Chinese Journal of Physiology 61(3): 152-162, 2018
DOI: 10.4077/CJP.2018.BAG551

Effects of Thyroid Dysfunction on Reproductive Hormones in Female Rats

Juan Liu[1, 4], Meng Guo[2], Xusong Hu[1], Xuechun Weng[1], Ye Tian[1], Kaili Xu[1], Dai Heng[1], Wenbo Liu[1], Yu Ding[1], Yanzhou Yang[3], and Cheng Zhang[1]

[1]College of Life Science, Capital Normal University, Beijing 100048
[2]Department of Laboratory Animal Science, School of Basic Medical Science, Capital Medical University, Beijing 100069
[3]Key Laboratory of Fertility Preservation and Maintenance, Ministry of Education, Key Laboratory of Reproduction and Genetics in Ningxia, Department of Histology and Embryology, Ningxia Medical University, Ningxia 750004
and
[4]State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China

Abstract

Thyroid hormones (THs) play a critical role in the development of ovarian cells. Although the effects of THs on female reproduction are of great interest, the mechanism remains unclear. We investigated the effects of TH dysregulation on reproductive hormones in rats. Propylthiouracil (PTU) and L-thyroxine were administered to rats to induce hypo- and hyperthyroidism, respectively, and the reproductive hormone profiles were analyzed by radioimmunoassay (RIA). Ovarian histology was evaluated with hematoxylin and eosin (H&E) staining, and gene protein level or mRNA content was analyzed by western blotting or reverse transcription polymerase chain reaction (RT-PCR). The serum levels of gonadotropin releasing hormone (GnRH) and follicle stimulating hormone (FSH) in both rat models were significantly decreased on day 21, although there were no significant changes at earlier time points. There were no significant differences in luteinizing hormone (LH) or progesterone (P_4) levels between the treatment and the control groups. Both PTU and L-thyroxine treatments downregulated estradiol (E_2) concentrations; however, the serum testosterone (T) level was increased only in hypothyroid rats at day 21. In addition, the expression levels of FSH receptor, cholesterol side-chain cleavage enzyme (P450scc), and steroidogenic acute regulatory protein (StAR) were decreased in both rat models. Moreover, the onset of puberty was significantly delayed in the hypothyroid group. These results provide evidence that TH dysregulation alters reproductive hormone profiles, and that the initiation of the estrous cycle is postponed in hypothyroidism.

Key Words: hyperthyroid, hypothyroid, ovary, rat, reproductive hormone

Corresponding authors: [1] Cheng Zhang, Ph.D., College of Life Science, Capital Normal University, Beijing 100048, People's Republic of China. Tel: +86-10-6890-3623; E-mail: zhch8012@163.com; [2] Dr. Yanzhou Yang, Key Laboratory of Fertility Preservation and Maintenance, Ministry of Education, Key Laboratory of Reproduction and Genetics in Ningxia, Department of Histology and Embryology, Ningxia Medical University, Ningxia 750004, People's Republic of China. E-mail: alnord820119@163.com
Received: July 31, 2017; Revised (Final Version): March 16, 2018; Accepted: March 19, 2018.
 ISSN : 0304-4920. http://www.cps.org.tw

Introduction

Ovarian folliculogenesis and follicular development are complex processes that are tightly regulated by endocrine, autocrine and paracrine factors (21, 36, 41). The coordinated biosynthesis of reproductive hormones during the reproductive cycle is critical for maintaining normal female reproductive function. Cross-talk among granulosa cells, theca cells, and oocytes is critical for folliculogenesis and steroidogenesis, which maintain follicular development and maturation in mammals (46, 47). Although gonadotropins are important for follicle development, thyroid hormones (THs) have been highlighted as possible endocrine factors involved in ovarian function. It is well known that the THs, triiodothyronine (T_3) and thyroxine (T_4), are involved in various biological processes, including growth, energy metabolism, maturation, embryonic development, and cellular differentiation. Studies have shown that thyroid disease is the second most common endocrine disorder in women of childbearing age (37), and THs are vital for normal reproductive function (33). Dysregulation of the hypothalamic-pituitary axis is associated with reproductive disorders, including impaired follicular development. Hypothyroidism causes impaired fertility, including attenuated follicle development in multiple species (25), and subclinical hypothyroidism often results in infertility and can cause miscarriage and stillbirth in humans (31). However, these reproductive abnormalities are markedly reversed by TH therapy, especially in the presence of gonadotropin although gonadotropin alone is effective (23-25, 29). In hyperthyroidism, both spontaneous abortion (early pregnancy) and stillbirth (late pregnancy) pregnancy losses are common (1). The number of ovarian follicles at different stages is reduced in both hypo- and hyperthyroid rats (13). Previous studies have also suggested that THs alter steroidogenesis in ovarian cells in various species, including pigs (2, 17), cattle (42), mouse (7), rat (16) and humans (44) *in vitro*. Dysregulation of THs suppresses ovarian follicular growth and function, which indicates that hypo- and hyperthyroidism are potentially related to the dysregulation of the hypothalamic-pituitary-gonadal axis. The negative effects of TH dysregulation on ovarian function may be due to disordered reproductive hormone profiles. Indeed, thyroidectomy influences the secretion of gonadotropins in ovariectomized cows (43); however, whether hypothyroidism affects the levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) remains controversial (14). The effects of hyperthyroidism on FSH and LH are also not well understood. Therefore, the main objectives of the present study were to induce hypo- and hyperthyroid states *via* administration of propylthiouracil (PTU) and exogenous L-thyroxine, respectively, and to subsequently evaluate their influence on reproductive hormones and puberty initiation in immature rats.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) excluding following reagents. The commercial radioimmunoassay (RIA) kits for hormones assay from Research Institute of North (Beijing, China). The enhanced chemiluminescence (ECL) detection kit was obtained from Amersham Life Science (Oakville, ON, Canada). Acrylamide (electrophoresis grade), N,N′-methylene-bis-acrylamide, ammonium persulfate, glycine, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) prestained molecular weight standards, nitrocellulose membranes, and horse radish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse immunoglobulin (Ig) G were products of Bio-Rad (Richmond, CA, USA). Rhodamine-conjugated goat anti-rabbit and mouse IgGs were product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-mouse FSH receptor (FSHR)/LH receptor (LHR) was from Bioworld Technology, Inc. (Bioworld Technology, USA). TRIzol Reagent was a product of life (USA). FastQuant Reverse Transcription Kit (RT Kit) (with gDNA) and SuperReal PreMix Plus (SYBR Green) kit were purchased from TIANGEN (Beijing, China). PCR primers for steroidogenic acute regulatory protein (StAR) CYP11A1, β-actin were from Sunbiotech (Beijing, China).

Animal Treatments

Since immature rats before puberty have lower GnRH pulsing with basic level of FSH, LH, estradiol (E_2) concentrations than those in mature rats, we selected Sprague-Dawley rats (21 day old) to perform the followed experiments. Rats were obtained from the Beijing Vital Laboratory Animal Technology Co. (Beijing, China). Rats were housed in a temperature (23 ± 2°C) and humidity (60 ± 2%) with a 12/12 h light/dark cycle room and received pathogen-free water and food for maintenance except special requirement. All procedures were approved by the Institutional Animal Care and Use Committee of Capital Normal University and in accordance to the Principles of the Care and Use of Laboratory Animals and China Council on Animal Care.

