Superovulation of immature hypothyroid *rdw* rats by thyroxine therapy and the development of eggs after *in vitro* fertilization

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The aim of this study was to examine the effect of thyroxine on ovulation in immature *rdw* rats and the fertilization and development of the eggs. Serum thyroxine concentrations at 30 days of age were significantly lower in *rdw* rats than in normal rats ($P < 0.001$), and greatly increased after thyroxine replacement therapy ($P < 0.001$). Although few eggs (1.5 ± 1.2) were obtained from immature *rdw* rats treated with gonadotrophins alone, females treated with gonadotrophins and thyroxine ovulated significantly more eggs (85 ± 5). As a control, normal littermates ovulated 21–45 eggs when treated with gonadotrophins alone, and 68 eggs when administered with gonadotrophins and thyroxine. Of the eggs collected from *rdw* rats treated with gonadotrophins and thyroxine, and inseminated with spermatozoa from mature F_1 males, 98% were penetrated and in almost all (99%) of these eggs, male and female pronuclei formed. Forty-seven per cent of the pronuclear eggs developed to the blastocyst stage *in vitro*. After transfer to recipients, 21% (14/66) of one-cell and 22% (8/37) of two-cell embryos developed to offspring, and 62% (8/13) of pups were of *rdw/rdw* genotype. The average body weight (6.9 versus 7.8 g) of offspring derived from one-cell embryos was lower than that for two-cell embryos. The morulae and blastocysts did not develop to term, although 41% implanted in the uterine horns of recipients. In conclusion, in immature *rdw* rats, superovulation was induced by gonadotrophins combined with thyroxine therapy and the superovulated oocytes were fertilized and developed *in vitro* and developed to term after embryo transfer.

Introduction

The *rdw* rat, a new hereditary dwarf animal, was discovered by Koto *et al.* (1988) in Chugai Pharmaceutical Co. Ltd, Tokyo. It was derived from an inbred colony of Wistar–Imamichi rats. The mutant was an autosomal recessive trait with infertility in both sexes, so it has been maintained by $F_1 \times F_1$ mating. The *rdw* rat is characterized by hypoplasia of the pars distalis (anterior pituitary), in particular the cells that secrete growth hormone and prolactin. This results in defects in several hormones, such as growth hormone, prolactin and thyroid hormones, but causes significantly high serum thyroid-stimulating hormone concentrations (Koto *et al.*, 1988; Umezu *et al.*, 1991, 1993, 1995, 1996). The *rdw* rat is useful in endocrinological research as an animal model of human pituitary dwarfism (Koto *et al.*, 1988) and may be an ideal model for studying the role of the thyroid in reproduction in hypothyroid animals, including humans (Cooke and Arambepola, 1997). Fertility in adult male *rdw* rats was partly restored by thyroxine therapy (Umezu *et al.*, 1997). Few adult female *rdw* rats

produced offspring when given thyroxine therapy for 1 month, and in those that did, litter sizes were small, nursing capacity was poor, and most of the pups died in the first few days after delivery. In immature female *rdw* rats, no induction of ovulation was achieved with standard gonadotrophin treatment procedures (Jiang *et al.*, 1996a).

Hypothyroidism results in impaired fertility in many animals (Werner, 1969), and subclinical hypothyroidism often causes infertility in humans (Mochizuki, 1977; Louvet *et al.*, 1979; Bohnet *et al.*, 1981; Maruo *et al.*, 1992). These reproductive abnormalities can be improved by thyroid hormone therapy. Hagino (1971) reported that in thyroidectomized rats, ovulation was erratic, occurred in a small percentage of animals and a small number of ova were produced. These animals showed normal ovulation after L-thyroxine administration. In women, successful clinical use of thyroid hormone in the induction of ovulation in patients with subclinical hypothyroxinaemia implied that concomitant Clomiphene treatment with thyroid hormone replacement therapy is of great value for ovulation induction in patients with subclinical hypothyroxinaemia (Maruo *et al.*, 1992).

The aim of the present study was to induce superovulation in immature *rdw* rats and to produce *rdw* rats by *in vitro* fertilization, *in vitro* culture and embryo transfer.

