

# Characteristics of infertility in female hypothyroid (*hyt*) mice

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Female hypothyroid (*hyt*) mice are infertile, but the reason for this infertility is not yet known. The present study was conducted to determine whether hypothyroidism induced infertility in immature and mature *hyt* mice. Furthermore, animals were treated with thyroxine and gonadotrophins at different times to determine whether infertility was due to failure of follicular development, implantation or pregnancy. There were no significant differences in the numbers of ovulated eggs induced by gonadotrophin treatment or the percentages of eggs developed *in vitro* among immature normal controls, *hyt* and thyroxine-treated *hyt* mice. Mature *hyt* mice showed continuous dioestrus, and ovulated significantly fewer eggs after

gonadotrophin treatment and failed to establish pregnancy after mating compared with mature control mice. Mature *hyt* mice had significantly fewer corpora lutea > 500 µm in diameter and significantly lower progesterone concentrations. Thyroxine treatment before mating in *hyt* mice resulted in well-developed corpora lutea, an increase in progesterone and normal pregnancy, regardless of subsequent thyroxine administration. In conclusion, infertility occurs in mature rather than immature *hyt* mice, is due to the failure of follicular development and pregnancy, and can be reversed by thyroxine treatment before mating.

## Introduction

The *hyt* mouse has an autosomal recessive, fetal onset, severe hypothyroidism that persists throughout life. The defective thyroid stimulating hormone (TSH) receptor and thyroidal agenesis make the *hyt/hyt* mouse a good model for this same condition in humans (Stein *et al.*, 1994; Biesiada *et al.*, 1996). *hyt* mice have been used to study the effects of thyroid hormones on the development and functions of many organs, including the brain (Sher *et al.*, 1998; Khan *et al.*, 1999), lung (Ansari *et al.*, 1997, 2000), kidney (Sole *et al.*, 1994, 1996) and male reproductive organs (Amador *et al.*, 1986; Kuroda, 1989). *hyt* mice of both sexes have been reported to be infertile but the infertility of males can be reversed by supplementing their feed with desiccated thyroid powder (Beamer *et al.*, 1981). In contrast, other studies have indicated that male *hyt* mice are fertile (Chubb and Nolan, 1985; Chubb and Henry, 1988). Although female *hyt* mice were described as infertile by Beamer *et al.* (1981), data to support this conclusion were not included in this study. Therefore, the aim of the present study was to investigate whether and to what extent female *hyt* mice are infertile in both immature and mature stages.

## Materials and Methods

### Preparation of animals

*hyt* mice were produced by mating adult C.RF-Tshr *hyt/+* males with females purchased from the Jackson Laboratory

(Bar Harbor, ME). In the present study, the term *hyt* mice always refers to *hyt/hyt* homozygotes, while normal littermates include both *hyt/+* and wild types (+/+) since there are no phenotypic differences between them. Animals were placed in polycarbonate cages (30 cm × 20 cm × 13 cm) with wood shavings on the floor in a room at a controlled temperature of 24 ± 2°C and humidity of 65 ± 5% with lights coming on at 7:00 h and going off at 19:00 h. Mice were given pelleted commercial food (MEQ: Oriental Yeast Co. Ltd, Chiba) and tap water *ad libitum*. *hyt* mice were distinguished according to their low body weight and retarded ear development at about 2 weeks of age (Adams *et al.*, 1989).

The present study was approved by the Ethics Committee for Care and Use of Laboratory Animals for Biomedical Research of the Graduate School of Agricultural Science, Tohoku University.

### Fertility in immature *hyt* mice

**Hormone administration.** Immature *hyt* mice and their normal littermates were divided randomly and treated as follows to examine the roles of gonadotrophins and thyroid hormones in ovulation in *hyt* mice: (i) gonadotrophins (eCG and hCG) alone in both *hyt* and normal mice; and (ii) thyroxine combined with gonadotrophins in *hyt* mice alone. *hyt* mice were administered thyroxine (L-thyroxine, Sigma Chemical Co., St Louis, MO) i.p. once a day at a dose of 10 µg per 100 g body weight from days 21 to 30 of age. Thyroxine was dissolved in 2 mol NaOH l<sup>-1</sup> and prepared in physiological saline solution (final pH 7.0). In both *hyt* and normal mice, 5 iu eCG (Sankyo Kabu

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Company, Tokyo) was injected i.p. at 18:00 h on day 28 and 5 iu hCG (Sankyo Kabu Company) was administered i.p. 48 h later.

