Effect of hypothyroidism on ovarian follicular development, granulosa cell proliferation and peripheral hormone levels in the prepubertal rat

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The aim of this study was to examine the effects of prepubertal hypothyroidism on ovarian development in rats. Therefore, from birth up to day 40 postpartum, rats were given 6-propyl-2-thiouracil (PTU) via the drinking water of mothers and pups. At ages ranging from 12 to 40 days, ovarian weights were measured and serum was collected to estimate thyrotrophin (TSH), follicle-stimulating hormone (FSH) and inhibin levels. Two hours before sacrifice the animals received an injection of bromodeoxyuridine (BrDU) to estimate the proliferative activity of the follicular granulosa cells. Ovaries were fixed in Carnoy’s fluid and follicle counts were performed on sections stained with anti-BrDU and with haematoxylin and eosin. The PTU treatment resulted in increased serum TSH levels, indicative of hypothyroidism, and markedly lower body and ovarian weights, whereas serum FSH and inhibin levels were hardly affected. At day 40, ovaries of PTU-treated animals contained relatively more secondary and less antral follicles, smaller non-atretic antral follicles and more atretic follicles when compared with untreated rats, while corpora lutea were absent. It is concluded that this disturbed folliculogenesis is due to inadequate thyroid hormone supply, which hampers the differentiation and not the proliferation of granulosa cells because diameters of antral follicles were significantly smaller whereas the BrDU-labelling index had not changed.

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In male rats, hypothyroidism induced during the neonatal period by administration of 6-propyl-2-thiouracil (PTU) results in a large increase of adult testis size (1) and sperm production (2). This increased testis size is caused by a prolongation of the period of Sertoli cell proliferation, resulting in an enhancement of the number of Sertoli cells (3). These authors also showed that the increase of the number of Sertoli cells is accompanied by a delay in morphological differentiation of Sertoli cells, and that the altered pattern of Sertoli cell proliferation and differentiation is most likely due to reduced triiodothyronine (T₃) levels.

Information about the effects of thyroid hormone on gonadal development in female rats is absent. In the pig, thyroid hormone, in synergism with follicle-stimulating hormone (FSH), exerts stimulatory effects on granulosa cell differentiation and function (4). Specific high-affinity binding sites with characteristics expected of a T₃ receptor are present in the nuclei of porcine granulosa cells from follicles in various stages (5) and in human granulosa cells (6). In women, an adequate circulating level of thyroid hormone is one of the factors necessary for successful induction of ovulation (7). In rabbits, thyroidectomy or treatment with thyroid suppressive drugs causes the arrest of follicular maturation (8, 9).

The present study was undertaken to examine whether, as in the male, hypothyroidism drastically affects gonadal development in the female rat. The effect of hypothyroidism in female prepubertal rats was determined by studying ovarian growth, follicular development and atresia, granulosa cell proliferation and corpus luteum formation. Animals were made hypothyroid by treatment with PTU. Peripheral TSH, FSH and inhibin levels were estimated to evaluate the efficiency of the PTU treatment, the effect of the treatment on gonadotrophic output and the differentiation of granulosa cells, respectively.

Materials and methods

Animals

Pregnant Wistar rats were obtained from the Central Animal Facilities of the University of Utrecht (The Netherlands). The animal cages were examined for litters twice a day. On the day of birth (day 1), the litters were designated randomly to the untreated or to the
PTU-treated group. The drinking water of the PTU group contained 0.1% PTU (a synthetic goitrogen from Merck, Schuchardt, Germany). At different ages, varying from 12 to 40 days per group, seven to nine females of four to six litters were killed by decapitation. Body and ovarian weights were measured and serum was collected.