Materials and Methods

Preparation of animals

rdw rats and normal littermates (Wistar–Imamichi) were produced in the Laboratory of Animal Reproduction, Tohoku University by mating adult F_1 males with F_1 females, the offspring of which were known to include *rdw* pups. In this study, the term *rdw* always refers to *rdw/rdw* homozygotes, whereas the term normal littermates includes F_1 (*rdw/+*) and wild-types (+/+), since there are no phenotypical differences between these animals. Animals were placed in polycarbon cages (25 cm × 40 cm × 20 cm) with wood shavings on the floor, in a temperature controlled room at $24 \pm 2^\circ\text{C}$, $65 \pm 5\%$ humidity and lights on at 5:00 h and off at 19:00 h. The animals were given bullet type commercial rat feed (High Pure Ace (P); Itochu Co., Ishinomaki) and tap water *ad libitum*. *rdw* rats were distinguished by low body weight and retarded development of the ears, as well as a small body size at about 2 weeks of age.

Hormone administration

Immature *rdw* rats and their normal littermates ($n = 5$ in each group) were randomly divided and treated as follows to examine the roles of gonadotrophins and thyroxine hormone in ovulation in *rdw* rats: (1) pregnant mares' serum gonadotrophin (PMSG) (10 iu); (2) PMSG and hCG (10 iu); (3) thyroxine and PMSG; and (4) thyroxine combined with PMSG and hCG. Animals were administered with thyroxine (L-thyroxine, Sigma Chemical Co., St Louis, MO) intraperitoneally once a day at 14:00 h at a dose of 10 µg per 100 g body weight from day 21 to day 30. The optimum dose of thyroxine was determined according to Jiang *et al.*, 1996b. Thyroxine was dissolved in 2 mol NaOH l^{-1} and prepared in physiological saline solution (pH 8.3). PMSG (10 iu; Sankyo Kabu Company, Tokyo) was injected subcutaneously at 10:00 h on day 28, and 10 iu hCG (Sankyo) was injected intraperitoneally 54 h later.

Blood sampling and thyroxine determination

Blood samples were collected from *rdw* rats, *rdw* rats administered with thyroxine and normal rats on day 30, 48 h after PMSG injection, and serum was obtained by centrifugation at 900 g for 15 min at 4°C . Serum was stored at -40°C until assay. Serum thyroxine concentrations were determined using a solid method radioimmunoassay and were expressed as ng ml^{-1} .

Egg collection

Animals were killed by cervical dislocation 14 h after administration of hCG. Ovaries and oviducts were transferred into a glass dish (60 mm × 60 mm) with PBS (Dulbecco's PBS; Nissui Pharmaceutical Co. Ltd, Tokyo). The adjunct fat was removed and ovary and oviduct were separated under a microscope. Oocyte–cumulus cell complexes were flushed out

of the oviducts with PBS, treated with 0.1% (w/v) hyaluronidase (Sigma), and the number of oocytes was counted. Only those oocytes with an intact zona pellucida and homogeneous cytoplasm were considered normal and were included in the analysis.

Medium

The culture medium used in this study was mR1ECM (Miyoshi *et al.*, 1994, 1995a) containing 76.7 mmol NaCl l^{-1} , 3.2 mmol KCl l^{-1} , 0.5 mmol $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ l^{-1} , 2.0 mmol $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ l^{-1} , 25.0 mmol NaHCO_3 l^{-1} , 7.5 mmol D-glucose l^{-1} , 0.5 mmol sodium pyruvate l^{-1} (all from Wako Pure Chemical Industries Ltd, Osaka), 10.0 mmol sodium lactate l^{-1} (Sigma), 0.1 mmol glutamine l^{-1} (Wako), 2% (v/v) minimal essential medium (MEM) essential amino acids solution (50×, No. 1000574; Gibco BRL, Grand Island, NY), 1% (v/v) MEM non-essential amino acids solution (100×, No. 1001447; Gibco BRL), 1.0 mg polyvinylalcohol (PVA) ml^{-1} (Sigma). The fertilization medium was mR1ECM containing 100 mmol NaCl l^{-1} and 4 mg BSA ml^{-1} (No. A-7638, Fraction V; Sigma) and omitting PVA (Oh *et al.*, 1997). Fertilization and culture media (400 µl per drop) were prepared in polystyrene culture dishes (35 mm × 14 mm; Sumitomo Bakelite Co. Ltd, Tokyo), covered with paraffin oil (No. 261-17; Nacalai Tesque Inc., Kyoto) and equilibrated overnight in a CO_2 incubator (5% CO_2 in air at 37°C).