**Egg collection and examination of fertilization.** After hCG injection, all female mice were paired with mature *hyt* males, which were treated with thyroxine from days 21 to 40 of age by injection as described in previous studies on immature *rdw* rats (Jiang *et al.*, 1999, 2000a) and were subsequently given drinking water supplemented with thyroxine (Jiang *et al.*, 2000b). The next morning, the animals were killed by cervical dislocation and eggs were collected by flushing oviducts with culture medium as described by Jiang *et al.* (1999). The culture medium was CZB (Chatot *et al.*, 1989; Wakayama *et al.*, 1998) supplemented with 5.56 mmol D-glucose l<sup>-1</sup> (Wako Pure Chemical Industries Ltd, Osaka) and 5 mg BSA ml<sup>-1</sup> (No. A-7638, Fraction V, Sigma). The eggs were counted, and only those eggs with two distinct pronuclei and a second polar body (Wakayama *et al.*, 1998) were considered to be fertilized and were included in the analysis.

**Embryo culture in vitro.** Embryo culture and examination of development were conducted as described by Wakayama *et al.* (1998) and Jiang *et al.* (1999). Briefly, after washing three to six times with culture medium, approximately ten fertilized eggs were transferred to 100 µl of culture medium and cultured in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> in air, for 5 days. Embryo development was examined every 24 h.

#### *Fertility in mature hyt mice*

**Hormone administration.** Eight-week-old *hyt* mice were divided into five groups and treated as follows to examine the characteristics of infertility and the effects of thyroxine treatment: (i) given no hormone treatment; (ii) given gonadotrophin (eCG and hCG) treatment alone; (iii) given gonadotrophin treatment with thyroxine after mating; (iv) given thyroxine before mating (from day 21 of age to the day of mating) alone; and (v) given thyroxine treatment before and after mating (from day 21 of age to the day of examination) alone. Normal littermates with gonadotrophin treatment alone were used as controls. Thyroxine was administered by i.p. injection each day from days 21 to 40 of age as described above and subsequently in drinking water supplemented with thyroxine solution at a final concentration of 3 µg ml<sup>-1</sup> as described by Jiang *et al.* (2000b). The stage of the oestrous cycle was evaluated each day by smear examination. eCG (5 iu) was injected i.p. at 18:00 h on day 58 of age and 5 iu hCG was administered i.p. 48 h later. Each female mouse in pro-oestrus or after hCG injection was paired with a male *hyt* mouse treated with thyroxine as described above.

**Egg collection and ovary sampling.** All female mice were checked for vaginal plugs the morning after pairing with males. Animals in corresponding groups were killed by

cervical dislocation and eggs were collected from the oviducts by flushing with CZB medium. Ovaries ( $n = 3$ ) in corresponding groups were collected for histological examination, performed as described by Jiang *et al.* (2000a). Briefly, ovaries were fixed in neutral buffered formalin solution, embedded in paraffin wax, sectioned at a thickness of 8 µm and stained with haematoxylin–eosin. Healthy and atretic follicles were examined according to the criteria of Hirshfield and Midgley (1978) and Braw and Tsafiriri (1980).

**Implantation and pregnancy.** Female *hyt* mice with vaginal plugs in corresponding groups were kept to examine whether normal implantation and pregnancy occurred. Animals were checked once a day by smear examination. Females that showed pro-oestrous smears, or that were pregnant but did not deliver offspring by day 24 of pregnancy, were killed and their uterine horns were examined for implantation sites.

**Blood sampling and examination of corpus luteum formation.** Four days after mating, females in corresponding groups were anaesthetized after their body weight had been measured. Blood was collected from the heart, and serum was immediately separated by centrifugation at 900 g for 15 min at 4°C and stored at -20°C until examination (Umezu *et al.*, 1998; Jiang *et al.*, 2000a,b). Ovaries were collected and examined for corpora lutea histologically using haematoxylin–eosin staining.

