

Effects of Maternal Hypothyroidism on the Pubertal Development in Female Rat Offspring

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Received: April 3, 2021
Revised: May 6, 2021
Accepted: June 1, 2021

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Conflict of interests

The authors declare no potential conflict of interest.

Acknowledgements

This research was funded by a 2020 research Grant from Sangmyung University (2020-A000-0172).

Authors' contributions

Conceptualization: Lee SH.
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Software: Park JS.
Validation: Lee SH, Park JS.
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Writing-review & editing: Lee SH, Park JS.

Abstract

The present study was performed to investigate the effect of maternal hypothyroidism and puberty onset in female rat pups. To do this, we employed propylthiouracil (PTU) to prepare a hypothyroid rat model. Pregnant rats were treated with PTU (0.025%) in drinking water from gestational day 14 to postnatal day 21 of offspring. Comparison of general indices such as body and tissue weights and puberty indices such as vaginal opening (VO) and tissue histology between control and PTU-treated rats were conducted. There was no significant difference in the date of VO between control and PTU group. The body weights of the PTU group were significantly lower, only 36.8% of the control group ($p < 0.001$). Although the absolute thyroid weight was not changed by PTU treatment, the relative weight increased significantly about 2.8 times ($p < 0.001$), indicating that hypothyroidism was successfully induced. On the other hand, the absolute weights of the ovary and uterus were markedly decreased by PTU administration ($p < 0.001$), and the relative weight was not significantly changed. The ovarian histology of PTU group revealed the advanced state of differentiation (i.e., presence of corpora lutea). Inversely, the uterine histology of PTU group showed underdeveloped structures compared those in control group. Taken together, the present study demonstrates that our maternal hypothyroidism model resulted in minimal effect on pubertal development symbolized by VO despite of huge retardation in somatic growth. More sophisticatedly designed hypothyroidism model will be helpful to achieve a better understanding of pubertal development and related disorders.

Keywords: Maternal hypothyroidism, Puberty, Ovary, Vaginal opening, Rat

INTRODUCTION

Thyroid hormones (THs) are vital for the maintenance of normal physiology in humans and animals. Hypothyroidism is a disorder of the endocrine system in which the thyroid does not produce enough THs, affecting up to 5% of the general population with a further estimated 5% being undiagnosed in USA (Chiovato et al., 2019). It can cause a wide range of symptoms, such as poor ability to tolerate cold, a feeling of tiredness, constipation, slow heart rate, depression, and weight gain (El-Shafie, 2003; Idris et al., 2005).

In older children and adolescents, the symptoms of hypothyroidism are similar to those in adult, and particularly, include irregular menstrual cycles and delayed puberty in girls (Anasti et al., 1995;

Ethics approval

The animal protocols were approved by the Animal Care and Use Committees at Sangmyung University (approval number R-2001-1).

Counts and Varma, 2009). In hypothyroid rodents, however, the delay of puberty onset in female is uncertain while it is prominent in male (Wilén et al., 1981; Shibutani et al., 2009). So far, this discrepancy has not been investigated in detail.

6-n-propyl-2-thiouracil (PTU) is a medication used to treat hyperthyroidism, also is widely applied to induce hypothyroidism in experimental rodents (Mandel and Cooper, 2001; Mallela et al., 2014). The present study was performed to understand the relationship between maternal hypothyroidism and puberty onset in female rat offspring. To do this, we employed PTU-induced hypothyroid rat model. Based on our pilot studies, we used slightly modified PTU model (Hapon et al., 2007; Zamoner et al., 2008; Tanaka et al., 2019). Comparison of general indices such as body and tissue weights, and puberty indices such as vaginal opening (VO) and tissue histology between control and PTU-treated rats were conducted.