Preparation of sperm suspension

Spermatozoa were obtained from F_1 males at about 8 months to 1 year as described by Miyoshi *et al.* (1997). Briefly, one drop of a dense mass of spermatozoa was introduced into pre-equilibrated insemination medium (400 µl). After about 5 min warming in the incubator, 10–60 µl of the sperm suspension was transferred into drops of insemination medium to give a final sperm concentration of 1×10^6 cells ml^{-1} . The diluted sperm suspensions were preincubated for 5 to 7 h in a CO_2 incubator.

Collection of cumulus–oocyte complexes and in vitro fertilization

Animals treated with thyroxine and PMSG were killed by cervical dislocation 13–14 h after hCG injection (Toyoda and Chang, 1974). Oviducts were isolated, placed on a piece of sterilized filter paper to remove the liquid and blood on the surface, and placed in the dishes containing the diluted sperm suspension. The cumulus–oocyte complexes in the oviducts were carefully released into the sperm suspension. The dishes were kept in a CO_2 incubator for 10 h.

Examination of fertilization

Methods for examining penetration and polyspermy were as described by Toyoda and Chang (1974) and Miyoshi *et al.*

(1995b, 1997). Briefly, after incubation, eggs were transferred into 100 μ l culture medium and freed from surrounding cumulus cells by repeated pipetting with a fine pipette. The denuded eggs were placed in the centre of four vaseline spots on a glass slide, compressed gently with a cover slip, fixed briefly with 2.5% (v/v) glutaraldehyde in phosphate buffer solution and in 10% (v/v) neutral formalin at room temperature for 4–6 h, stained with lacmoid and examined under a phase-contrast microscope. Eggs were considered penetrated when a spermatozoon was observed inside the perivitelline space or when the eggs had pronuclei with sperm tails in the vitellus (Toyoda and Chang, 1974). Eggs containing two or more enlarged sperm heads or male pronuclei in the vitellus with corresponding tails were considered polyspermic (Miyamoto and Chang, 1973).

Embryo culture in vitro

Embryo culture and examination of development were conducted as described by Miyoshi *et al.* (1995b). Briefly, 10 h after insemination, eggs were separated from cumulus cells, washed three to six times with culture medium and examined under a phase-contrast microscope for evidence of fertilization. Approximately 10–20 eggs with female and male pronuclei with corresponding tails were transferred to 400 μ l culture medium and cultured in a CO₂ incubator for 5 days.

Embryo transfer

The procedures for inducing pseudopregnancy and transfer were as described by Toyoda and Chang (1974), with the exception that two-cell embryos were transferred to day 1 (day of ovulation) rather than day 2 pseudopregnant recipients. Briefly, 4- to 5-month-old rats (200–280 g) that were in oestrus for at least three consecutive regular 4 day periods were stimulated by inserting a plastic rod connected to two electrodes (20 V) into the vagina and switching it on and off for 3 seconds three times on the morning of day 4 (pro-oestrus, the day before ovulation) and day 1. Seven to ten embryos at the one- or two-cell stage were transferred to the oviducts of each recipient on day 1. After 4–5 days of

culture *in vitro*, morulae or blastocysts were transferred to the uterine horns of recipient rats whose oestrous cycles were 1 day later (day 2) than those receiving one- or two-cell embryos. All transferred embryos were derived from eggs of immature *rdw* (*rdw/rdw*) rats fertilized *in vitro* with epididymal spermatozoa of mature F₁ (*rdw/+*) males. Recipients were checked once a day by smear examination. Recipients that showed pro-oestrous or oestrous smears, or were pregnant but did not deliver offspring by day 24 of pregnancy, were killed and their uterine horns were examined for implantation sites. The number of young born was counted on the day of parturition. The young were weighed on the day of birth. *rdw* rats were identified as described earlier.