**Progesterone assay.** Progesterone concentrations were measured using a kit (DELFLIA-Progesterone Reagents R066-101), according to the manufacturer's instructions (Pharmacia Biotech KK, Tokyo). The limit of sensitivity of the assay was 0.25 ng ml<sup>-1</sup>. The interassay and intra-assay variations were 8.1 and 4.2%, respectively. Data are shown as means ± SEM ( $n = 5$ ).

#### *Statistical analysis*

Student's *t* test was used to compare differences in the number of corpora lutea in mature *hyt* mice given gonadotrophins alone and those given thyroxine alone. ANOVA and Duncan's multiple-range test were used for other comparisons. Differences were considered significant at  $P < 0.05$ .

## **Results**

Immature *hyt* mice given a single injection of eCG and hCG on days 28 and 30 of age, respectively, ovulated as many eggs as did normal controls on day 31 of age. Pre-treatment with thyroxine did not affect ovulation in immature *hyt* mice. Fertilization rates were not significantly different among *hyt* mice, *hyt* mice given thyroxine and normal controls (Table 1).

There were no significant differences in the development *in vitro* of eggs derived from *hyt* mice, *hyt* mice given thyroxine and normal controls (Table 2).

**Table 1.** Ovulation and fertilization in immature hypothyroid mice (*hyt*) and normal littermates (normal) given gonadotrophins alone, and in *hyt* mice given thyroxine and gonadotrophins (T4-*hyt*)

Females	Number of mice ovulating/tested	Number of eggs collected		Number (%)* of eggs fertilized
		Total	Mean ± SEM	
<i>hyt</i>	7/7	123	17.6 ± 2.9	101 (82.1)
T4- <i>hyt</i>	6/6	115	19.2 ± 1.9	101 (87.8)
Normal	22/22	377	17.1 ± 1.1	336 (89.1)

\*Percentage of the number of eggs collected.

After injection with hCG, all females were paired with mature male *hyt* mice treated with thyroxine from 21 days of age.

**Table 2.** Development *in vitro* of eggs derived from immature hypothyroid mice (*hyt*) and normal littermates (normal) given gonadotrophins alone, and in *hyt* mice given thyroxine and gonadotrophins (T4-*hyt*)

Females	Number of eggs cultured	Number (%)* of eggs developed to			
		≥ Two-cell (48) <sup>†</sup>	≥ Four-cell (72) <sup>†</sup>	≥ Morula (96) <sup>†</sup>	Blastocyst (120) <sup>†</sup>
<i>hyt</i>	75	75 (100)	66 (88.0)	57 (76.0)	37 (49.3)
T4- <i>hyt</i>	79	79 (100)	67 (84.4)	58 (73.4)	44 (55.7)
Normal	216	216 (100)	185 (85.6)	134 (62.0)	106 (49.1)

\*Percentage of the number of eggs cultured.

<sup>†</sup>Numbers in parentheses indicate time (h) after hCG injection.

After hCG injection, all females were paired with mature male *hyt* mice treated with thyroxine from 21 days of age.

**Table 3.** Ovulation in mature hypothyroid (*hyt*) mice with or without treatment with gonadotrophins

Females	Gonadotrophins (eCG and hCG)	Number of mice			Number of eggs collected per mouse
		Tested	Oestrus	Ovulation	
<i>hyt</i>	–	5	0	0	0 <sup>a</sup>
<i>hyt</i>	+	6	ND	ND	9.8 ± 1.3 <sup>b</sup>
Normal	+	5	5	5	20.8 ± 1.7 <sup>c</sup>

Oestrus: number of animals showing oestrus; ovulation: number of animals ovulating eggs.

ND: not determined.

Number of eggs collected per mouse are expressed as mean ± SEM.

<sup>a-c</sup>Values with different superscripts within the same column are significantly different ( $P < 0.001$ ).