MATERIAL & METHODS

1. Chemicals and animals

6-Propyl-2-thiouracil (PTU; CAS no. 51-52-5, purity >99.0%) was purchased from Tokyo chemical industry (Tokyo, Japan). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pregnant Sprague-Dawley rats were purchased from DBL (Eumseong, Korea) at gestational day (GD) 12. The animal rooms were maintained in air-conditioned temperature $22 \pm 1^\circ\text{C}$, relative humidity $40 \pm 5\%$ with 12-hour light/dark cycle. Dam and offspring were fed *ad libitum* with Purina Mouse Diets, purchased from Biopia (Gunpo, Korea). The animal protocols were approved by the Animal Care and Use Committees at Sangmyung University (approval number R-2001-1). All the animals received humane care in accordance with the guides for animal experiments of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2. PTU treatment and tissue preparations

Dams were given drinking water containing 0.025% of PTU w/v or water alone from GD14 to at postnatal day (PND) 21 as previously reports with slightly modification, for longer exposure period and lower dose than original procedures (Hapon et al., 2007; Zamoner et al., 2008). The start date of PTU treatment was determined to be the 13th day of gestation (GD13) according to the report that thyroid hormone receptors were first expressed in the fetus on the GD14 of pregnancy (Dowling et al., 2000). PTU containing water was freshly changed every two days. Pups of control group were weaned at PND 21, and dams in PTU-exposed group were further maintained for additional 7 days without PTU-treatment for further nursing of offspring showing severe growth suppression due to hypothyroidism (Tanaka et al., 2019). Body weights and the dates of VO were measured until PND 36. Animals were sacrificed, and the tissue weights were measured between 18:00–19:00 pm. The tissues were fixed in paraformaldehyde (4%) for histological study.

3. Paraffin section

Fixed tissues (thyroids, ovaries, uteri and ovarian fat fads) were dehydrated in graded concentrations of ethanol (70%, 80%, 90%, 95%, and 100%; Duksan, Korea) for 1 hour 30 minutes in each with gentle shaking and soaked in absolute ethanol overnight. The tissues were immersed in xylene (Samchun Chemical, Seoul, Korea) for 30 minutes, 3 times and in paraffin (Avantik Biogroup, Pine Brook, NJ, USA) at 56°C for 30 minutes, 3 times. The tissues were embedded in paraffin and sectioned (Microm, Walldorf, Germany) at $5\ \mu\text{m}$. The samples were attached on

microscope slides (Marienfeld, Lauda-Königshofen, Germany) and the slides were stained with hematoxylin (Sigma-Aldrich) and eosin (Across, Carson, CA, USA) for 5 minutes, respectively. The width of the thyroid follicle lumen was measured using Image-J software, and other histological parameters such as thickness of the follicular cells were measured.

4. Statistical analysis

Values were expressed as mean \pm SE. Data were analyzed using Student's *t*-test and Mann-Whitney U test as indicated. $p < 0.05$ was considered statistically significant. Calculations were performed using Graphpad Software Prism version 5.

RESULTS

1. Changes in body weights

Fig. 1 shows that maternal PTU administration significantly inhibited weight gain in hypothyroid pups ($p < 0.001$). The offspring weights of two groups differed significantly from PND7. The gap in average weight continued to increase thereafter until PND 35.

2. Analyses of vaginal opening

There was no significant difference in the date of VO, which is a hallmark of the puberty onset, between control and PTU group (Fig. 2). In both groups, VO of the most objects were observed at PND36, but some animals of PTU group had checked relatively later.

3. The body weights at vaginal opening day

The average body weight at the time of VO showed a huge difference between the two groups. The body weights of the PTU group were significantly lower, only 36.8% of the control group ($p < 0.001$, Table 1).

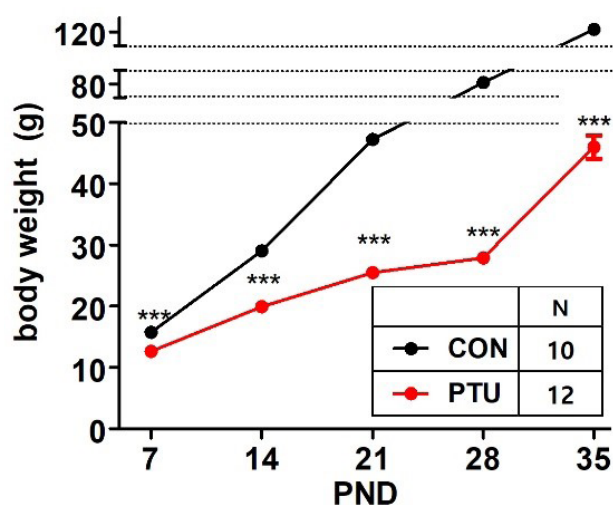


Fig. 1. PTU-induced maternal hypothyroidism inhibits offspring growth until postnatal day (PND) 36. Mothers of PTU group received PTU in drinking water from GD 14 to PND 21 and then Tap water until PND 28. Pups of control group were weaned at PND 21. *** $p < 0.001$, compared to CON group by Mann-Whitney or unpaired *t*-test. PTU, propylthiouracil; PND, postnatal day.