Statistical analysis

Serum thyroxine concentrations and data for egg counts (Table 1) were expressed as mean $\pm$ SEM and assigned for one-way or three-way analysis of variance (ANOVA), respectively. When ANOVA revealed a significant treatment effect, the treatments were compared by Duncan's new multiple-range test. The weight of newborns (Table 2) was analysed by paired Student's *t* test.

Results

Serum thyroxine concentrations at 30 days of age were significantly lower in *rdw* rats (13.6 ± 1.1 ng ml⁻¹) than in normal rats (32.8 ± 0.7 ng ml⁻¹, $P < 0.001$), and were significantly higher (58.5 ± 3.0 ng ml⁻¹, $P < 0.001$) after thyroxine replacement therapy from day 21 to day 29.

hCG increased the ovulation rate in both *rdw* rats and normal littermates (Table 1). When PMSG was administered alone or with thyroxine, ovulation rates were 40 and 20% in *rdw* rats and 40 and 60% in normal rats, respectively, and increased to 100% in both groups when hCG was administered in the presence of PMSG alone or with thyroxine. hCG also increased the egg counts in normal rats (45 versus 21) but not in *rdw* rats (5 versus 3) pre-stimulated with PMSG. However, no additive effect was observed with thyroxine in the presence of PMSG. The combined treatment

Table 1. Effect of gonadotrophins and thyroxine on ovulation in immature *rdw* rats and normal littermates

Hormones administered			<i>rdw</i>		Normal	
Thyroxine	PMSG	hCG	Number of rats ovulating	Number of eggs collected per rat	Number of rats ovulating	Number of eggs collected per rat
–	+	–	2/5	3 $\pm$ 2 ^a	2/5	21 $\pm$ 13
–	+	+	5/5	5 $\pm$ 2 ^{a*}	5/5	45 $\pm$ 12
+	+	–	1/5	1 $\pm$ 1 ^a	3/5	26 $\pm$ 15
+	+	+	5/5	85 $\pm$ 5 ^b	5/5	68 $\pm$ 12

^aValues with different superscripts within each column are significantly different ($P < 0.01$).

^{*}The number of eggs collected in *rdw* rats is significantly lower than in normal littermates given the same treatment ($P < 0.05$). PMSG, pregnant mares' serum gonadotrophin.

Table 2. Pregnancies and offspring derived from oocytes of *rdw/rdw* rats fertilized *in vitro* with epididymal spermatozoa from *rdw/+* males and transferred to pseudopregnant recipients

Stage of embryos transferred	Number (%) of pregnancies	Number (%) of litters	Number of embryos transferred	Number of offspring				Average weight (range) of newborns (g)
				Total (%) [*]	Male (<i>rdw</i>)	Female (<i>rdw</i>)	Dead [†]	
One-cell	7/7 (100)	5/7 (71)	66	14 (21)	7 (4)	6 (4)	1	6.9 (5.9–8.2) ^a
Two-cell	5/5 (100)	4/5 (80)	37	8 (22)	nt	nt	8	7.8 (6.9–9.4) ^b
Morulae and blastocysts	10/10 (100)	0/10 (0)	85	0 (0)	–	–	–	–

^{a,b}Values with different superscripts within each column are significantly different ($P < 0.01$).

^{*}Percentage of the embryos transferred.

[†]Died within 2 weeks after birth.

nt, not tested.

of PMSG and hCG with thyroxine therapy significantly increased the number of ovulated eggs in both *rdw* rats (85, $P < 0.001$, low variation) and normal littermates (68, $P < 0.05$, high variation) compared with other treatments.

In the *in vitro* fertilization experiment, 89 (97.8%) of 91 inseminated eggs were penetrated *in vitro* and most (98.9%) had pronuclei. A small percentage (3.4%) of fertilized eggs were polyspermic; 2.2% of eggs showed fragmentation 10 h after insemination.

In the eggs fertilized *in vitro*, 100% (45/45), 78% (35/45), 69% (31/45) and 47% (21/45) developed *in vitro* to two-cell, beyond four-cell, morula and blastocyst stages, respectively. After transfer to the recipients, 21% of one-cell and 22% of two-cell embryos developed to offspring. However, none of the morulae or blastocysts developed to term. Four male and four female pups were determined to be *rdw* rats. The average body weight for newborns derived from two-cell embryos (7.8 g) was significantly greater than those from one-cell embryos (6.9 g) (Table 2).