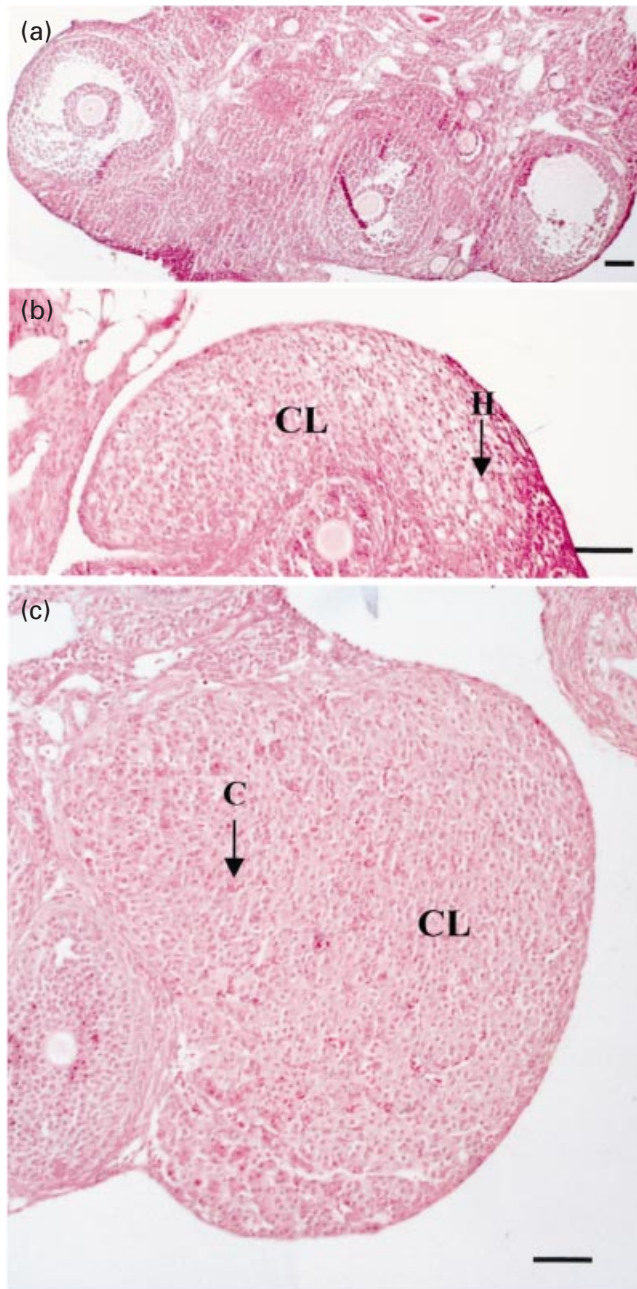
In untreated mature *hyt* mice, vaginal cytology showed continuous dioestrus and no eggs were collected (Table 3). Most follicles > 200 µm in diameter (62.3%, 43/69) were degenerative, and few healthy follicles > 400 µm in diameter (1 ± 0) and no corpora lutea were observed in the ovaries of these animals (Fig. 1a). Although treatment with gonadotrophins induced ovulation in mature *hyt* mice, the number of eggs ovulated was significantly lower than that in normal controls ( $P < 0.001$ , Table 3).

Although there were no significant differences in the number of corpora lutea < 500 µm in diameter, corpora lutea in mature *hyt* mice given gonadotrophins alone were poorly developed and possessed fewer capillaries but many small hollows (Fig. 1b). Significantly lower numbers of corpora lutea > 500 µm in diameter and no implantation sites were observed in non-treated female *hyt* mice given gonadotrophins and mated at 8 weeks of age, regardless of thyroxine treatment after mating. However, mature *hyt*

females given thyroxine before mating had well-developed corpora lutea in the ovaries (Fig. 1c), established pregnancies and delivered offspring (Table 4). Concentrations of progesterone in non-treated female *hyt* mice were significantly lower than in treated *hyt* mice and non-treated normal controls (Table 4,  $P < 0.01$ ).