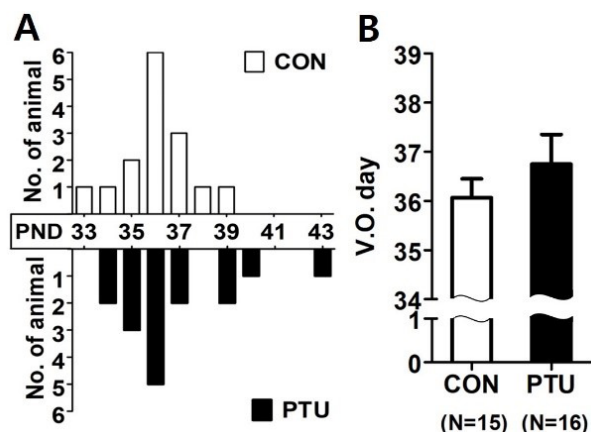


Fig. 2. Effect of maternal hypothyroidism on puberty onset of female offspring. Vaginal opening and smears were examined daily after weaning. (A) Comparison of mean vaginal opening (VO) day between control and PTU-treated groups. (B) Comparison of the daily VO profiles between control and PTU-treated groups. In both groups, VO occurred the most on the PND 36. PTU, propylthiouracil.

Table 1. Effect of maternal PTU exposure during GD 13 through PND 21 on offspring body weight at vaginal opening day

	CON	PTU
Body weight (g)	138.0±3.69	50.8±3.15***

Values are expressed as mean±SE. CON (n=12), PTU (n=13).

Differences between groups were considered as significant at $p < 0.05$ and analyzed with Mann-Whitney test (** $p < 0.001$).

PTU, propylthiouracil.

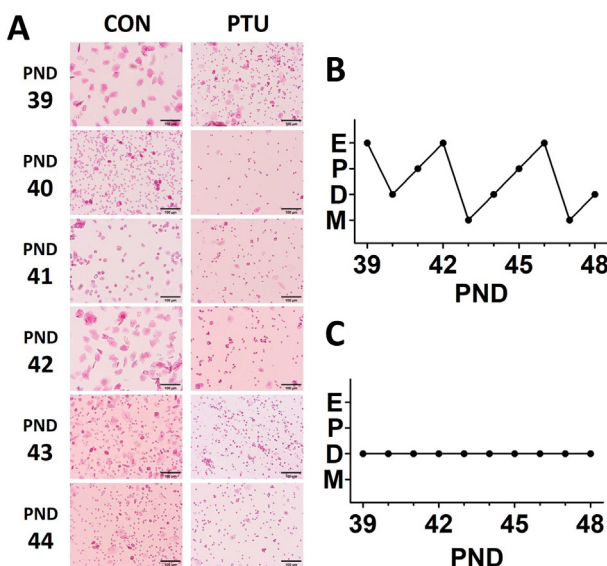


Fig. 3. Daily analysis of estrous cyclicity in CON and PTU rats after VO. (A) Representative patterns of vaginal smears for 6 days post VO from CON (left) and PTU (right) rats. Smears were stained with 0.1% Eosin solution (Bar = 100 μ m). The vaginal smear contains only keratinocytes at estrus (PND39 and 42 of CON group). Metestrus is characterized by the mix of lymphocytes (small-nucleated cells) and keratinocytes in the vaginal fluid (PND43 of CON group). Diestrus is characterized by the presence of lymphocytes in the vaginal fluid (PND 44 of CON group). During the proestrus stage, nucleated epithelial cells are observed in the vaginal smear (PND41 of CON group). On the other hand, vaginal smears collected from PTU rat reveal the prolonged presence of lymphocytes (i.e., diestrus), indicating a complete alteration of the estrous cycle. The bar represents 100 μ m (B and C). Representative samples of the cyclicity in CON (B) and PTU (C) groups, respectively. PTU, propylthiouracil; PND, postnatal day; E, estrus; P, proestrus; D, diestrus; M, metestrus; VO, vaginal opening.