Rats were divided into two experimental groups

and their respective controls: hypothyroid rats (HypoT, n = 36) and untreated rats (EUT, n = 18); hyperthyroid rats (HyperT, n = 36) and vehicle-treated rats (VEH, n = 18). The day of drug administration was considered as day 0 of the experiment.

The hypo- and hyperthyroidism rats model were induced as previous report (13, 18). Briefly, the hypothyroidism group was treated with 0.1 g/L PTU in drinking water for 21 days (PTU, Sigma-Aldrich). The hyperthyroidism rats were induced by subcutaneous L-thyroxine (L-thyroxine was dissolved in 0.1 mm NaOH (sodium hydroxide) solution and diluted in physiological saline) injection (250 μg/kg body weight) for 21 consecutive days (3, 13) (T_4: Sigma-Aldrich). The vehicle-treated rats were injected with 0.9% (w/v) sodium chloride solution. Since no statistical differences were observed between the EUT and VEH groups in any of the studied variables, we grouped and analyzed them as a single control group.

On days 0, 7, 14 and 21 during treatment, nine rats in each time point were sacrificed and blood were collected.

Serum Collection and Hormone Level Analysis

The serum was collected after clotting and centrifuging (4,000 × *g*, 10 min) at room temperature (RT). And then serum was used to determine the concentrations of GnRH, FSH, LH, testosterone (T), progesterone (P_4) and E_2 using commercial RIA kits (Research Institute of North Beijing, China). The sensitivity of the GnRH, FSH, LH, T, P_4 and E_2 were 5 pg/ml, <1.0 mIU/ml, 1.0 mIU/ml, 0.02 ng/ml, <0.2 ng/ml, <5 pg/ml, respectively. The intra-assay coefficients of variation were <10% for hormones (13).

Protein Extraction and Western Blot

Ovaries were collected and western blot analysis was performed as described previously (47). Briefly, the samples were homogenized in buffer containing tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl 20 mM, pH 7.5), sucrose (0.25 M), $MgCl_2$ (2.5 mM), Ethylenediaminetetraacetic acid (EDTA, 2.5 mM), KCl (10 mM), thimerosal (0.02%), and a protease inhibitor cocktail (Sigma-Aldrich). And then, the homogenate was centrifuged for 10 min at 800 g and collected the supernatant. The supernatant was centrifuged again (14,000 × *g*, 4°C, 30 min). All protein extraction was carried out on ice and protein concentrations were determined by the Bradford assay (Bio-Rad). 15 μg of protein of cell lysates were subjected to SDS-PAGE (10%) and then electrophoretically transferred to a nitrocellulose membrane for 2 h. After transfer, the membranes were blocked with 5% (wt/vol) nonfat dry milk in Tris buffered saline with Tween 20 (TBST, 20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20; pH 7.5) buffer for 1 h (RT). Antibodies against FSHR (1:1000, Catalog#:ab75200) was from Abcam (Cambridge, Mass., USA), LHR (1:1000; Catalog#:sc-25828) and StAR (1:1000; Catalog#:sc-25806) purchased from Santa Cruz Biotechnology (Santa Cruz, Beijing, China), CYP11A1 (1:1000; Catalog#:14217S) was from Cell Signaling (Danvers, MA, USA) and β-actin (1:10000; Catalog#:sc-81178) purchased from Santa Cruz Biotechnology were used to incubate (4°C, overnight) with membranes. The membranes were then treated with secondary antibody (1:5000, 1:10000, respectively) in TBST buffer containing 5% nonfat milk for 1 h at RT. After washing three times with TBST buffer, immunoreactive bands were visualized with the ECL kit according to the manufacturer's instructions, and protein content was determined by densitometrically scanning the exposed x-ray film. Immunoreactions signals were analyzed using gel-pro Analyzer 4.0.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA of ovaries were isolated with TRIzol Reagent. After DNAase I treatment, 1.5 ug total RNA were reverse transcribed into cDNA. Quantitative RNA analysis for StAR, CYP11A1 and β-actin were performed by using a LightCycler 2.0 System (Roche Diagnostic, Indianapolis, IN, USA). The StAR primers used for amplification were a 5' forward primer (5'-GGCTGTCTTCTGTCCCTA-3') and a 3' reverse primer (5'-GGTCCACCAGTTCTCATA-3'). The CYP11A1 primer sequences were 5'-TCAACCGCTCTTGTCTTT-3' (5' forward primer) and 5'-TCTCCCTACCACTTCCCT-3' (3' reverse primer). The β-actin primer sequences used were a 5' forward primer (5'-AACCCTAAGGCCAACCGTGAAAAG-3') and a 3' reverse primer (5'-CGACCAGAGGCATACAGGGACAAC-3'). The SuperReal PreMix Plus (SYBR Green) kit was subsequently used to amplify the related genes. The heat cycling reactions were comprised by an initial denaturation step (95°C, 15 min) and then, the amplification reaction comprised 40 cycles (95°C for 10 s, 60°C for 20 s, and 72°C for 32 s, separately) for StAR, CYP11A1 and β-actin and the PCR products were followed by melted at 60°C for 30 s. The melting curve analysis at the described PCR working conditions shows a single peak without primer-dimers for the StAR, CYP11A1 and β-actin primer sets, respectively. Each gene includes a standard curve. PCR without reverse-transcribed cDNA were used as negative controls. The results were normalized to the expression levels of β-actin, a housekeeping gene, by the $2^{-\Delta\Delta Ct}$ method (27).