Discussion

Numerous studies have examined the effects of hypothyroidism on reproduction in female animals. However, most of these have used thyroidectomy (Hagino 1971; Pepler *et al.*, 1975; Ruiz *et al.*, 1989; Mattheij *et al.*, 1995) or chemical induction (Baksi, 1973; Lee *et al.*, 1991; Bagavandoss *et al.*, 1998). The congenital hypothyroid *rdw* rat parallels more closely the human clinical syndrome, as increased testicular size in hypothyroid boys may be more useful as a model for the human condition than propylthiouracil-treated rats (Cooke and Arambepola, 1997). In hypothyroid women with weight loss amenorrhoea, no ovulation was induced by Clomiphene therapy, but ovulation increased markedly after combined treatment with thyroid hormone replacement and Clomiphene citrate (Maruo *et al.*, 1992). In *rdw* rats, superovulation was not induced by gonadotrophins alone, but was induced by a combined treatment of thyroxine replacement therapy and gonadotrophin administration. These similarities imply that female *rdw* rats are a useful model for the condition in women.

Thyroid hormone deficiency can cause a number of

disorders in reproduction such as disruption of ovulation, sterility and abortion (Werner, 1969). In hypothyroid animals, these abnormalities can be partially or completely reversed by thyroxine replacement therapy. Reproduction and lactation in dwarf female mutant *dw* and *df* mice can be induced with a diet supplemented with desiccated thyroid powder (Bartke, 1965a,b). Hypothyroid mice respond to thyroid hormone therapy with improved growth and fertility (Beamer *et al.*, 1981). In women, the induction rate of ovulation with Clomiphene citrate in patients with weight loss amenorrhoea increased as serum triiodothyronine concentrations increased from 80 to 140 ng dl⁻¹ (Maruo *et al.*, 1992). Hagino (1971) reported that in thyroidectomized rats, both vaginal cornification and ovulation (with a small number of ova) were delayed. When PMSG was administered at 28 days of age, ovulation was erratic, occurred in a small percentage of animals and a small number of ova were produced on both day 30 and day 31. However, thyroxine administration with PMSG injection allowed normal ovulation on day 31 (14 ova per animal). The results of the present study also show that thyroxine replacement therapy combined with gonadotrophin administration plays an important role in inducing ovulation in *rdw* rats. However, there were large differences in the number of oocytes in these two studies, which may be due to the different states of hypothyroidism and different doses of thyroxine in the replacement therapy. The tendency of *rdw* rats to have low body weights at birth indicates that spontaneous hypothyroidism began at birth or earlier, whereas in thyroidectomized rats, hypothyroidism did not commence before 24 days of age at which time the thyroid gland was removed. In addition, *rdw* rats were given 10 µg thyroxine per 100 g body weight once a day, whereas 3.5 µg per 60 g body weight (about 5.8 µg per 100 g) was administered to thyroidectomized rats (Hagino, 1971).

The induction of transient hypothyroidism from birth to day 25 in neonatal male rats with propylthiouracil and subsequent recovery to euthyroidism resulted in an increase in adult testis mass and daily sperm production of 80 and 140%, respectively (Cooke and Arambepola, 1997). These results were attributed to the extended proliferation and greater final population of Sertoli cells (Cooke *et al.*, 1991; van Haaster *et al.*, 1992; Hess *et al.*, 1993). In females, the stimulatory action of thyroid hormone on granulosa cell

