CEOG Clinical and Experimental Obstetrics & Gynecology

Effects of levothyroxine on pregnancy outcomes and caveolin-1 expression in rat models of thyroid dysfunction

B.J. Du[1,2], C.H. Wang[1], C. Huang[2], Z.Q. Li[1]

[1] Shenzhen Maternal and Children Health Care Hospital of Southern Medical University, Shenzhen
[2] Obstetrics Department, the Second Affiliated Hospital of Guangzhou Medical University, Guangzhou (China)

Summary

To investigate the effect of thyroid hormone levothyroxine (L-T_4) on pregnancy outcomes and expression of caveolin-1 in rat models of thyroid dysfunction. *Materials and Methods:* One hundred Wistar rats (SPE grade, 60 female and 40 male) were randomly divided into three groups of control, model (hypothyroidism), and treatment (hypothyroidism + therapy with L-T_4) groups. Pregnant rats were investigated for pregnancy outcomes (litter size, number of dead fetus, placental index, body weight and length, and tail length of neonatal rat). Expression of caveolin-1 in placenta was analyzed using qRT- PCR and Western blot. *Results:* The modeling success rate was 75%. Investigations showed that the litter size, number of dead fetus, placental index, body and length, and tail length of neonatal rat in the models were significantly lower than those in control rats ($p < 0.05$), and significantly different from those in the treatment group ($p < 0.05$) except for the tail length ($p > 0.05$). The expression level of caveolin-1 in placenta was significantly higher in models than that in control ($p < 0.05$). *Conclusion:* Propylthiouracil (PTU) can successfully induce hypothyroidism in rat and hypothyroidism can seriously hinder the pregnancy process, resulting in developmental disorders in fetal rat.

Key words: Thyroid hormone; Thyroid function; Caveolin-1; Gene expression; Pregnancy fate.

Introduction

Thyroid dysfunction or hypothyroidism is a systemic low metabolic syndrome due to reduced thyroid hormone synthesis and secretion or insufficient physiological effects. Clinically, hypothyroidism or subclinical hypothyroidism can cause infertility and spontaneous abortion. It has been associated with pregnancy hypertension diseases, premature birth, placental abruption, fetal distress, and low infant weight. Diseased infants often have stunt brain and bone development, mental disorders, and short stature, known as cretinism [1, 2]. Studies show that the prevalence of hypothyroidism and subclinical hypothyroidism in young women is 0.77% to 5.32%, respectively [3, 4]. Therefore, hypothyroidism is considered to have great negative impact on pregnancy fate. Thyroid hormone is a collection name for hormones secreted by thyroid glands, including thyroxine 3 and 4 (T_3 and T_4). Chemically, T_4 or thyroxine is tetraiodothyronine. It promotes tissue metabolism, improves nerve excitability, and physical development. Clinically, it is used for treatment of diseases such as thyroid dysfunction, myxedema, and cretinism [5, 6]. Currently, the clinical diagnosis of hypothyroidism relies mainly on thyroid function tests. Primary hypothyroidism often results in significantly elevated level of thyroid stimulating hormone (TSH) companied with reduced with free thyroxine 4 (FT_4). Thyroid peroxidase antibody (TPO-Ab) is also used to diagnose the disease.

Although hypothyroidism has been well established for its clinical implications, its occurrence and development mechanisms are still largely unknown. Animal models of subclinical hypothyroidism play important role in studying the mechanisms and impacts on pregnancy [7, 8]. At present, hypothyroidism models are mainly induced by iodine deficiency, surgical resection of thyroid tissue, and propylthiouracil (PTU) [9, 10]. PTU inhibits peroxidase production in thyroid, thereby preventing tyrosine iodination and condensation of iodinated tyrosine, resulting in inhibition of thyroxine synthesis. In this study, the authors used PTU to induce hypothyroidism rats and investigated the effect of thyroxine on pregnancy fate as well as expression of caveolin-1 at mRNA and protein levels to gain insight into the molecular mechanism underlying the effect.

Materials and Methods

Ethics statement

The current investigation conforms to the standard ethical procedures and policies approved by Ethical Committee for Animal Experimentation at Southern Medical University.

Animal modeling and treatment

After seven days of the adaptive feeding, female rats were randomly divided into three groups (20 rats in each group). In addition to feed, rats were intragastric administration with saline in the control group, PTU one mg/kg body weight) in hypothyroidism group and PTU (one mg/kg body weight) and L-T_4 (0.75

Revised manuscript accepted for publication December 14, 2015

mg/kg body weight) in the therapy group for four weeks. The modeling process was as described [11] and monitored using assay kits for FT_4, TPO-Ab, and TSH. Male rats were fed with normal diet. The randomly selected estrus female and male rats were housed overnight in the same cages at 2:1 ratio. Early next morning, vagina suppository desquamated-females were selected as pregnant rats (day one) and fed as above. On day 20 (before delivery), the pregnant rats were fasted for 12 hours and sacrificed by intraperitoneal injection of chloral hydrate. The fetal rats were collected and counted for the stillbirths and alive rats and measured for body weight and length of body and tail. The placenta were weighed to calculate the placenta index (placental weight / body weight x 100%) and rinsed with RNase-free PBS and flash-frozen in liquid nitrogen.