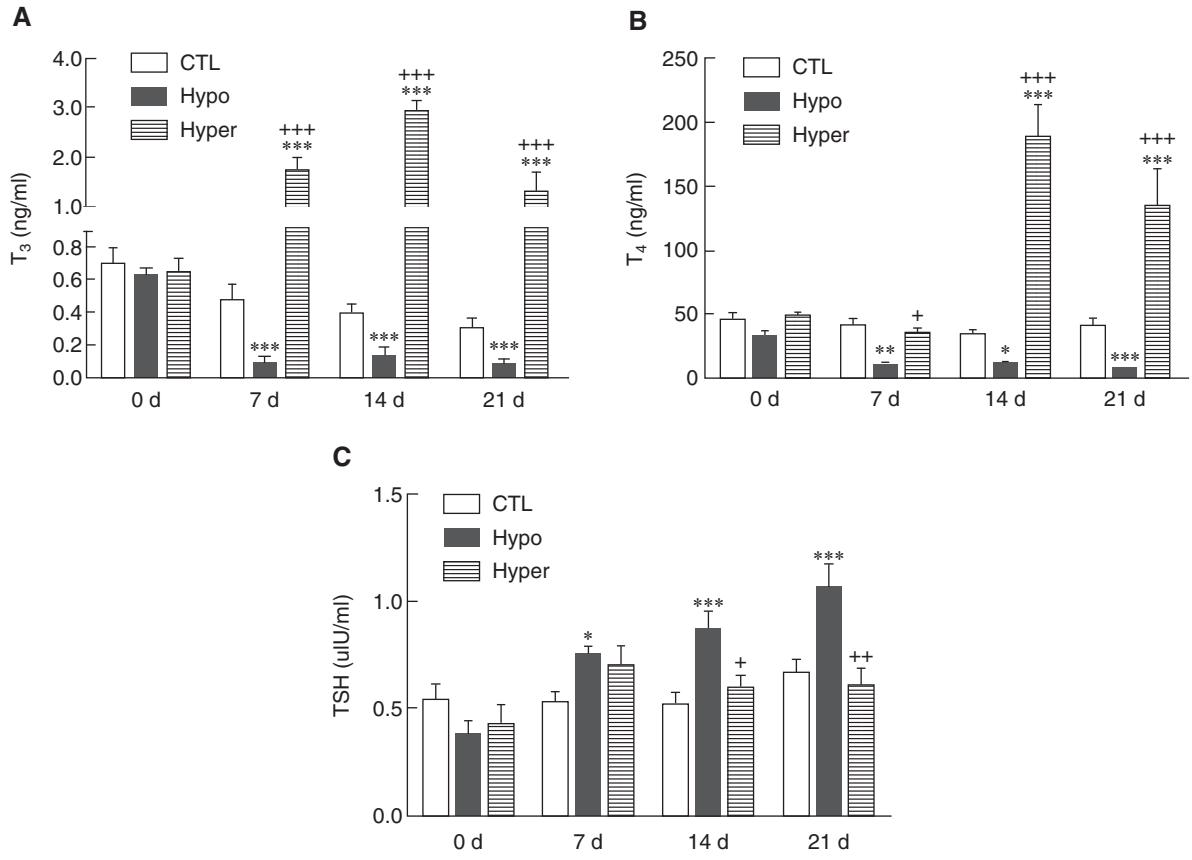

Fig. 1. Effect of thyroid dysfunction on serum concentration of T_3, T_4 and TSH. The immature rats (21 days) were treated with 0.1 g/L PTU in drinking water or subcutaneously injected with L-thyroxine injection (250 μg/kg body weight) for 21 consecutive days to construct hypo- and hyperthyroidism rats model. The serum was collected for detecting hormones by RIA method. (A) In contrast to changes in the hypothyroidism group, the serum T_3 concentration was significantly increased in the hyperthyroidism group. (B) The serum T_4 levels showed the same tendency with T_3 in both the hypo- and hyperthyroidism groups. (C) TSH concentration in the hypothyroidism group was significantly increased although TSH level was not dramatically changed in the hyperthyroidism group. * indicates significant difference from the control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), + indicates significant difference between hypo- and hyperthyroid rats (+ $P < 0.05$, ++ $P < 0.001$, +++ $P < 0.001$). T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone.

Histology Examination

After fixation in paraformaldehyde, rat ovarian tissue specimens were subjected to conventional dehydration followed by paraffin embedding and sectioning (5 μm thick). Hematoxylin and eosin (H&E) staining was performed to observe general histology of the rat ovary.

Onset of Puberty Monitoring

In order to determine whether THs treatment affected the onset of puberty, the vaginal opening of was checked daily. The estrous cycle was examined daily and identified under a microscope (200 ×) using a vaginal smear flushed with physiological saline after vaginal opening (39, 40). One mature rat was used as a positive control.

Statistical Analysis

All the experiments were independently replicated at least three times, and the data were presented as means ± standard error of the mean (SEM). All proportional data were analyzed using one way (repeated-measure) analysis of variance (ANOVA) (Prism 5.0 statistical software; GraphPad Software, Inc., San Diego, CA). Significant differences between treatment groups were determined by the Dunnett's test. Statistical significance was defined at $P < 0.05$.

Results

Relative Hormone Concentration of Thyroid Dysfunction

To facilitate our investigation of the reproductive

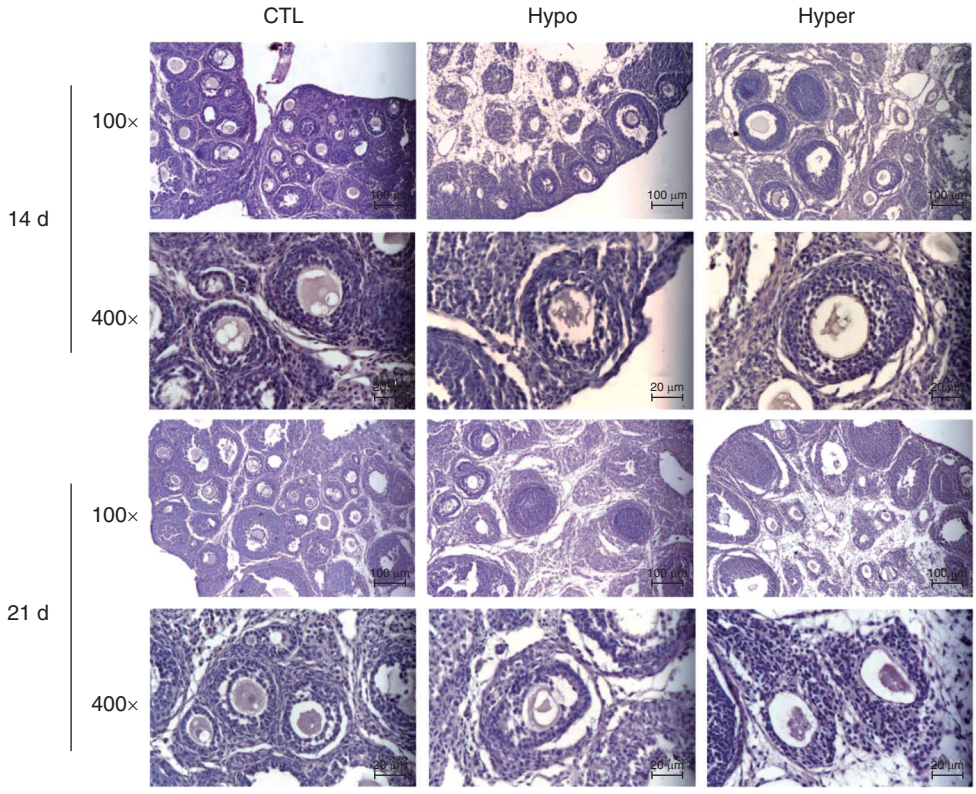

Fig. 2. H&E staining in the rat ovarian follicles. As described in the Materials and Methods, the paraffin sections of the ovaries were prepared. H&E staining was performed to compare the rat ovarian follicle development among the three groups. Magnification, 100× and 400×, Scale bar, 100 μm and 20 μm.

hormones underlying dysregulated THs, we utilized rodent models of hypo- and hyperthyroidism, induced by PTU and L-thyroxine injection, respectively (11, 13). As expected, circulating T_3 (Fig. 1A) and T_4 levels (Fig. 1B) were significantly lower than in the control group. Consistent with the previous report (14), thyroid stimulating hormone (TSH) values were increased in the hypothyroid group beginning at day 7 (Fig. 1C). In the hyperthyroid group, T_3 values were dramatically increased after L-thyroxine treatment (Fig. 1A) on days 14 and 21. Meanwhile, T_4 levels were significantly higher from day 14 ($P < 0.001$; Fig. 1B) to day 21 ($P < 0.001$; Fig. 1B) although the TSH concentration had no significantly changed.