**Immunohistochemical staining**

Two hours before sacrifice the animals received a subcutaneous injection of 150 mg/kg bromodeoxyuridine (BrdU, Sigma, St Louis, MO). Ovaries of five or six animals per group were fixed in Carnoy's fluid and embedded in paraffin wax. Step sections (5 μm) were mounted at 50-μm intervals onto microscope slides. Sections were incubated in 1% periodic acid (Merck, Darmstadt, Germany) for 30 min at 60°C, washed in tap water for 10 min, rinsed in distilled water and in 0.01 mol/l phosphate-buffered saline (PBS, pH 7.4), blocked with 2.5% normal horse serum (Vector Laboratories, Burlingame, CA) for 20 min and incubated with a monoclonal antibody against BrdU (1: 80; Becton & Dickinson, San Jose, CA) for 60 min. Normal horse serum and anti-BrdU were diluted in PBS containing 1% bovine serum albumin (BSA) (BDH, UK). After washing in PBS, endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 10 min. After this incubation the slides were washed in PBS and incubated with biotinylated horse-antimouse IgG (H + L) (Vector Laboratories) diluted 1: 100 in PBS containing 1% normal horse serum for 60 min. After washing in PBS the sections were incubated with the avidin–biotin complex Elite (Vector Laboratories) diluted 1: 500 in PBS for 60 min. Immunoreactivity was visualized by incubating the slides with a 0.5 mg/ml solution of 3,3′-diaminobenzidine (DAB) (Sigma, St Louis, MO) in 0.05 mol/l TRIS.HCl (pH 7.6) with 0.02% hydrogen peroxide for 10 min. All incubations were carried out at room temperature. The sections were counterstained with haematoxylin and eosin.

**Morphological analysis**

Based on micromorphological criteria (10, 11), follicles were classified as non-atretic or atretic. Non-atretic follicles were classified as secondary (with two or more layers of granulosa cells) and antral (having many layers of granulosa cells with either scattered areas containing fluid or a single cavity of fluid) follicles. Atretic secondary follicles had several (at least five) pyknotic granulosa cells, or a degenerated oocyte surrounded by either a disorganized granulosa or a few granulosa cells, and hypertrophied theca cells. In atretic antral follicles the oocyte was often found to be intact, whereas the layers of granulosa cells had become disorganized, pyknotic nuclei are found and, as atresia proceeded, the granulosa cells were lost.

In three sections of both ovaries (at a quarter, half and three-quarters of the ovary), all follicles were inspected and counted. Because of the large differences in absolute numbers of different follicle categories between the animals, and because the counted numbers reflect only part of the total follicle population in an ovary, in each group of rats the mean percentages of non-atretic and atretic follicles, as well as the mean percentages of secondary and antral follicles within these populations, were calculated and analysed statistically. Primordial and primary follicles were often arranged in small or large clusters. The number of follicles in these clusters varied considerably. Therefore, primordial and primary follicles were excluded from calculations.

As a parameter for cell proliferation, the BrdU-labelling index of the granulosa cells was determined by counting the BrdU-labelled and the total number of granulosa cells in the granulosa layer of all non-atretic follicles in the three sections.

In six sections of both ovaries, the category of secondary follicles was further subdivided into follicles with 2, 3, 4, 5, 6 and 7 or more granulosa cell layers to determine the percentage of each of these classes in 40-day old animals. The diameter of these secondary follicles and the diameter of their oocyte (only if the nucleolus was visible), as well as the diameter of early antral (with scattered granulosa lacunae) and advanced antral (with a single cavity) follicles and their oocyte were measured using a computer-assisted morphometric program (TIM-Histology, DIA, Breda The Netherlands). For each class of follicles, the mean diameter was estimated in each animal. Subsequently, the mean diameters were calculated for untreated and treated rats.

**Radioimmunoassays**

Serum of the PTU-treated animals and of the 12-day-old control animals was pooled to collect sufficient amounts of serum to determine the levels of FSH, inhibin and TSH, in such a way that all groups consisted of at least five measuring points.

Both FSH and inhibin were estimated by radioimmunoassay (RIA) as described previously (12). Results were expressed in terms of NIDDK-rFSH-RP-3 and of a bovine follicular fluid standard preparation, respectively. The lower limits of detection were 0.1 ng/ml FSH and 0.5 U/ml inhibin; intra-assay coefficients of variation were below 4% and 12% respectively.

Thyrotrophin was measured by RIA using the materials and procedure supplied by the NIDDK, and the reference preparation was NIDDK-rTSH-RP-2. The lower limit of detection in this assay was 0.3 ng/ml TSH. The intra-assay coefficient of variation was lower than 12%.
Statistics

Data on body and ovarian weights, follicle diameters, mean numbers of granulosa cell layers in secondary follicles and FSH and inhibin serum levels in PTU-treated and untreated rats were analysed with nested ANOVA, followed by the independent t-test. Likewise, the non-parametric Mann–Whitney U test was used to compare data on TSH serum levels. Data on non-atretic and atretic follicle percentages and BrdU incorporation in granulosa cells of follicles were analysed with logistic regression, followed by the Mann–Whitney U test. Differences were considered significant when \( p < 0.05 \). Data are expressed as means ± SEM.

