Author’s response to reviews

Title: Effect of hypothyroidism on the hypothalamic-pituitary-ovarian axis and reproductive function of pregnant rats

Authors:
jianran sun (824534133@qq.com)
Datong Deng (13855134251@163.com)
Cancan Hui (584099642@qq.com)
Tongjia Xia (daydayupxia@163.com)
Min Xu (okayxm@163.com)
Faming Pan (famingpan@ahmu.edu.cn)
Youmin Wang (971359183@qq.com)
Chunlin Zuo (zuochl@163.com)

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Author’s response to reviews:

Response letter

Dear Editors and Reviewers:

Thank you for your letter and suggestions. Your comments and those of the reviewers were very valuable for improving our manuscript entitled "Effect of hypothyroidism on the hypothalamic–pituitary–ovarian axis and reproductive function of pregnant rats" (BEND-D-17-00125). Based on your suggestions, we wrote a point-by-point response letter to acknowledge your help and we listed the areas in which we made revisions. Revised portions are indicated in the text by using track changes in the paper and another revised manuscript without using track changes is also uploaded. The main corrections in the paper and the responses to your and the reviewer’s comments are appended below.
Responds to the reviewers’ comments:

Reviewer #1:

1. In this study hypothyroidism has been developed by PTU though there are several other means for doing the same. This point requires to be explained.

Response: Thank you for your comments. We extensively searched the literature in this area[1]. There are probably four main methods to induce the rat model of hypothyroidism, which include restriction of iodine in the diet, using antithyroid drugs[1–4](PTU or MMI), thyroidectomy, and the use of radioactive iodine[5]. Gilbert et al.[6] and others maintained female long Evans rats with diets deficient in iodine to induce the model of hypothyroidism, and they assessed the effect of hypothyroidism on neurodevelopmental outcomes. There are too many examples to be listed here, but you can refer to references[1]–[6]. Based on the current research, the most popular method is the use of propyl-thiouracil (PTU) to induce the model of hypothyroidism, as it is a controllable, noninvasive method. Methods for inducing developmental hypothyroidism compared with the use of anti-thyroid drugs are listed below, which are shown in supplementary file 1.

References


2. In Table 2 indicating the pregnancy and miscarriage rates is not easily understandable.

Response: Thank you for your comments. The data were checked and double-checked, and some mistakes were corrected. The numbers of rats of each group in Table 2 are in accordance with the numbers of rats in Figure 1. In the experimental count, the number of pregnancy rats (n=28) includes the number of miscarriage rats (n=10).

In the hypothyroidism pregnancy group:

(1) Pregnancy rate (%) = (number of pregnancy rats)/ total number of pregnancy rats = (28/39) × 100% = 71.4%.

(2) Miscarriage rate (%) = (number of miscarriage rats)/ total number of pregnancy rats = (10/39) × 100% = 25.6%.

In the normal pregnancy group:
(1) Pregnancy rate(%)=(number of pregnancy rats)/ total number of pregnancy rats = (24/30) ×100%=80.0%.

(2) Miscarriage rate(%)=(number of miscarriage rats)/ total number of pregnancy rats = (0/30)×100%=0

The revised Table 2 is shown in supplementary file 2.

3. GnRH itself is released from hypothalamic nuclei then the reason for studying GnRHR distribution in hypothalamus requires to be explained.

Response: In the classic theory, GnRH itself is released from hypothalamic nuclei. However, studies show that GnRH neurons are not located in a single discrete brain nucleus or region, and instead are dispersed among a variety of regions in the forebrain, ranging from the olfactory bulb, rostrally, to the hypothalamus, caudally[1]. That is to say, whether any specific nucleus of the hypothalamus secretes GnRH is still unclear. In this regard, studying GnRHR distribution in the hypothalamus is used as a negative control group; we aimed to identify any difference in the distribution of the GnRHR among the four groups(normal control group, normal pregnancy group, hypothyroidism pregnancy group, and hypothyroidism group). We did not find any difference in the distribution of the GnRHR among the four groups.

Reference


4. The last line in the introduction (L 75-78) is unclear and thus these lines should be rewritten properly.

Response: Thank you for pointing this out. The last line of the introduction (L 75–78) was modified.