Ovarian Histologic and Morphometric in Thyroid Dysfunction Rat

As shown in Fig. 2, the internal structure of ovaries was scattered in the hypo- and hyperthyroid groups compared with that the control group. And histologic observation demonstrated the presence of fewer follicles in thyroid dysfunction rat.

Effect of THs on Serum GnRH

On day 14 the serum gonadotropin releasing hormone (GnRH) levels in both the hypo- ($P > 0.05$; Fig. 3A) and hyperthyroid ($P > 0.05$; Fig. 3A) groups were not changed significantly compared with the controls ($P > 0.05$; Fig. 3A). However, on day 21, reductions were observed in both experimental groups (Fig. 3A).

Dysregulation of THs Affect FSH and LH Concentration

Although serum FSH concentration was downregulated in either hypo- and hyperthyroidism rat at day 14, there was no significant difference. Serum FSH was downregulated by L-thyroxine treatment ($P < 0.01$; Fig. 3B) at day 21. Reduced serum FSH concentration was also observed in the hypothyroid group ($P < 0.05$; Fig. 3B). Although the LH concentration was not significantly influenced by the different treatments, the LH level was lower in the hyper- versus hypothyroid group at day 14 ($P < 0.05$; Fig. 3C).

Steroid Hormones Concentration in Control, Hypo- and Hyperthyroid Rat

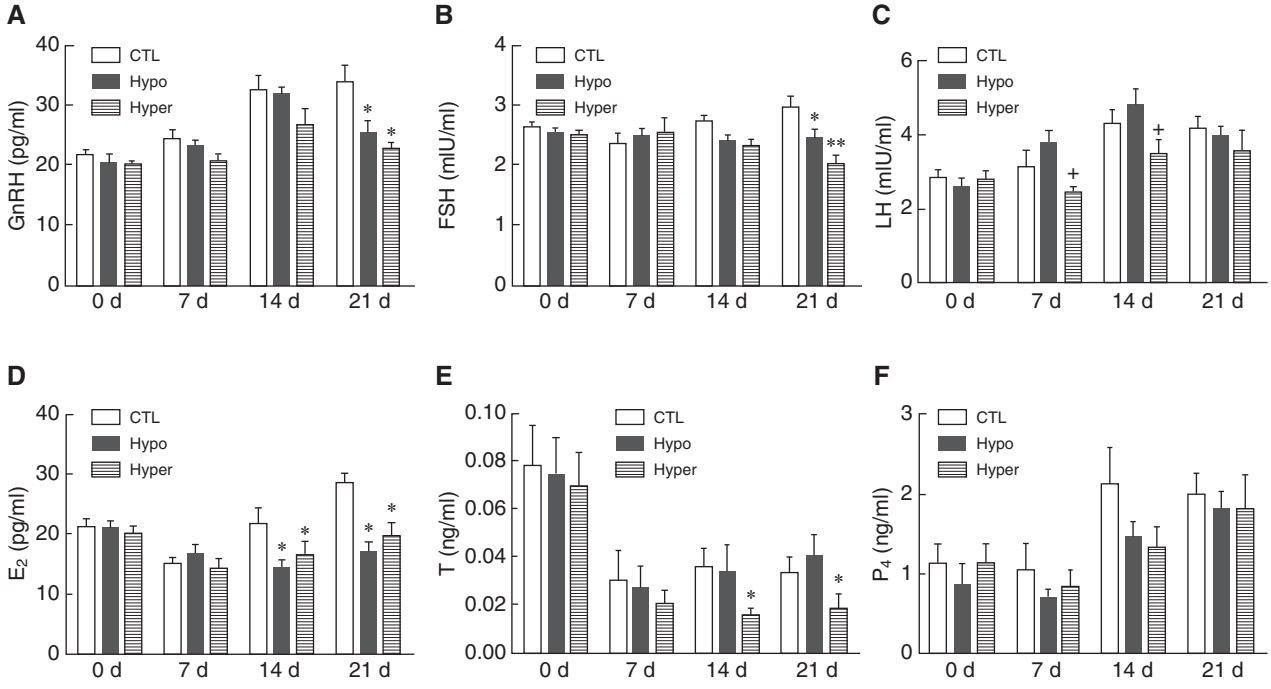

Fig. 3. Effect of thyroid dysfunction on serum reproductive hormones. The serum was collected after clotting and centrifuging (4,000 × *g*, 10 min) at room temperature. And reproductive hormones concentrations were detected by RIA method. (A) Significant reduction of the serum levels of GnRH in both the hypo- and hyperthyroid rats on day 21. (B) Significant reduction of FSH concentrations but not serum FSH levels in the earlier period in both the hypo- and hyperthyroid groups compared with control group on day 21. (C) No significant differences were detected between the hypo- and hyperthyroidism with control group respectively. (D) The serum E_2 concentrations were reduced in the hypo- and hyperthyroid groups compared with control group on day 14 and 21 respectively. (E) The serum T level was only decreased in the hyperthyroid rats on day 14 and 21. (F) There were no significant differences in serum P_4 concentration among three groups. * indicates significant difference from control (* $P < 0.05$; ** $P < 0.01$). + indicates significant difference between hypo- or hyperthyroid rats (+ $P < 0.05$). T: Testosterone; E_2: Estradiol; P_4: Progesterone.

To assess the ovarian steroidogenic capability of TH dysregulation rats, the levels of serum E_2, T and P_4 were also measured. The E_2 concentrations were reduced in both hypo- and hyperthyroid groups compared with the control group ($P < 0.05$; Fig. 3D). Moreover, the down-regulation of E_2 began on day 14. In hyperthyroid rats, the serum T concentration was significantly decreased compared with euthyroid controls ($P < 0.05$; Fig. 3E) at day 14, whereas the serum T level was not significantly altered in the hypothyroid group ($P > 0.05$; Fig. 3E). Moreover, the changes were not significant in either group at day 21 ($P > 0.05$, Fig. 3E). The concentration of P_4 was not significantly affected by either treatment ($P > 0.05$, Fig. 3F).

Deregulation of THs Affects FSHR and LHR Proteins Expression in Ovary

We conducted western blotting to test whether TH dysregulation affects the expression of FSHR and LHR in the rat ovary. Protein levels of FSHR in ovarian tissue were not significant changed in in both hypo- and hyperthyroid rats at day 14 (Fig. 4A). However, FSHR was decreased in both hypo- ($P < 0.05$; Fig. 4B) and hyperthyroid rats ($P < 0.05$; Fig. 4B) at day 21. LHR expression was decreased in hyperthyroid rats ($P < 0.05$; Fig. 4C) at day 14, whereas it was not significantly altered in the hypothyroid group ($P > 0.05$; Fig. 4D).