4. Analyses of estrous cyclicity

Fig. 3 demonstrated the estrous cyclicity observed after VO. Each stage of the cycle was determined by the predominant presence of either lymphocytes, nucleated epithelial cells, or keratinocytes (see figure legend for details). Serial microphotographs of the smeared cells from control animals revealed the good cyclicity while no cyclicity was found in the cells from PTU-treated animals (Fig. 3A). The normal estrous cycle has a characteristic periodicity, with an estrus every 4–5 days. The representative patterns of vaginal smears for 10 days post VO also showed the four stages in control animals, while only one persistent stage was found in PTU-treated animals (Fig. 3B and C, respectively).

5. Changes in tissue weights

Table 2 showed the effect of PTU administration on changes in tissue weight at VO day. The absolute weight of all tissues except for the thyroid gland decreased significantly, and the relative weights increased significantly except for the ovary and uterus (Table 2). Although the absolute thyroid weight was not changed by PTU treatment, the relative weight increased significantly about 2.8 times ($p < 0.001$), indicating that the hypothyroidism pursued by this experimental model was successfully induced. On the other hand, the absolute weights of the ovary and uterus were markedly decreased by PTU administration ($p < 0.001$), the relative weights were not significantly changed. This phenomenon is interesting, because it suggests that the pubertal growth of thyroid gland might be proceeded regardless of somatic growth, but not in other organs.

6. Histological analysis

At PND 36, when VO was not checked in control while VO was recorded in PTU animal, tissue samplings for histological study were performed. Although the absolute weights of the thyroid gland of both control and PTU group were similar (Table 2), histological analysis revealed that the area of thyroidal follicular lumen and the width of follicular epithelial cells were greatly reduced in the PTU group. As expected, the size of the ovaries was markedly reduced in PTU group (Table 2). There were many primary and secondary follicles and no corpus luteum in the control group, but relatively few primary and secondary follicles and many tertiary follicles and corpus luteum-like structures were found in the PTU group (Fig. 4). The size of the uterus was relatively small in the PTU group (Table 2), however, the endometrium, lumen, and muscle layers of the PTU group were underdeveloped states compared to those of control group. In case of ovarian fat pad, more cell nuclei and reductions in cell area were observed in the PTU group.

Table 2. Effect of maternal PTU administration on tissue weight change

	Absolute tissue weight (mg)		Relative tissue weight	
	CON	PTU	CON	PTU
Thyroid	4.5±0.25	4.7±0.29	3.6±0.22	10.2±0.73***
Ovary	20.4±1.48	7.3±0.32***	16.4±1.21	15.4±0.75
Uterus	206.7±23.22	100.1±27.85*	16.4±1.71	21.9±6.88
Adrenal	15.0±0.69	8.4±0.39***	12.0±0.62	19.1±0.90***
Kidney	661.9±19.07	306.7±8.26***	53.0±1.59	69.5±1.78***
Spleen	466.2±17.78	250.1±15.08***	37.3±1.17	56.5±2.76***

Relative tissue weight calculations are as follows, depending on the tissue.

Thyroid, ovary, adrenal: relative tissue weight=[Tissue weight (mg)/Bodyweight (g)]×100.

Uterus, kidney, spleen: relative tissue weight=[Tissue weight (mg)/Bodyweight (g)]×10.

Values are expressed as mean±SE. Groups marked with an asterisk are significantly different from control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

PTU, propylthiouracil.