differentiation was demonstrated by *in vitro* and *in vivo* studies. Thyroid hormone plays an important role in the functional differentiation of granulosa cells. However, treatment of cultured granulosa cells with thyroid hormone alone was incapable of inducing the stimulatory effects, thus synergism between thyroid hormone and FSH seems to play a vital role in granulosa cell differentiation (Maruo *et al.*, 1987; Maruo, 1988; Mochizuki and Maruo, 1988). In rats, prepubertal hypothyroidism induced by 6-propyl-2-thiouracil from birth to day 40 post partum interfered with differentiation of granulosa cells and resulted in a greater number of secondary follicles, fewer antral follicles, smaller non-atretic antral follicles, and more atretic follicles in the ovaries at day 40 compared with untreated rats (Dijkstra *et al.*, 1996). In addition, the number of ovulations decreased, since hypothyroidism reduced the number of follicles that were able to ovulate (Mattheij *et al.*, 1995). The evidence that thyroxine administration in the presence of PMSG increased the number of healthy large and small follicles may explain the marked results for oocytes in immature *rdw* rats (85 eggs per animal) compared with their normal counterparts (68 eggs per animal). It is clear that neonatal hypothyroidism resulted in an enlarged pool of secondary follicles, and thyroxine and PMSG administration allowed granulosa cell differentiation of secondary follicles and improved cell growth. As a result, large antral follicles developed and these were triggered to ovulate by hCG, which was shown to be necessary for superovulation in this study. The mechanism by which hCG affects superovulation in *rdw* rats remains to be elucidated.

In immature rats, a single injection of PMSG was effective for superovulation and egg counts of 33–34 were observed in treated Sprague–Dawley rats (Walton *et al.*, 1983; Armstrong and Opavsky, 1988). In the present study, fewer eggs were produced in PMSG-primed normal Wistar–Imamichi rats, indicating that these animals were less sensitive to PMSG than those used in the previous studies. The protocol that combines 10 iu hCG and 10 iu PMSG is widely used to obtain eggs for *in vitro* fertilization (Miyamoto and Chang, 1973; Toyoda and Chang, 1974; Miyamoto and Ishibashi, 1975; Niwa and Chang, 1975; Vanderhyden and Armstrong, 1989). hCG treatment also enhances the ovulation in adult rats pretreated with high doses of PMSG (Welschen and Rutte, 1971; Greenwald and Terranova, 1988). In the present study, normal immature littermates administered with 10 iu hCG ovulated 45 eggs, indicating that the superovulation was effective. In contrast, only a few eggs were obtained in immature *rdw* rats that received the same treatment, and no improvement was observed after combined treatment with hCG, indicating that hormone therapy is necessary for normal superovulation. The fact that the combined treatment of PMSG and hCG with thyroxine therapy induced superovulation (85 eggs per rat) in *rdw* rats similar to that in normal rats after FSH treatment (60–85 eggs per rat; Armstrong and Opavsky, 1988) supports this proposal.

Eggs produced after treatment with PMSG at appropriate concentrations developed normally with (Toyoda and Chang, 1974; Walton and Armstrong, 1983; Vanderhyden *et al.*, 1986; Vanderhyden and Armstrong, 1989) or without transfer (Nuti *et al.*, 1975). In the present study, the eggs

derived from PMSG-primed *rdw* rats had a high *in vitro* fertilization rate (97.8%). After fertilization, 47% of one-cell embryos developed to blastocysts, which was low compared with naturally ovulated eggs fertilized *in vivo* or *in vitro* and cultured *in vitro* (Miyoshi *et al.*, 1994, 1995a,b, 1997; Oh *et al.*, 1997), indicating that eggs derived from immature *rdw* rats have reduced developmental ability.

Miyoshi *et al.* (1997) reported that 25% of late fetuses or pups were produced after transfer of morulae or blastocysts to recipients. The reason why no pups were derived from morulae or blastocysts in the present study is unclear. However, 21 and 22% of one- and two-cell embryos, respectively, developed to term after transfer. This supports the findings of Toyoda and Chang (1974) that in immature rats, 21% of two-cell embryos derived from embryos fertilized *in vitro* developed to fetuses and newborn young. The fact that some of the offspring derived from one-cell embryos in the current study were *rdw* rats shows that this procedure can be used to produce *rdw* pups from infertile immature *rdw* rats for research purposes.

In conclusion, in infertile immature *rdw* rats, superovulation was induced by gonadotrophins with thyroxine replacement therapy. The superovulated eggs developed to blastocysts *in vitro* and to term after *in vitro* fertilization with spermatozoa from F₁ males and transfer of early stage embryos. About 50% of the offspring produced in this system were *rdw*, thus the method could be useful in endocrinological research as well as for the production of *rdw* rats.

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