Effect of TH Deregulation on CYP11A1 and StAR Content in Ovary

To test whether TH dysregulation affects CYP11A1 and StAR expression in the rat ovary, western blotting was used. Protein levels of CYP11A1 in ovarian tissue were decreased in both hypo- ($P < 0.01$; Fig. 5B) and hyperthyroid rats, ($P < 0.01$; Fig. 5B) at day 21. However, CYP11A1 protein levels were not significantly affected at day 14 (Fig. 5A). Decreased StAR expression was also detected in both rat models at days 14 ($P < 0.05$; Fig. 5C) and 21 ($P < 0.05$; Fig. 5D). The mRNA levels of CYP11A1 and StAR were

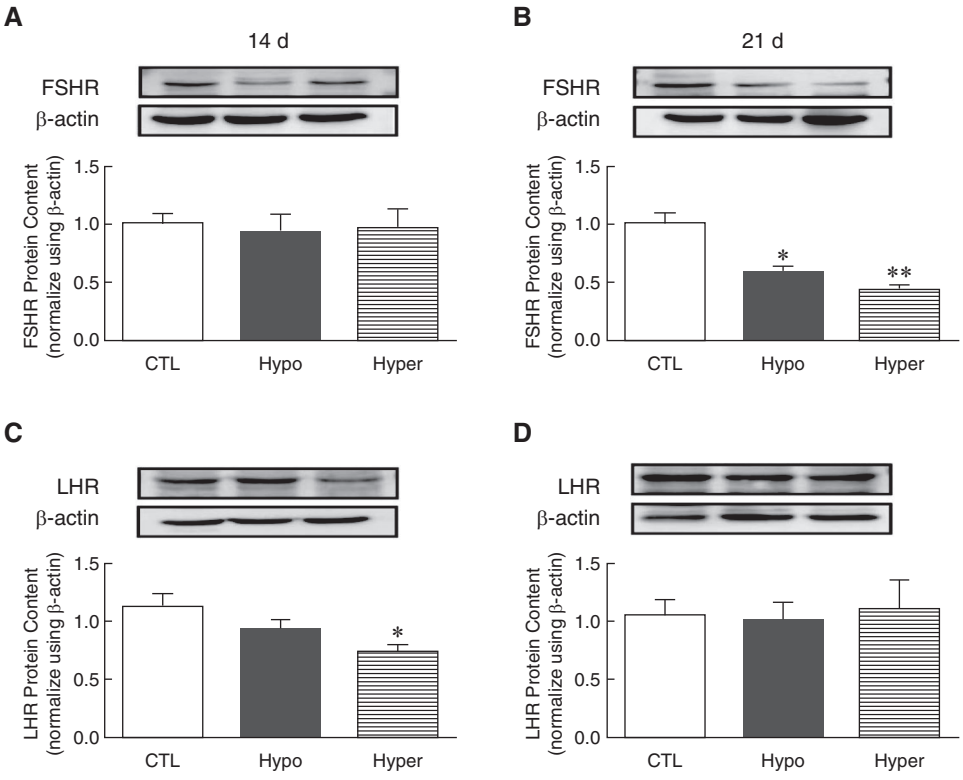

Fig. 4. Effect of THs on FSHR and LHR expression. The total proteins were extracted from rats' ovaries and FSHR, LHR proteins were detected by western blot. The protein contents were normalized by β-actin. (A) No significant differences were detected among three groups on day 14. (B) Significant reduction of FSHR protein content in both the hypo- or hyperthyroid rats on day 21. (C) Expression of LHR was decreased in the hyperthyroid rats on day 14. (D) There were no significant differences in LHR expression among three groups. * indicates significant difference from control (* $P < 0.05$; ** $P < 0.01$).

also assessed. Transcript levels of CYP11A1 in ovarian tissue were not significantly altered in either hypo- or hyperthyroid rats ($P > 0.05$; Fig. 5E, F). However, StAR mRNA relative expression was significantly increased in the hypothyroid rats at day 21 ($P < 0.01$; Fig. 5H).

The Effect of THs on the Onset of Rat Puberty

Puberty onset in the rat is typically characterized by vaginal opening. The estrous cycle is divided into proestrus, estrous, metestrus and diestrus phases, which are determined by vaginal smear collections (9, 15, 26, 39, 40). Our results showed that vaginal opening was observable at day 11 [11.50 ± 0.27] in the control group after treatment as day 0. There were no significant differences between the control rats and hyperthyroid rats ($P > 0.05$; Fig. 6). However, the onset of puberty was significantly delayed by PTU treatment in the hypothyroid group ($P < 0.05$).

Discussion

In the present study, hypo- and hyperthyroidism were induced in rats by treatment with PTU and exogenous L-thyroxine. The serum levels of T_3 and T_4 in the hypo- and hyperthyroidism groups were significantly different, which indicates the rat models are reliable. The endocrine levels of pituitary hormones (FSH and LH) and steroid hormones (E_2 and T) were dysregulated in the hypo- and hyperthyroid rats. Additionally, the onset of the estrus cycle in hypothyroid rats was delayed.

The pulsated secretion of GnRH is a prerequisite for normal mammalian follicular development and ovulation. GnRH regulates the synthesis and secretion of FSH and LH from the pituitary, which are important in ovarian cell development (4, 6). Our results in Sprague-Dawley rats showed that, while treatment with PTU or exogenous L-thyroxine had little immediate effect on GnRH, serum GnRH concentrations were significantly decreased in both models at day 21. We observed a decrease in FSH levels, which may have been due to decreased GnRH. However, serum LH concentrations were not significantly affected. Similarly, another study showed that the serum LH level was not significantly altered in hypothyroid dwarf rats treated with thyroxine and equine chori-