Results

Body and ovarian weights

Body weights of the PTU-treated rats were significantly lower than those of untreated animals (Fig. 1). Up to and including 16 days after birth, ovarian weights of both groups were not different. From 21 days onward, ovarian weights of PTU-treated animals were significantly lower as compared to untreated rats.

Morphology

With increase in age, the percentage of non-atretic (secondary plus antral) follicles decreased (Table 1). Within this population of non-atretic follicles, the percentage of secondary follicles decreased with increase of age in favour of the percentage of antral follicles (Table 2). Propyl-2-thiouracil treatment resulted in a higher percentage of non-atretic follicles at day 21 and in a lower percentage at day 40 (Table 1). Compared to untreated rats, the population of non-atretic follicles in PTU-treated rats consisted of a lower percentage of antral and a higher percentage of secondary follicles from day 21 onwards (Table 2). At 21, 26 and 40 days of age these differences were significant. Corpora lutea were found in the ovaries of two out of five untreated animals at day 35 and in the ovaries of all five untreated rats at day 40. In the PTU-treated animals, however, no corpora lutea could be observed at any age.

Up to and including 16 days after birth, atretic follicles were absent or hardly present (Table 1). From day 21 onward, the population of atretic follicles in PTU-treated rats consisted of a higher percentage of secondary and a lower percentage of antral follicles than in untreated rats (Table 2). These differences, however, were significant only at 21 days of age. Up to and including 21 days of age, atretic secondary follicles showed pyknotic nuclei in the granulosa layer, both in PTU-treated and in control rats. In older animals, atresia of secondary follicles was mainly characterized

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Treatment} & \text{Age (days)} & \text{Follicles} & \text{Atretic} \\
 & & \text{Non-atretic (mean no.)} & \text{Non-atretic (mean %)} \\
\hline
\text{Untreated} & 12 & 118.6 ± 19.1 & 0.0 ± 0.0 \\
 & 16 & 110.6 ± 7.2 & 0.3 ± 0.2 \\
 & 21 & 167.6 ± 18.2 & 25.8 ± 5.6 \\
 & 26 & 201.2 ± 18.8 & 56.8 ± 2.0 \\
 & 30 & 202.4 ± 12.8 & 63.4 ± 3.1 \\
 & 35 & 178.6 ± 12.6 & 68.5 ± 3.2 \\
 & 40 & 167.8 ± 11.7 & 71.3 ± 1.5 \\
\hline
\text{PTU} & 12 & 132.4 ± 20.6 & 0.0 ± 0.0 \\
 & 16 & 1602.2 ± 17.8 & 0.8 ± 0.5 \\
 & 21 & 183.6 ± 17.9 & 3.7 ± 1.2* \\
 & 26 & 222.0 ± 27.8 & 5.5 ± 2.5 \\
 & 30 & 161.4 ± 23.9 & 6.5 ± 0.7 \\
 & 35 & 193.8 ± 21.2 & 73.8 ± 2.1 \\
 & 40 & 222.2 ± 29.0 & 78.2 ± 1.8* \\
\hline
\end{array}
\]

\* \( p < 0.05 \) compared with untreated animals of the same age.

![Graph showing body and ovarian weights](Fig. 1. Body and paired ovarian weights of untreated and propyl-2-thiouracil (PTU)-treated rats. Data are expressed as means ± SEM. Stars indicate significant differences compared with untreated animals of the same age: \( p < 0.05 \), independent t-test.)
Table 2. Mean number of non-atretic and atretic follicles (in three sections per ovary) in rats (N = 5) and the effect of propyl-2-thiouracil (PTU) treatment on the mean percentage of secondary and antral follicles within these populations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age (days)</th>
<th>Non-atretic follicles</th>
<th>Atretic follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-antral (mean no.)</td>
<td>Antral (mean %)</td>
</tr>
<tr>
<td>Untreated</td>
<td>12</td>
<td>118.6 ± 19.1</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>110.2 ± 7.1</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>126.0 ± 21.1</td>
<td>87.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>87.8 ± 10.8</td>
<td>80.5 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>74.0 ± 7.7</td>
<td>78.3 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>56.2 ± 6.6</td>
<td>75.2 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>48.0 ± 4.0</td>
<td>64.6 ± 2.9</td>
</tr>
<tr>
<td>PTU</td>
<td>12</td>
<td>132.4 ± 20.6</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>159.2 ± 18.5</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>177.4 ± 18.1</td>
<td>95.0 ± 1.9*</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>97.6 ± 12.2</td>
<td>91.2 ± 0.9*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>55.6 ± 7.9</td>
<td>85.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>50.2 ± 6.2</td>
<td>83.8 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>46.8 ± 3.8</td>
<td>83.2 ± 3.3*</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with untreated animals of the same age.

by a degenerated oocyte, few granulosa cells and hyperthrophied theca cells.