## Discussion

The results of the present study indicate that ovulation can be induced by gonadotrophin treatment in immature *hyt* mice. In mature *hyt* mice, infertility occurs, characterized by a lack of an oestrous cycle, and a response to gonadotrophin treatment followed by a decrease in the number of ovulated eggs, poor corpus luteum formation, lower progesterone concentrations and no implantation. The infertility of mature *hyt* mice can be reversed by thyroxine treatment even if given before mating. These



**Fig. 1.** Ovarian section of an untreated mature hypothyroid (*hyt*) 61-day-old mouse (a) and a corpus luteum (CL) from a mature *hyt* mouse treated with gonadotrophins (eCG on day 58 and hCG on day 60) alone (b) or with thyroxine alone from day 21 (c). Each female mouse in pro-oestrus or after hCG injection was paired with a male *hyt* mouse treated with thyroxine. Ovaries were collected 4 days after mating and stained with haematoxylin and eosin. C: capillary; H: hollow that may be the site of a lipid droplet that has been removed by the alcohol-xylene treatments. Scale bars represent 100 µm.

findings indicate that infertility in *hyt* mice is different from that observed in *rdw* rats, another congenital hypothyroid model. Immature *hyt* mice responded to gonadotrophin treatment alone and ovulated as many eggs as normal controls. However, immature *rdw* rats treated with

gonadotrophins did not ovulate (Jiang *et al.*, 1999). In immature *rdw* rats, eCG treatment alone or in combination with eCG and thyroxine, partially and completely revived follicular development, respectively (Jiang *et al.*, 2000a). Indeed, a point mutation that occurs in the thyrotrophin receptor of the *hyt* mice was not observed in *rdw* rats (Stein *et al.*, 1994; M. Umezumi and J. Y. Jiang, unpublished). In contrast, a novel missense mutation in thyroglobulin that causes hypothyroidism in *rdw* rats has been reported (Hishinuma *et al.*, 2000). The different gene mutations reported in these two models may result in endocrinological differences, for example in the concentrations of thyroid hormones at different ages, which result in varied infertile characteristics. However, both *hyt* mice and *rdw* rats have reduced concentrations of thyroid hormones at both immature and mature stages. *hyt* mice have congenital hypothyroidism of fetal onset from 15 days after conception and reduced concentrations of thyroid hormones at neonatal, immature and mature stages (Beamer and Cresswell, 1982). Neonatal *hyt* mice had reduced serum thyroxine ranging from one fifth to one sixth of normal concentrations (Adams *et al.*, 1989). The thyroxine values of *hyt* mice were 0.34, 0.32 and 0.8 µg dl<sup>-1</sup> compared with those of normal littermates (> 6 µg dl<sup>-1</sup>) at 20 days, 5–6 weeks and 6 months of age, respectively (Stein *et al.*, 1989). Reduced concentrations of thyroxine were also found in immature and mature *rdw* rats (Jiang *et al.*, 1999, 2000b). Therefore, the mechanisms leading to the differences in infertility between immature *hyt* mice and *rdw* rats remain unclear and require further investigation.

When female mice are rendered hypothyroid, their ovaries show degenerative changes (Dalton *et al.*, 1945), and the oestrous cycle is altered (Morris *et al.*, 1946). Similarly, rats with hypothyroidism have longer and variable oestrous cycles (Krohn and White, 1950). Hypothyroid cows do not show the usual signs of oestrus (Longcope, 1986). In the present study, untreated mature *hyt* mice also showed no signs of oestrus and did not ovulate. Histological examination indicated that most follicles were atretic and no corpora lutea were observed. Treatment with gonadotrophins induced ovulation, but the number of ovulated eggs was lower than that in normal controls. These findings were in agreement with the results of previous studies on hypothyroid rats and hens, indicating that hypothyroidism hampered follicular development and ovulation (Longcope, 1986; Jiang *et al.*, 1999, 2000a).