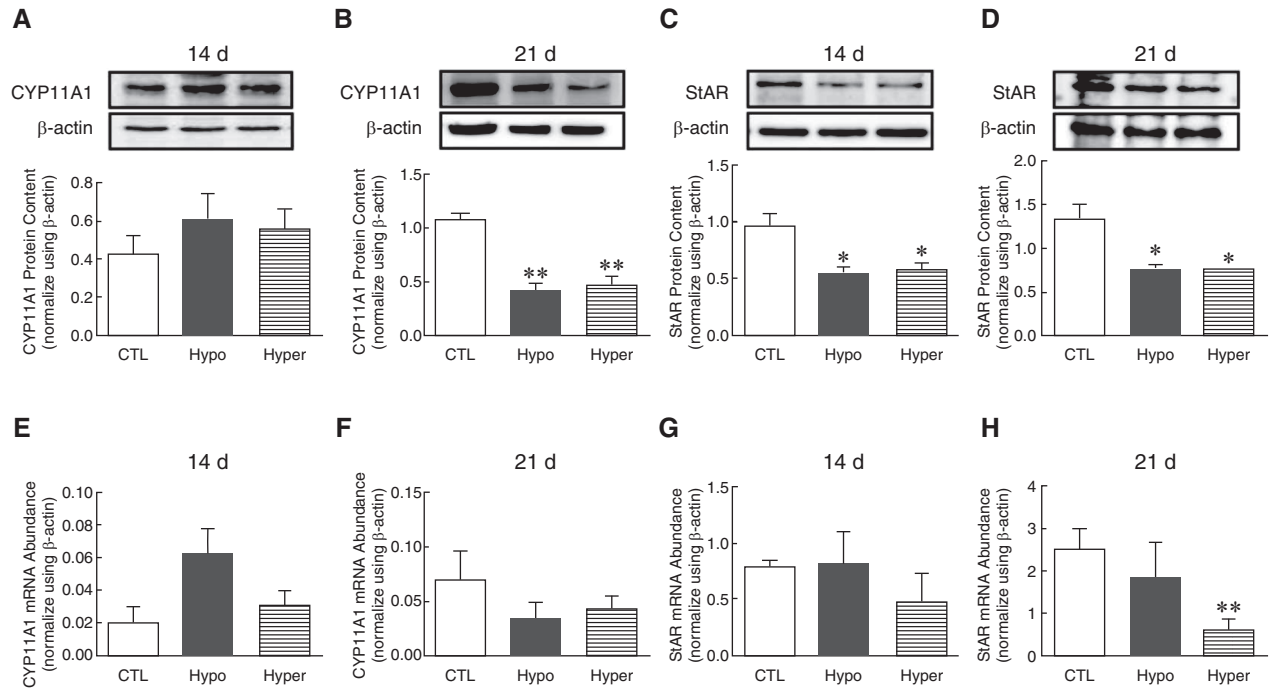

Fig. 5. Effect of THs on CYP11A1 and StAR content. The StAR, CYP11A1 proteins and mRNA in ovaries were detected by western blot and quantitative RT-PCR, respectively. (A) No significant differences of CYP11A1 protein content in both the hypo- or hyperthyroid groups compared with control group on day 14. (B) Significant reduction of CYP11A1 protein content in both the hypo- or hyperthyroid rats on day 21. (C) Protein levels of StAR in ovary tissue were decreased in both the hypo- or hyperthyroidism rats on day 14. (D) The change manners of StAR protein in both model rats on day 21 were similar with that on day 14. (E) No significant change of CYP11A1 mRNA level in both the hypo- or hyperthyroid groups compared with control group on day 14. (F) No significant change of CYP11A1 mRNA level in both the hypo- or hyperthyroid groups compared with control group on day 21. (G) No significant change of StAR mRNA level in both the hypo- or hyperthyroid groups compared with control group on day 14. (H) Significant reduction of StAR mRNA level in the hyper groups compared with control group on day 21. * indicates significant difference from control (* $P < 0.05$; ** $P < 0.01$).

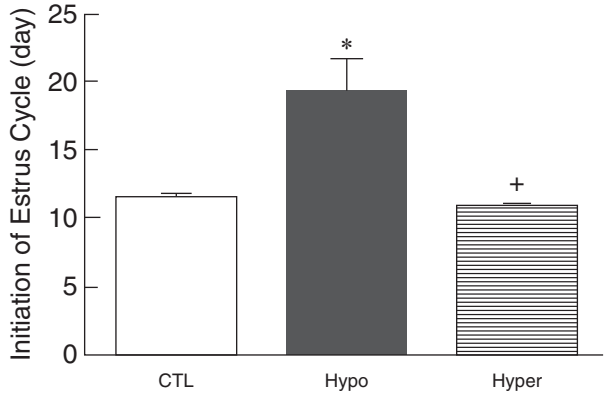

Fig. 6. Effect of thyroid dysfunction on rat puberty onset. Vaginal opening and smears were examined daily after PTU or L-thyroxine treatment. The mature rats were also used as a positive control. * indicates significant difference from control (* $P < 0.05$). + indicates significant difference between hypo- and hyperthyroid rats (+ $P < 0.05$).

onic gonadotropin (eCG) (25).

It has been reported that hypo- and hyperthyroidism are associated with dysregulation of the hypothalamic-pituitary axis and suppressed ovarian follicular growth and function (10, 13, 25). Therefore, dysregulation of TH prevents follicle from growing further mainly *via* down-regulating FSH concentration and or FSHR expression than LH level and LHR. We also detect the expression of LHR in ovary, and found that LHR expression was only down-regulated on day 14 in hyperthyroidism group. However, FSHR expression was decreased in both hypo- and hyperthyroidism groups. It is possible that disorder of TH mainly regulated FSH synthesis since FSH and TSH share the same glycoprotein alpha chain (CGA) as part of their protein structure (5, 35). It is possible that TH disorder impair the synthesis and secretion of FSH by regulating CGA expression (5). E_2 is one of the most important ovarian hormones and is derived from T (33). E_2 and T are both steroid hor-

mones, which are synthesized using cholesterol as the raw material. The cytochrome P450scc enzyme CYP11A1 is the only enzyme that catalyzes the conversion of cholesterol to pregnenolone, which is required for the biosynthesis of all steroid hormones. During steroid hormone biosynthesis, StAR plays a vital role as the rate-limiting step regulating cholesterol transfer within the mitochondria (22, 28). If these processes are impaired, synthesis of E_2 is also disrupted. Estrogens from pre-ovulatory follicles are used as an index of follicular maturation (25). Decreased expression of CYP11A1 in hypo- or hyperthyroid rats may affect the process of E_2 synthesis. In the present study, the lower serum concentration of E_2 on days 14 and 21 in hyperthyroid rats could have resulted from the observed decrease in T concentration, as the source material of E_2. And the lower T concentration was induced partially by the decreased expression of StAR on days 14 and 21. Moreover, TH inhibits FSH-induced aromatase activity in granulosa cells, which can also decrease E_2 levels (8). Although CYP11A1 mRNA abundance in the hypo and hyper rats in 21 d was not significantly changed, the protein expression of CYP11A1 was significantly decreased, suggesting that the regulation of CYP11A1 protein occurs at the translation level or *via* post-translational processing. The regulation of StAR in dysregulation of TH rats may be similar with CYP11A1. Additionally, decreased FSHR expression in hyperthyroid rats may also reduce ovarian sensitivity to FSH, leading to lower serum E_2 concentrations and retarded follicular development. However, the serum T level was not significantly altered in our hypothyroid rats, although E_2 levels remained low. These results are consistent with a previous report, in which spontaneous hypothyroidism was indicated by decreased secretion of E_2 (25). T_4 treatment completely removes the symptoms of hypothyroidism, including decreased E_2 levels (25), which indicates that insufficient TH affects the secretion of E_2. Moreover, lower serum E_2 is correlated with the level of sex hormone binding globulin (SHBG) and its binding activity (33), and even the conversion rate of T to Δ4-androstenedione (34). In hyperthyroidism, E_2 levels may also be related to the metabolic clearance rate of E_2 (38).

Rats with thyroid dysregulation exhibit lower serum E_2 levels, indicating that TH affects steroid biosynthesis in growing follicles, or decreases the number of receptors for gonadotropins in growing follicles. Decreased E_2 levels may be induced by lower FSH concentrations, causing decreased responsiveness of granulosa cells to FSH and retarded follicular growth and steroid production in these follicles (19, 30). Previous reports have shown that THs (T_4 and T_3) in the physiological range synergize with FSH to increase FSH-mediated induction of P_4 in granulosa cells in multiple species (20, 30, 44). Although P_4 levels were downregulated at days 7 and day 14 in both the hypo- and hyperthyroid groups in our study, there were no significant differences compared with the control group. It is possible that lower FSH and E_2 levels retard follicular development and decrease ovulation, which correlates with decreased LHR expression. In addition, it is well known that LH mainly induces ovulation and corpus luteum development during the latter stage of follicular development (45). And P_4 concentration is also gradually increased. However, serum LH concentrations were not significantly changed in both rat models in the present study. It partially explains that P_4 levels were not significantly changed. Inadequate corpus luteum P_4 secretion occurs when LH is delayed *in vivo*. Additionally, differences in the *in vitro* culture systems used may have been responsible for the discordant observations between different studies.