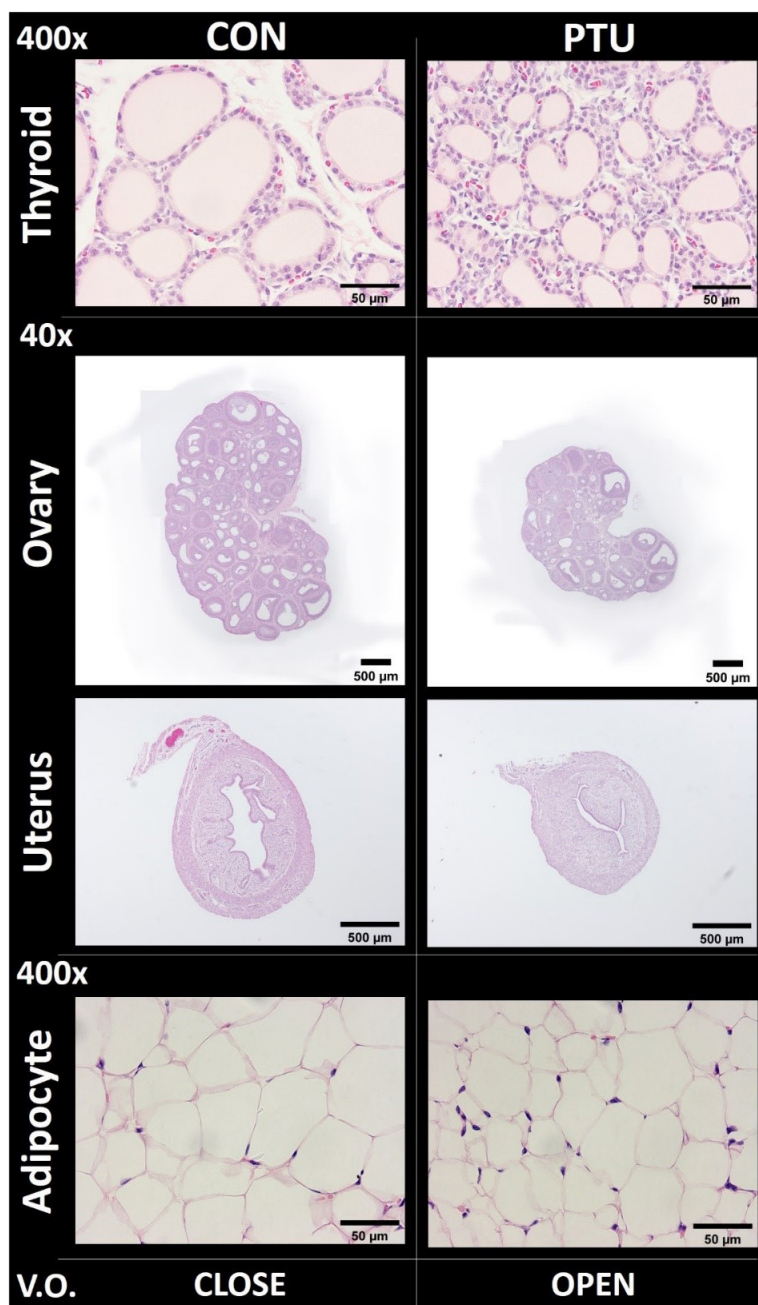


Fig. 4. Histological analyses of the tissues from female offspring exposed to maternal treatment with PTU. At PND36, animals were sacrificed and the thyroids, ovaries, uteri, ovarian fat pads were immediately collected and fixed in 4% paraformaldehyde. The samples were applied to the standard paraffin section and hematoxylin & eosin staining method. Representative microphotographs ($\times 40$; ovaries, uterus, $\times 400$; thyroid, ovarian fat pad-adipocytes). PTU, propylthiouracil; PND, postnatal day.

7. Histological parameter analysis

Table 3 showed histological parameters such were luminal area, epithelial cell width, gland number, follicle number, and adipocyte cell number. In the thyroid of PTU group, follicle luminal and epithelial width were significantly reduced ($p < 0.001$). In the uterus of PTU group, widths of perimetrium and myometrium, and epithelial area were significantly reduced ($p < 0.01$ and $p < 0.001$, respectively). In the ovary of PTU group, numbers of primary, secondary and Graafian follicles were

Table 3. Effects of maternal PTU treatment on histological parameters of thyroid, ovary, uterus and adipocyte at PND36

		CON	PTU
Thyroid	Area of follicle lumen (μm^2)	3,106.60 \pm 130.025	1,366.92 \pm 34.059***
	Width of epithelium (μm)	7.73 \pm 0.166	5.33 \pm 0.103***
Uterus	Width of perimetrium (μm)	75.60 \pm 5.476	53.82 \pm 3.804**
	Width of myometrium (μm)	83.04 \pm 4.664	64.22 \pm 3.651**
	Number of endometrium gland	4.67 \pm 0.667	4.67 \pm 1.453
	Area of epithelium (μm^2)	29.45 \pm 3.196	12.97 \pm 1.098***
Ovary	Number of primary follicles	21.67 \pm 3.383	32.67 \pm 9.333
	Number of secondary follicles	24.67 \pm 6.386	37.33 \pm 4.177
	Number of Graafian follicles	6.00 \pm 2.517	3.67 \pm 1.333
	Number of corpus lutea	0	2.33 \pm 2.333
Area of adipocyte (μm^2)		1,861.93 \pm 69.279	1,318.18 \pm 58.123***

Mother rats were supplied with water or 0.025% propylthiouracil from gestation day 14 to offspring postnatal day (PND) 21. After PND 21, offspring rats were supplied with non-propylthiouracil water until sacrifice. The parameters were measured by ImageJ software.