Hypothyroidism has been associated with a wide range of reproductive abnormalities including frequent abortions in many female animals, including women (Maruo *et al.*, 1992; Jiang *et al.*, 2000a). In mature female rats, hypothyroidism apparently does not result in sterility but does interfere with gestation and absorption of the embryos, and subsequently results in reduced litter size (Krohn and White, 1950). Hypothyroidism present at conception interferes with pregnancy and can cause subsequent absorption of the embryos (Longcope, 1986). In adult women, when hypothyroidism is diagnosed during pregnancy, thyroxine

**Table 4.** Pregnancy in mature hypothyroid (*hyt*) mice with or without treatment with gonadotrophins and thyroxine

Females	Treatment			Number of mice pregnant/tested	Number of corpora lutea			Progesterone concentrations (ng ml <sup>-1</sup> )
	Gonadotrophins (eCG and hCG)	Thyroxine before mating*	Thyroxine after mating		Total	≤ 500 μm	> 500 μm	
<i>hyt</i>	+	-	-	0/5	6.7 ± 0.3	6.3 ± 0.3	0.3 ± 0.3 <sup>a</sup>	4.4 ± 1.7 <sup>c</sup>
<i>hyt</i>	+	-	+	0/5	ND	ND	ND	ND
<i>hyt</i>	-	+	-	5/5	ND	ND	ND	ND
<i>hyt</i>	-	+	+	5/5	9.3 ± 2.0	6.0 ± 2.7	3.3 ± 0.7 <sup>b</sup>	28.4 ± 6.3 <sup>d</sup>
Normal	-	-	-	5/5	ND	ND	ND	36.8 ± 6.6 <sup>d</sup>

\*From 21 days of age until mating.

ND: not determined.

The number of corpora lutea in ovaries ( $n = 3$ ) and concentrations of progesterone are expressed as mean ± SEM.

Values with different superscripts within the same column are significantly different (<sup>a</sup> $P < 0.05$ ; <sup>c</sup> $P < 0.01$ ).

therapy should be commenced promptly to increase the chances of a normal pregnancy. Women requiring thyroid hormone therapy before pregnancy should continue with such therapy throughout pregnancy (Longcope, 1986). The need for thyroxine increases in many women with primary hypothyroidism during pregnancy (Mandel *et al.*, 1990; Tamaki *et al.*, 1990). Hypothyroidism also interferes with the formation and function of corpora lutea, resulting in the failure of normal pregnancy, since the corpus luteum is the primary source of progesterone secretion, which is crucial for maintaining pregnancy to parturition in mammals (Anderson *et al.*, 1999). Luteal-phase defect (LPD), leading to delayed endometrial maturation, may arise as the result of various factors affecting ovarian function. It has been suggested that abnormalities in the follicular phase may be responsible for LPD (Di Zerega and Hodgen, 1981; Soules *et al.*, 1989; Maruo *et al.*, 1992). Although the corpus luteum secretes several species of proteins and sex steroids, the best-characterized manifestation of LPD is a decrease in the concentration of circulating progesterone. Clinically, women with recurrent LPD may present with either infertility or habitual first-trimester abortion (McNeely and Soules, 1988). Experimental findings *in vitro* indicate that presence of an adequate concentration of thyroid hormone is needed for appropriate FSH-mediated granulosa cell differentiation (luteinization). Since the adequate differentiation of granulosa cells is the pivotal event in formation of the normal corpus luteum, hypothyroxinaemia may be assumed to be one condition responsible for deficient corpus luteum formation (Maruo *et al.*, 1987). Consistent with the possible involvement of thyroid hormones in corpus luteum function, thyroid hormone replacement therapy has been found to be very effective for the treatment of LPD associated with subclinical hypothyroxinaemia. In the present study, both poor corpus luteum formation and insufficient circulating concentrations of progesterone in female *hyt* mice were improved markedly by thyroxine treatment. Poor corpus luteum formation with lower progesterone concentrations and no pregnancy were observed in mature *hyt* mice after treatment with gonadotrophins alone. However, thyroxine treatment

before mating supported corpus luteum formation and pregnancy, irrespective of treatment with thyroxine after mating. This finding was in agreement with a previous report in women, in which treatment with thyroid hormones in patients with infertility caused by LPD increased the incidence of pregnancy, indicating that a course of thyroid hormone therapy should be administered before more aggressive therapy with chorionic gonadotrophins or Clomid (Naficy and Behjatnia, 1975).

In conclusion, ovulation was induced by gonadotrophin treatment in immature female *hyt* mice. In mature female *hyt* mice, infertility occurred that was characterized by a lack of an oestrous cycle, and failure of follicular development, ovulation and pregnancy that could be reversed by thyroxine treatment before mating.

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