Immature rats (21 d old) were used in the present study, which partially eliminated the potential confounding effects of endogenous hormones, although puberty was initiated in most of the rats during the later stage of treatment. Immature rats with hypothyroidism have delayed sexual maturity. The present study indicates a possible relationship between thyroid function and the onset of puberty in rats. Delayed puberty in hypothyroid rats could be the direct result of a decreased interaction between THs and the hypothalamic-pituitary-gonadal axis, or of indirect effects mediated through altered metabolism since lower GnRH and FSH levels were present in hypothyroid rats. Prepubertal rats with hypothyroidism have more secondary, and fewer antral, follicles, smaller non-atretic antral follicles and more atretic follicles in the ovaries due to the impaired differentiation of granulosa cells (10). Moreover, hypothyroidism reduced the number of follicles, which resulted in a decrease in the number of ovulations (32). This clearly suggests a pathway whereby TH influences gonadal function, which is tightly correlated with imbalanced cross-talk between the hypothalamic-pituitary-gonadal axis and the hypothalamic-pituitary-thyroid axis (12).

In conclusion, our findings demonstrate that TH dysregulation alters reproductive hormonal profiles, and the initiation of the estrous cycle is delayed in hypothyroidism. This study significantly improves our understanding of the role of THs in female reproduction. Further studies are needed to elucidate the precise mechanisms underlying altered serum reproductive hormone profiles in rats with dysregulated THs.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No.31671555; No. 31300958), and Beijing Natural Science Foundation (No.5142003). This project was also supported by Scientific Research Program of Beijing Municipal Commission of Education (KM201610028011) and National Natural Science Foundation of China (No.81560259). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of Interest

The authors have nothing to declare.

References

1. Andersen, S.L., Olsen, J., Wu, C.S. and Laurberg, P. Spontaneous abortion, stillbirth and hyperthyroidism: a danish population-based study. *Eur. Thyroid. J*. 3: 164-172, 2014.
2. Asahara, S., Sato, A., Aljonaid, A.A. and Maruo, T. Thyroid hormone synergizes with follicle stimulating hormone to inhibit apoptosis in porcine granulosa cells selectively from small follicles. *Kobe J. Med. Sci*. 49: 107-116, 2003.
3. Ayala, C., Valdez, S.R., Morero, M.L.N., Soaje, M., Carreño, N.B., Sanchez, M.S., Bittencourt, J.C., Jahn, G.A. and Celis, M.E. Hypo- and hyperthyroidism affect NEI concentration in discrete brain areas of adult male rats. *Peptides* 32: 1249-1254, 2011.
4. Baird, D. Role of FSH and LH in follicle development. *J. Gynecol. Obstet. Biol. Reprod.* (*Paris*) 35: 2S24-2S29, 2006.
5. Bargi-Souza, P., Romano, R.M., Goulart-Silva, F., Brunetto, E.L. and Nunes, M.T. T_3 rapidly regulates several steps of alpha subunit glycoprotein (CGA) synthesis and secretion in the pituitary of male rats: Potential repercussions on TSH, FSH and LH secretion. *Mol. Cell. Endocrinol*. 409: 73-81, 2015.
6. Bartlewski, P.M., Vanderpol, J., Beard, A.P., Cook, S.J. and Rawlings, N.C. Ovarian antral follicular dynamics and their associations with peripheral concentrations of gonadotropins and ovarian steroids in anoestrous Finnish Landrace ewes. *Anim. Reprod. Sci*. 58: 273-291, 2000.
7. Cecconi, S., Rucci, N., Scaldaferri, M.L., Masciulli, M.P., Rossi, G., Moretti, C., D'Armiento, M. and Ulisse, S. Thyroid hormone effects on mouse oocyte maturation and granulosa cell aromatase activity. *Endocrinology* 140: 1783-1788, 1999.
8. Chan, W.K. and Tan, C.H. Inhibition of follicle-stimulating hormone induction of aromatase activity in porcine granulosa cells by thyroxine and triiodothyronine. *Endocrinology* 119: 2353-2359, 1986.
9. Davis, L.K., Murr, A.S., Best, D.S., Fraites, M.J., Zorrilla, L.M., Narotsky, M.G., Stoker, T.E., Goldman, J.M. and Cooper, R.L. The effects of prenatal exposure to atrazine on pubertal and postnatal reproductive indices in the female rat. *Reprod. Toxicol*. 32: 43-51, 2011.
10. Dijkstra, G., de Rooij, D.G., de Jong, F.H. and van den Hurk, R. Effect of hypothyroidism on ovarian follicular development, granulosa cell proliferation and peripheral hormone levels in the prepubertal rat. *Eur. J. Endocrinol*. 134: 649-654, 1996.
11. Ding, Y., Tian, Y., Guo, M., Liu, J., Heng, D., Zhu, B., Yang, Y. and Zhang, C. Regulation of glucose transport by thyroid hormone in rat ovary. *Cell Tissue Res*. 366: 455-466, 2016.
12. Dittrich, R., Beckmann, M.W., Oppelt, P.G., Hoffmann, I., Lotz, L., Kuwert, T. and Mueller, A. Thyroid hormone receptors and reproduction. *J. Reprod. Immunol*. 90: 58-66, 2011.
13. Fedail, J.S., Zheng, K., Wei, Q., Kong, L. and Shi, F. Roles of thyroid hormones in follicular development in the ovary of neonatal and immature rats. *Endocrine* 46: 594-604, 2014.
14. Fitko, R., Kucharski, J., Szlezyngier, B. and Jana, B. The concentration of GnRH in hypothalamus, LH and FSH in pituitary, LH, PRL and sex steroids in peripheral and ovarian venous plasma of hypo- and hyperthyroid, cysts-bearing gilts. *Anim. Reprod. Sci*. 45: 123-138, 1996.
15. Gereltsetseg, G., Matsuzaki, T., Iwasa, T., Kinouchi, R., Nakazawa, H., Yamamoto, S., Kuwahara, A., Yasui, T. and Irahara, M. Delay in the onset of puberty of intrauterine growth retarded female rats cannot be rescued with hypernutrition after birth. *Endocr. J*. 59: 963-972, 2012.
16. Grasso, P. and Crisp, T.M. *In vitro* responses of luteinizing rat granulosa cells to human thyroid-stimulating hormone. *Biol. Reprod*. 32: 935-945, 1985.
17. Gregoraszczuk, E.L. and Skalka, M. Thyroid hormone as a regulator of basal and human chorionic gonadotrophin-stimulated steroidogenesis by cultured porcine theca and granulosa cells isolated at different stages of the follicular phase. *Reprod. Fertil. Dev*. 8: 961-967, 1996.
18. Hapon, M.B., Varas, S.M., Jahn, G.A. and Gimenez, M.S. Effects of hypothyroidism on mammary and liver lipid metabolism in virgin and late-pregnant rats. *J. Lipid Res*. 46: 1320-1330, 2005.
19. Hatsuta, M., Abe, K., Tamura, K., Ryuno, T., Watanabe, G., Taya, K. and Kogo, H. Effects of hypothyroidism on the estrous cycle and reproductive hormones in mature female rat. *Eur. J. Pharmacol*. 486: 343-348, 2004.
20. Hayashi, M., Maruo, T., Matsuo, H. and Mochizuki, M. Effect of thyroid hormone on steroidogenic enzyme induction in porcine granulosa cells cultured *in vitro*. *Jpn. J. Endocr*. 63: 1231-1240, 1987.
21. Hunter, M.G. and Paradis, F. Intra-follicular regulatory mechanisms in the porcine ovary. *Soc. Reprod. Fertil. Suppl*. 66: 149-164, 2009.
22. Jahromi, M.S., Tehrani, F.R., Noroozzadeh, M., Zarkesh, M., Ghasemi, A. and Zadeh-Vakili, A. Elevated expression of steroidogenesis pathway genes; CYP17, GATA6 and StAR in prenatally androgenized rats. *Gene* 593: 167-171, 2016.
23. Jiang, J.Y., Miyabayashi, K., Nottola, S.A., Umezu, M., Cecconi, S, Sato, E. and Macchiarelli, G. Thyroxine treatment stimulated ovarian follicular angiogenesis in immature hypothyroid rats. *Histol. Histopathol*. 23: 1387-1398, 2008.
24. Jiang, J.Y., Miyoshi, K., Umezu, M. and Sato, E. Superovulation of immature hypothyroid rdw rats by thyroxine therapy and the development of eggs after *in vitro* fertilization. *J. Reprod. Fertil*. 116: 19-24, 1999.
25. Jiang, J.Y., Umezu, M. and Sato, E. Improvement of follicular development rather than gonadotrophin secretion by thyroxine treatment in infertile immature hypothyroid rdw rats. *J. Reprod. Fertil*. 119: 193-199, 2000.
26. Lie, M.E., Overgaard, A. and Mikkelsen, J.D. Effect of a postnatal high-fat diet exposure on puberty onset, estrous cycle regularity, and kisspeptin expression in female rats. *Reprod. Biol*. 13: 298-308, 2013.
27. Livak, K.J. and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
28. Lombardi, L.A., Simoes, R.S., Maganhin, C.C., Baracat, M.C., Silva-Sasso, G.R., Florencio-Silva, R., Soares, J.M.Jr. and Baracat, E.C. Immunohistochemical evaluation of proliferation, apoptosis and steroidogenic enzymes in the ovary of rats with polycystic ovary. *Rev. Assoc. Med. Bras*. (1992) 60: 349-356, 2014.
29. Macchiarelli, G., Palmerini, M.G., Nottola, S.A., Cecconi, S., Tanemura, K. and Sato, E. Restoration of corpus luteum angiogenesis in immature hypothyroid rdw rats after thyroxine treat-