Values are shown as mean \pm SE.

Significant differences are marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; compared with the CON group).

PTU, propylthiouracil.

not significantly changed, while the number of the corpus luteum was 2.33 \pm 2.333 in PTU group and 0 in CON group. In the ovarian fat pad adipocytes of PTU group, cell areas were significantly reduced ($p < 0.001$).

DISCUSSION

The primary function of the thyroid is the production of the iodine containing THs, triiodothyronine (T₃) and thyroxine (T₄) and the peptide hormone calcitonin (Hall and Guyton, 2011). THs have a wide range of effects on the human body in metabolism, cardiovascular function, development and play a role in maintaining normal sexual function (Colledge et al., 2010). Two main TH-associated thyroid disorders are hyperthyroidism and hypothyroidism; hyperthyroidism is characterized by excessive secretion of THs, and hypothyroidism is characterized by a deficient secretion of THs (Taylor et al., 2018). In the present study, the absolute weight of the thyroid from female offspring was not changed by PTU treatment from GD14 through PND 21. However, the relative weight was increased significantly about 2.8 times ($p < 0.001$), indicating that the hypothyroidism pursued by this experimental model was successfully induced.

Pituitary TSH, hypothalamic TRH, and thyroid THs form the hypothalamic-pituitary-thyroid hormonal axis (HPT), and in general, elevated blood levels of THs inhibit the release of TRH and TSH, whereas the opposite effect occurs when serum TH levels decrease (Choksi et al., 2003). Furthermore, HPT and the hypothalamic-pituitary-gonadal axis (HPG) are physiologically related and act together as a unified system in some pathological conditions (Doufas and Mastorakos, 2000). There are several reports that speculate how thyroid disorders induce abnormal puberty based on interaction between the two neuroendocrine axes (Castañeda Cortés et al., 2014; Fekete and Lechan, 2014; Kiyohara et al., 2017). The junction of the two axes is estimated to be hypothalamic gonadotropin-inhibitory hormone (GnIH, also known as RF amide-related peptide coded by the NPVF gene); TH-mediated HPG regulation may be initiated by decrease of GnIH expression,

which acts at the most upstream level of the HPG axis by inhibiting the activity of GnRH neurons to reduce circulating levels of gonadotropins (LH and FSH) and gonadal sex steroids (Tsutsui et al., 2018). The precise mechanism of TH action, however, on puberty onset is still unclear. In addition, there is conflicting results regarding the effect of abnormal thyroid status on reproductive development. For example, hypothyroidism in girls can cause alterations in the pubertal process, usually a delay, but occasionally it can result in pseudo-precocious puberty (Doufas and Mastorakos, 2000). In the present study, we found that there was no significant difference in the date of VO between control and PTU group. It is intriguing that, the body weight and ovarian weight of PTU group were greatly reduced compared to the weights of control group while VO could be checked almost simultaneously in both groups. The ovary of PTU group might have equivalent ability to induce VO, despite of the tiny size. At this time, we tentatively concluded that there is no strong correlation between body/ovarian weights and VO when maternal hypothyroidism model is employed. To confirm this idea, one should carefully compare the available data from several precocious and/ or delayed puberty of rat models (Lee et al., 2009; Sun et al., 2021).

Regarding the determination of the puberty onset in female rodents, date of VO has been accepted as most reliable phenotypic signal. We often found that incomplete opening of vagina still collection of epithelial cells is possible particularly in PTU group, and these cases could make a serious error. Therefore, some auxiliary index will be helpful, and serum hormone levels and histology might be served as final confirming index at postmortem.

Taken together, the present study demonstrates the maternal hypothyroidism induced by PTU administration exerts minimal effect on pubertal development symbolized by VO despite of huge retardation in somatic growth. Further studies using more sophisticatedly designed hypothyroidism model will be especially important, and it will help to advance our understanding of pubertal development and related disorders where and when HPT and HPG axis are mutually controlled.

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