In 40-day-old rats, 60–80 non-atretic secondary follicles were found in six sections of both ovaries. The mean number of granulosa cell layers in these follicles in PTU-treated animals (4.50 ± 0.11) was not significantly different from that of untreated rats (4.62 ± 0.24). As shown in Table 3, PTU treatment resulted in 13.1% more secondary follicles with only two or three layers of granulosa cells in 40-day-old rats. Mean diameters of the various classes of secondary follicles were not affected by PTU treatment (data not shown). Similarly, the diameters of early atretic follicles were not changed (289 ± 15 vs 296 ± 8 µm in controls and PTU-treated animals, respectively). However, the diameter of the advanced antral follicles in PTU-treated rats was significantly larger than in the controls (437 ± 14 vs 378 ± 15 µm; p < 0.05). Oocyte diameters of secondary (32–55 µm), early antral (55–57 µm) and advanced antral (55–57 µm) follicles were not influenced by PTU treatment.

**Proliferation of granulosa cells**

The BrdU-labelling indices of granulosa cells in secondary and antral follicles varied, respectively, from 19.7 to 21.6 and 25.6 to 27.9 in untreated rats and from 17.6 to 19.8 and 26.6 to 30.7 in PTU-treated animals. However, values belonging to untreated and PTU-treated rats of corresponding age did not differ significantly (data not shown).

**Serum hormone levels**

Propyl-2-thiouracil treatment resulted in a significant increase of serum follicle-stimulating hormone (FSH) concentrations, as shown in Table 3. The effect of propyl-2-thiouracil (PTU) treatment on the percentage of various classes of non-atretic secondary follicles (in six sections per ovary) in 40-day-old rats (N = 5).

<table>
<thead>
<tr>
<th>% Follicles</th>
<th>Untreated</th>
<th>PTU-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 gran. layers</td>
<td>15.9 ± 1.2</td>
<td>24.5 ± 3.1</td>
</tr>
<tr>
<td>3 gran. layers</td>
<td>18.3 ± 2.4</td>
<td>22.8 ± 2.0</td>
</tr>
<tr>
<td>4 gran. layers</td>
<td>22.4 ± 0.9</td>
<td>16.3 ± 2.6</td>
</tr>
<tr>
<td>5 gran. layers</td>
<td>16.4 ± 2.0</td>
<td>8.2 ± 2.1</td>
</tr>
<tr>
<td>6 gran. layers</td>
<td>11.1 ± 2.7</td>
<td>8.4 ± 1.0</td>
</tr>
<tr>
<td>&gt; 6 gran. layers</td>
<td>15.8 ± 1.7</td>
<td>19.9 ± 3.7</td>
</tr>
</tbody>
</table>

*Data are expressed as means ± sxe; gran. = granulosa.
increase in serum TSH levels during the whole period of investigation (Fig. 2).

High FSH levels were found at 12 and 16 days of age in both groups. After this age, FSH levels decreased sharply and remained low (Fig. 2). At the age of 16 days, FSH levels in the PTU-treated rats were significantly but slightly lower than in untreated animals.

During the period of investigation, serum inhibin levels varied from approximately 20 to 30 U/ml and from approximately 15 to 30 U/ml in untreated and PTU-treated rats, respectively, the values being significantly but slightly different at 12, 16, 30 and 35 days of age (Fig. 2). Inhibin levels were low at days 12 and 16, increased between days 16 and 20 and stayed higher during the remaining period of investigation.

Discussion

Ovarian weight in untreated prepubertal female rats increased abruptly between day 35 and day 40 after birth. This observation is in accordance with findings of Knudsen et al. (13), who found a similar increase between day 34 and day 36, together with an increase in the proportion of antral follicles and in uterine weight, indicative of enhanced oestrogen secretion. Like Knudsen et al. (13), we observed an increased percentage of healthy antral follicles from one month after birth onwards, which explains the increase in gonadal weight.