ment: morphologic and molecular evidence. *Theriogenology* 79: 116-126, 2013.
30. Maruo, T., Hayashi, M., Matsuo, H., Yamamoto, T., Okada, H. and Mochizuki, M. The role of thyroid hormone as a biological amplifier of the actions of follicle-stimulating hormone in the functional differentiation of cultured porcine granulosa cells. *Endocrinology* 121: 1233-1241, 1987.
31. Maruo, T., Katayama, K., Barnea, E.R. and Mochizuki, M. A role for thyroid hormone in the induction of ovulation and corpus luteum function. *Horm. Res*. 37 (Suppl 1): 12-18, 1999.
32. Mattheij, J.A., Swarts, J.J., Lokerse, P., van Kampen, J.T. and Van der Heide, D. Effect of hypothyroidism on the pituitary-gonadal axis in the adult female rat. *J. Endocrinol*. 146: 87-94, 1995.
33. Medenica, S., Nedeljkovic, O., Radojevic, N., Stojkovic, M., Trbojevic, B. and Pajovic, B. Thyroid dysfunction and thyroid autoimmunity in euthyroid women in achieving fertility. *Eur. Rev. Med. Pharmacol. Sci*. 19: 977-987, 2015.
34. Mintziori, G., Kita, M., Duntas, L. and Goulis, D.G. Consequences of hyperthyroidism in male and female fertility: pathophysiology and current management. *J. Endocrinol. Invest*. 39: 849-853, 2016.
35. Patrick, A.W., Atkin, S.L., MacKenzie, J., Foy, P.M., White, M.C. and MacFarlane, I.A. Hyperthyroidism secondary to a pituitary adenoma secreting TSH, FSH, alpha-subunit and GH. *Clin. Endocrinol. (Oxf.)* 40: 275-278, 1994.
36. Quesnel, H. Nutritional and lactational effects on follicular development in the pig. *Soc. Reprod. Fertil. Suppl*. 66: 121-134, 2009.
37. Ramprasad, M., Bhattacharyya, S.S. and Bhattacharyya, A. Thyroid disorders in pregnancy. *Indian J. Endocrinol. Metab*. 16: S167-S170, 2012.
38. Ridgway, E.C., Longcope, C. and Maloof, F. Metabolic clearance and blood production rates of estradiol in hyperthyroidism. *J. Clin. Endocrinol. Metab*. 41: 491-497, 1975.
39. Samuel, J.B., Stanley, J.A., Princess, R.A., Shanthi, P. and Sebastian, M.S. Gestational cadmium exposure-induced ovotoxicity delays puberty through oxidative stress and impaired steroid hormone levels. *J. Med. Toxicol*. 7: 195-204, 2011.
40. Samuel, J.B., Stanley, J.A., Roopha, D.P., Vengatesh, G., Anbalagan, J., Banu, S.K. and Aruldhas, M.M. Lactational hexavalent chromium exposure-induced oxidative stress in rat uterus is associated with delayed puberty and impaired gonadotropin levels. *Hum. Exp. Toxicol*. 30: 91-101, 2011.
41. Silva, J.R., Figueiredo, J.R. and van den Hurk, R. Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. *Theriogenology* 71: 1193-1208, 2009.
42. Spicer, L.J., Alonso, J. and Chamberlain, C.S. Effects of thyroid hormones on bovine granulosa and thecal cell function *in vitro*: dependence on insulin and gonadotropins. *J. Dairy Sci*. 84: 1069-1076, 2001.
43. Stewart, R.E., Stevenson, J.S. and Minton, J.E. Serum hormones in response to estradiol and (or) progesterone in ovariectomized cows after thyroidectomy. *Domest. Anim. Endocrinol*. 11: 13-24, 1994.
44. Wakim, A.N., Polizotto, S.L. and Burholt, D.R. Augmentation by thyroxine of human granulosa cell gonadotrophin-induced steroidogenesis. *Hum. Reprod*. 10: 2845-2848, 1995.
45. Wei, Q., Fedail, J.S., Kong, L., Zheng, K., Meng, C., Fadlalla, M.B. and Shi, F. Thyroid hormones alter estrous cyclicity and antioxidative status in the ovaries of rats. *Anim. Sci. J*. 89: 513-526, 2017.
46. Zhang, C., Wang, X., Wang, Z., Niu, W., Zhu, B. and Xia, G. Effect of different culture systems and 3, 5, 3'-triiodothyronine/follicle-stimulating hormone on preantral follicle development in mice. *PLoS One* 8: e61947, 2013.
47. Zhang, C., Xia, G. and Tsang, B.K. Interactions of thyroid hormone and FSH in the regulation of rat granulosa cell apoptosis. *Front. Biosci*. (Elite Ed) 3: 1401-1413, 2011.