was not statistically significant, possibly because of the small number in the group ($N = 5$).

The abnormalities observed in the progeny in these studies are unlike those present in the fathers (Table 4) and also they present a different pattern than seen in the progeny of neo-T_4 and Tx mothers.[8,9,11] However, the F_1 of Tx mothers and Tx fathers both had increased thyroid weights and decreased testicular weights.

In spite of extensive literature on the teratologic consequences of maternal metabolic abnormalities, there is very little reported on the progeny of abnormal fathers. Spergel, Levy, and Goldner[12] and Goldner and Spergel[13] reported that the untreated progeny of rats treated with subdiabetogenic doses of alloxan developed a persistant state of carbohydrate intolerance. The untreated progeny had latent diabetes even when only the fathers had received alloxan. They point out that because the male parent was capable of transmitting the defect this ruled out the possibility that the conceptus was affected by an abnormal intrauterine environment as might be the case when the mothers were treated with alloxan. Reluctant to suggest that the alloxan treatment had been mutagenic, the authors suggest the possibility of paramutation[14] to explain their results. This may or may not be relevant to the claims of Jackson[15] and Kellock[16] that the birthweight of children of diabetic and prediabetic fathers are above normal. However, Malins et al.[17] failed to confirm this observation so the matter remains unsettled.

Friedler[18,19] performed experiments in which morphine was injected into young male mice twice a day for 5 days and then they were bred with normal females 5 days later. Their progeny weighed significantly less than comparable control groups. Although the author could not demonstrate any consistent abnormalities in chromosomal morphology, she suspected that the morphine might be mutagenic.

Lutwak-Mann[20] treated male rabbits of proved fertility with thalidomide and 2–10 wk later mated them to normal does. They found a significant impairment in fertility, smaller litters and increased malformations such that in 27 out of 40 matings there appeared to be deleterious effect on progeny ascribable to the paternal treatment with thalidomide. Later Lutwak-Mann et al.[21] presented evidence that thalidomide was concentrated both in the sperm and semen, but they did not demonstrate this to be the mechanism producing abnormal progeny. Jones et al.[22] have shown in man that older paternal age is associated with a significant increase in congenital abnormalities. It is known that the mammalian spermatozoon contain mitochondria estimated to number at least 72 in each bull sperm[23] and that these mitochondria enter the egg at the time of fertilization. It is not known whether or not these paternal mitochondria replicate at a rate equal to maternal mitochondria. An attempt to demonstrate this by Hutchison et al.[24] failed to detect paternal mitochondrial DNA in the progeny using a system that should have demonstrated their presence if they constituted more than five percent of the total cytoplasmic mitochondrial DNA.

Another possibility to be considered is that paternal metabolic abnormalities (such as hypothyroidism) might alter sperm metabolism in such a way that a morphologically or biochemically abnormal population of sperm might be given a preferential opportunity to succeed in fertilizing the egg, giving rise to abnormal progeny on this basis without invoking any mutagenic event or the transfer of abnormal cytoplasmic factors.

The importance of the male gamete in fertility disturbances has recently been reviewed.[25] Although most abnormalities in sperm morphology and biochemistry are thought to be genetic in origin, most have not yet been proven so, and the possibility of acquired metabolic abnormalities affecting the progeny must be considered. There is evidence that aging of human spermatozoa in the female genital tract is associated with an increased frequency of abortion and, presumably, birth defects.[26]

The four experiments presented herein constitute all of our studies on the effects of paternal hypothyroidism on their offspring. Although of sufficient size to produce statistically significant results within a single experiment, and there is some agreement between different experiments, there is still sufficient unexplained variability to leave the thesis in question. These studies are reported with the hope that others will pursue similar studies since, if our thesis is confirmed, the biologic implications are far reaching.

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Free and Total Insulin Levels in a Patient With Insulin-resistant Diabetes Mellitus and Chronic Lymphatic Leukemia: Effect of Prednisone Therapy

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An insulin-resistant diabetic patient who also has chronic lymphocytic leukemia and very high plasma levels of free and total insulin along with high levels of insulin antibodies is described. In response to prednisone therapy, his insulin requirement decreased, but the total and free insulin concentrations increased as insulin antibody measured as the maximal insulin-binding capacity of plasma remained unchanged. In insulin resistance, persistent hyperglycemia, in spite of high levels of immunoreactive free insulin, presumably reflects peripheral tissue unresponsiveness to insulin. The beneficial effect of prednisone treatment in this patient is discussed, and it is postulated to be the result of either increased availability of free insulin or an increased responsiveness of the tissues to insulin or both.

INSULIN-RESISTANT DIABETES MELLITUS has been arbitrarily defined as a state in which 200 or more U of insulin per day are required for more than 48 hr to maintain adequate control of hyperglycemia in the absence of ketoacidosis, infection, or endocrine disorders.[1] Most pancreatectomized subjects require 30–60 U of insulin each day to maintain normal levels of blood glucose.[2] All patients treated continuously with insulin develop insulin antibodies within a few weeks after the institution of insulin therapy.[3] The insulin-binding capacity of these antibodies in noninsulin resistant patients has been found to be < 10 U/liter.[3] The insulin wastage by immune mechanisms in insulin-sensitive patients treated with insulin has been estimated to be between 10 and 15 U/day.[3] The exact cause of insulin resistance is not known. While most patients with insulin resistance have been found to have significantly elevated levels of circulating antibodies to insulin,[4,5] the role of these insulin antibodies in the pathogenesis of insulin resistance has not been established conclusively. Insulin-resistant patients who did not have increased levels of circulating insulin antibodies have been reported.[6,7] Moreover, a reduction in insulin antibodies has not always been observed to parallel the decreased insulin requirements following corticosteroid therapy of insulin resistance.[8] Although corticosteroid treatment is usually effective in patients with high levels of circulating insulin antibodies,[9] the exact mechanism of its beneficial effect is not known.

The present study offers some insight into whether or not the beneficial effect of corticosteroids in insulin resistance is a reflection of an increase in the avail-

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