In previous studies of our research group (3), treatment of rats with PTU resulted in reduced serum T4 levels that led to an increased serum TSH content. In the present study, increased serum TSH levels in PTU-treated female rats confirmed the effectiveness of the treatment.

In serum of untreated prepubertal female rats, FSH levels are high before day 20 of development, which corresponds with previous studies (14, 15). According to the latter authors, this high FSH level is essential for normal folliculogenesis. In the present study FSH values are hardly influenced in hypothyroid rats. The reduction in peripheral FSH at day 16 may be statistically significant but it is too small to account for abnormal folliculogenesis including anovulation at day 40. In previous studies (15, 16), even total suppression of the high FSH levels in immature rat did not prevent the occurrence of first ovulations at day 39 as normal. Likewise, it is unlikely that the observed impairment in folliculogenesis in hypothyroid rats results from the significantly but slightly lowered inhibin levels that occur prior to day 21. On the contrary, the low body weight of our prepubertal female rats can be considered as a determining factor for both abnormal follicular development and the prevention of corpus luteum formation, because body weight is a well-known initiating factor for puberty in rats (17, 18). Hypothyroidism leads to lethargy and stunted growth, due to diminished calorigenesis and oxygen consumption (19, 20). First ovulation occurs only in rats with a body weight over 90 g (21). At the end of the PTU treatment, at day 40, rat body weights appeared far lower than 90 g. This may explain the absence of corpora lutea in our hypothyroid rats.

As in food-restricted immature rats (18), folliculogenesis in hypothyroid immature rats becomes disturbed. In both cases, atretic follicles were more abundantly present. Both after PTU treatment (3) and after food restriction (22), decreased thyroid hormone levels were observed in the peripheral blood. This suggests that, in both cases, effects on folliculogenesis are due to a shortage of thyroid hormone. In rabbits, thyroid hormones have been associated previously with follicular development (8, 9). The finding of Maruo et al. (7) that women are anovulatory in the presence of low serum T4 levels may point to a role of thyroid hormones also in the final step of folliculogenesis, i.e. maturation of oocytes, ovulation and luteinization of the follicle wall. The observed effects of PTU treatment are the result of direct ovarian action of reduced thyroid hormone levels rather than direct action of increased TSH levels, because thyroid hormone receptors in human (6) and pig (7) and, in human, their mRNAs are clearly expressed in granulosa cells, whereas only minimal amounts of TSH receptor mRNAs could be demonstrated in rat ovaries (23).

Hypothyroidism seems to affect further follicle development via an influence on differentiation and not on proliferation of granulosa cells, because the proliferative activity of the granulosa cell, as reflected by the BrdU-labelling index, does not change. Differentiation of granulosa cells includes the production of follicular fluid, inhibin, oestrogens and other cytokines (24–29). Indeed, significantly smaller mean diameters of antral

![Fig. 2. Serum TSH, FSH and inhibin levels of untreated and propyl-2-thiouracil (PTU)-treated rats. Data are expressed as means ± SEM. Stars indicate significant differences compared with untreated animals of the same age: p < 0.05: Mann-Whitney U test for TSH and independent t-test for FSH and inhibin.](image-url)
follicles at day 40 of age may be indicative of reduced follicular fluid production in hypothryroid rats. Although in PTU-treated rats inhibin levels have hardly been influenced, hypothryoidism may have adversely influenced the capacity of granulosa cells to form oestrogens, because a higher proportion of atretic follicles was found. Previously, oestradiol has been demonstrated to rescue follicles from atresia (3). Unfortunately, the amounts of blood that could be collected were too small to establish serum oestrogen levels or other hormone levels, apart from those of inhibin, FSH and TSH.

In summary, hypothryoidism, brought about by a successful PTU treatment of immature female rats from birth onward, resulted in lowered body and gonadal weights and abnormal folliculogenesis leading to anovulation in the presence of hardly affected peripheral FSH and inhibin levels. We conclude that the disturbed folliculogenesis is due to reduced thyroid hormone output, which inhibits the normal differentiation of granulosa cells but not their proliferation. Further studies with isolated and in vitro cultured rat follicles will be carried out to investigate direct effects of thyroid hormones.

References

1. Cooke PS, Meisami E. Early hypothryoidism in rats caused increased adult testis and reproductive organ size but does not change testosterone levels. Endocrinology 1991;119:237–43
20. Glass CK, Holloway JM. Regulation of gene expression by the thyroid hormone receptor. Biochim Biophys Acta 1990;1032:157–76

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