Revised contents in the manuscript: “The foregoing considerations led us to use a hypothyroid pregnant rat model to block any structural remodeling in the hypothalamus, which is in
accordance with the activation of the two switches governing the pattern of pulsatile GnRH release during postnatal development.” was replaced by “The TH deficiency during infancy also fails to have an effect on the timing of the pubertal resurgence of gonadotropin secretion[1].”

Reference


5. Vaginal plug is a standard method that is used for the occurrence of pregnancy by many workers (though there is controversy about it); there are other methods by mating the female rats during their pro-estrous phase with male followed by presence of sperm in the vagina found to be more accurate. How miscarriage rate has been evaluated should be explained clearly.

Response: In the hypothyroidism pregnancy group:

Miscarriage rate (%)=(number of miscarriage rats)/ total numberof pregnancy rats =(10/39)×100%=25.6%.

In the normal pregnancy group:

Miscarriage rate (%)=(number of miscarriage rats)/ total number of pregnancy rats =(0/30)×100%=0

6. 'Establishment of the hypothyroid pregnant rat model' - this part is not clearly understandable (Line 108 to 125).

Response: The revision of Figure 1 may clarify this issue. Briefly, there are two main steps to explain. Firstly, we measured the levels of T3, T4, and TSH to determine whether the model of hypothyroidism was successfully or not. Secondly, the female and normal male rats were mated by 1:2 for the establishment of the pregnancy model. The day in which an avaginal plug was secreted and detected was considered to be day 1 of gestation (G-1). One day after G-21, 11 rats
that were not pregnant from the hypothyroidism pregnancy group and six rats that were not pregnant from the normal pregnancy group were humanely killed.

Revision of Figure 1 is shown in supplementary file 3.

References


7. In the discussion section, line 325 - 336, a study by Lazaraus has been mentioned but its relevancy in this context is not clear.

Response: In the context of the manuscript, lines 325–336, a study by Lazaraus was mentioned and this was not accurate; there is a weak correlation between the study by Lazaraus and our experiment and we replaced this example. “A study performed by Lazarus et al. [14] in 2012 investigated women who were pregnant in the 16th week. Patients were allocated to a screening group, in which the patients’ thyrotropin and free thyroxine (FT4) levels were instantly measured, and a control group in which the patients’ sera were stored and measured shortly after delivery. TSH levels >97.5% and/or FT4<2.5% were considered to be a positive screening result. In the screening group, women with positive findings were treated with 150 µg levothyrocine per day. There was no significant difference in IQ between the two groups. However, the percentage of children with an IQ <85 was higher in the screening group than in the control group.” is replaced by “Maraka et al.[1] assessed (a) the effect of Subclinical hypothyroidism (SCH)
during pregnancy on maternal and neonatal outcomes and (b) the impact of levothyroxine replacement therapy in these patients. They found that compared with euthyroid pregnant women, pregnant women with SCH were at a higher risk for pregnancy loss (risk ratio: 2.01, confidence interval: 1.66–2.44), placental abruption (risk ratio: 2.14, confidence interval: 1.23–3.70), premature rupture of membranes (risk ratio: 1.43, confidence interval: 1.04–1.95), and neonatal death (risk ratio: 2.58, confidence interval: 1.41–4.73). The authors concluded that SCH during pregnancy is related to multiple adverse maternal and neonatal outcomes. The value of levothyroxine therapy in preventing these adverse outcomes remains uncertain”.

Reference


8. In the discussion line 337-354 requires to be explained more clearly. Based on the distribution of GnRHR in hypothalamus, pituitary and ovary on immuno-histochemical analysis is a gross study without any proper labelling and on the basis of this author tried to conclude that in hypo-thyroid condition the distribution of those receptors are uneven and this is responsible for interfering the pregnancy and increasing abortion rate.

Response: Semi-quantitative analysis of staining results[1]: Results are comprehensively scored by the combination of staining intensity and the percentage of positive cells. The cytoplasm in tissue sections with a color scale from pale yellow to brown indicates a positive cell. Staining intensity is scored by the staining property of most cells (staining shades are compared with the background color): 0 point for no coloring, 1 point for pale yellow color, 2 points for brownish yellow color, and 3 points for brown color. The percentage of positive cells for each type of cell is calculated as the average number of positive cells in five fields (100 such cells are counted every 400× high power field): 0 point for 0–5%, 1 point for 6%–25%, 2 points for 26%–50%, 3 points for 51%–75%, and 4 points if the percentage is >75%. For each slide, five 400× high power fields are selected randomly for scoring of staining intensity and positive cell percentage scoring. Points for the products of staining intensity are multiplied by the positive cell
percentage: 0 point for a negative result (-), 1–4 points for a weakly positive result (+), 5–8 points for a moderately positive result (++), and 9–12 points for a strongly positive result (+++).

However, immunohistochemistry is only semi-quantitative at best[2, 3]. Limitations of this study were the inability to detect the frequency and amplitude of GnRH release and obtain electron micrographs of a GnRH neuroendocrine cell in the rat brain, which would be of value for reviewers and readers to locate the regional distribution of GnRH neuroendocrine cells. We intend to perform these experiments in a future study.

References


9. The paragraph (lines 354 to 360) is not clear.

Response: I added some content in the revised manuscript.

The following paragraph (lines 354–360) was added in the discussion of the revised manuscript: "In the classic theory, GnRH itself is released from hypothalamic nuclei. However, three different forms of GnRH have been reported: hypothalamic GnRH or GnRH-I, mid brain GnRH or GnRH-II, and GnRH-III, which are present in various species of protochordates and vertebrates[1]. Although the hypothalamus and pituitary are the principal sources and target sites for GnRH, several recent reports suggested the presence of extra-hypothalamic GnRH and
GnRH receptors in various reproductive tissues such as ovaries, placenta, endometrium, oviducts, testes, prostate, and mammary glands[2, 3]. GnRH in non-hypothalamic reproductive tissues may have interfered with our experimental results; therefore, we were unable to determine the effect of hypothyroidism on GnRH mRNA expression.” As a supplement for our experiment, sequences of PCR primers of GnRH are universal and do not have any specificity for GnRH-I, GnRH-II, and GnRH-III.

References


10. It has been mentioned GnRH excretion in lieu of secretion in several sites. Hormones are secreted from the specific cells of the endocrine glands but is not excreted.

Response: Thank you for pointing this out. To be more precise, I have changed “excretion” to “secretion”.

11. It has already been mentioned in Reference 21 that 'hypothyroidism affects the distribution of the pituitary GnRHR’ (1984) then what is the novelty of this study.

Response: Reference 21 is a review. According to the author (not listed in the original) temporal changes in hormone secretion are considered on a scale from circpheral (or ultradian) through circadian, menstrual, and seasonal to age-related and are well recognized, especially in relation
to reproduction. The pulsed generator is in the central nervous system, in the medial basal region of the hypothalamus. The novelty of this study is that we showed that hypothyroidism has an adverse impact on pregnancy in rats related to the regulation of the hypothalamic-pituitary-ovarian axis.

12. The overall improvement in the language likely to be developed.

Response: My manuscript was re-edited by International Science Editing and I appreciate your suggestions.

13. The mRNA expression of GnRHR as shown using Melt curves followed by its analysis are understandable.

Response: You maybe mistaking “The mRNA expression of GnRH” (lines 532–543) for “The mRNA expression of GnRHR”. The melting curves for every reaction were recorded to confirm the purity of the amplified product[1](lines 160–161). Based on your suggestion, I added the following histograms in the revised manuscript. The mRNA expression of GnRH is presented as a histogram[2,3], which is shown in supplementary file 4.

References


Comments from the Editor:

1. My submissions to BMC Endocrine Disorders have a Declarations section which includes Ethics approval and consent to participate, Consent to publish, Availability of data and materials, Competing interests, Funding Authors' Contributions, Acknowledgements, Authors' Information.

2. If my revised manuscript still has some minor problems that are not in accordance with BioMed Central editorial policies and formatting guidelines, please point them out and I will revise it as best as I can. I hope my manuscript is now suitable to be published in BMC Endocrine Disorders.