Enzyme-linked immunosorbant assay (ELISA)

After four weeks of PTU treatment, blood was collected from the tail vein and the serum was separated by centrifugation and used for quantification of TPO-Ab, TSH, and FT_4 using ELISA kits according to the manufacturer's protocols.

qRT-PCR

Total RNA was extracted using a RNA extraction kit according to the manufacturer's protocol. After qualification using Qubit fluorometer, the RNA was reversely transcripted into cDNA using a RNA reverse transcription kit and subjected to qRT-PCR using SYBR Green real time PCR Premix using primers based on the mRNA sequence of the caveolin-1 gene (Table 1) and GAPDH (used as internal control) (Table 1). Reverse transcription was performed with 200 ng of RNA in a total volume of ten µl using the High Capacity cDNA Transcriptase Reverse kit according to manufacturer's recommendations. qRT-qPCR was performed on the 7900HT Fast Real-Time PCR system using TaqMan gene expression assays probes. The PCR was carried out in a total volume of ten µl containing 1.5 µl of diluted and pre-amplified cDNA, one µl of TaqMan Gene Expression Master Mix and one µl of each fluorescence TaqMan probe. The cycling conditions were pre-denaturing at 95ºC for ten minutes, followed by 40 cycles, each one consisting of denaturing at 95ºC for ten seconds, annealing at 54.4ºC for 20 seconds (for caveolin-1) or 55.2 ºC for 20 seconds for GAPDH, and elongation at 72ºC for 33 seconds. The data were managed using the RQ Manager software v1.2.1. Relative expression was calculated by using comparative Ct method according to previously described protocol [12].

Western blot

Placental tissue was ground in liquid nitrogen and extracted using ReadyPrep protein extraction kit according to the manufacturer's instructions. The supernatant was measured for protein content using BCA protein kit.

Proteins were separated using SDS-PAGE electrophoresis and then transferred to the PVDF membrane (0.22-µm pore size). The membrane was incubated with caveolin-1 and GAPDH monoclonal antibodies washed with TBST, and then with secondary antibody (horseradish peroxidase-labeled goat anti-rabbit Ig). The membranes were then washed, added with ECL developer solution and exposed in ChemiDocTM XRS gel imaging system. The gray values of band were measured using the Quantity One software (v4.62).

Statistical analysis

All measurements were repeated three times and expressed as mean ± SEM. One way analysis of variance (ANOVA) was used for comparing difference between groups. Pair-wise comparisons were done using the Tukey-HSD test. All analyses were performed using SPSS 18 and differences were considered statistically significant at $p < 0.05$ for all tests.

Table 1. — *Primers used in real-time PCR.*

Gene	GenBank accession no.	Primer sequence (5'- 3')
Caveolin-1	AB029929	Forward: ATGTCTGGGGGCAAATACGTG
		Reverse: CGCGTCATACACTTGCTTCT
GAPDH	NM_014364	Forward: TGTGGGCATCAATGGATTTGG
		Reverse: ACACCATGTATTCCGGGTCAAT

Table 2. — *FT_4, TPO-Ab, and TSH level in rats.*

Group	n	FT_4 (pM)	TSH (µIU/ml)	TPO-Ab (IU/ml)
Control	20	12.06 ± 1.06	1.21 ± 0.67	14.51 ± 2.01
Model	15	4.26 ± 0.95*	6.37 ± 0.37*	51.04 ± 5.33**
Therapy	17	9.17 ± 1.22	2.06 ± 0.18Δ	12.85 ± 2.57ΔΔ

*$p < 0.05$, **$p < 0.01$ (*vs.* control); Δ$p < 0.05$, ΔΔ$p < 0.01$ (*vs.* model).

Results

Hypothyroidism modeling

Four weeks after feeding, surviving female rats were assayed for hypothyroidism using blood TSH, FT_4, and TPO-Ab levels. The results showed that TSH level in the hypothyroidism model group was significantly higher than that in the control group ($p < 0.05$), and similar to that in the therapy (PTU). At the same time, FT_4 level in the model group was significantly lower than that in the control group ($p < 0.05$), indicating that hypothyroidism modeling was successful. Among the 20 animals in model group, 15 showed typical symptoms of hypothyroidism giving a success rate of 75%. On the other hand, the levels of TPO-Ab, FT_4 and TSH in therapy group were close to those in control rats (Table 2).

Effect of thyroid hormone on the pregnancy outcomes in rats with hypothyroidism

After modeling, pregnant rats (14 in control group, 11 model group, and 13 therapy group) were investigated for pregnancy outcomes on day 20. Results are shown in Table 3. The litter size, number of dead fetus, placental index, body weight and length, and tail length of neonatal rats in the model group were significantly lower than those in the control group ($p < 0.05$), and significantly different from those in the therapy group ($p < 0.05$), except for the tail length ($p > 0.05$).

Effect of thyroid hormone on expression of caveolin-1 in rats with hypothyroidism

Caveolin-1 mRNA level in placenta tissue isolated on day 20 is shown in Figure 1A. As shown, the level in the model

Table 3. — *Pregnancy outcomes in each group ($\bar{x} \pm s$)*

Group	No	Litter size	No. of dead fetus	Placental index (%)	Infant		
					Weight (g)	Body length (cm)	Tail length (cm)
Control	14	12.22 ± 2.10	2.06 ± 0.70	0.12 ± 0.01	5.27 ± 0.14	3.85 ± 0.10	1.18 ± 0.21
Model	11	4.27 ± 0.94*	3.16 ± 1.04	0.08 ± 0.01*	3.29 ± 0.23**	2.51 ± 0.34*	1.06 ± 0.43*
Therapy	13	9.64 ± 1.16$^{\Delta\Delta}$	1.46 ± 0.85$^{\Delta}$	0.14 ± 0.02$^{\Delta}$	4.47 ± 0.64$^{\Delta}$	3.61 ± 0.36	1.13 ± 0.37$^{\Delta}$

$^{*}p < 0.05$, $^{**}p < 0.01$ (*v.* control); $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ (*vs.* model).

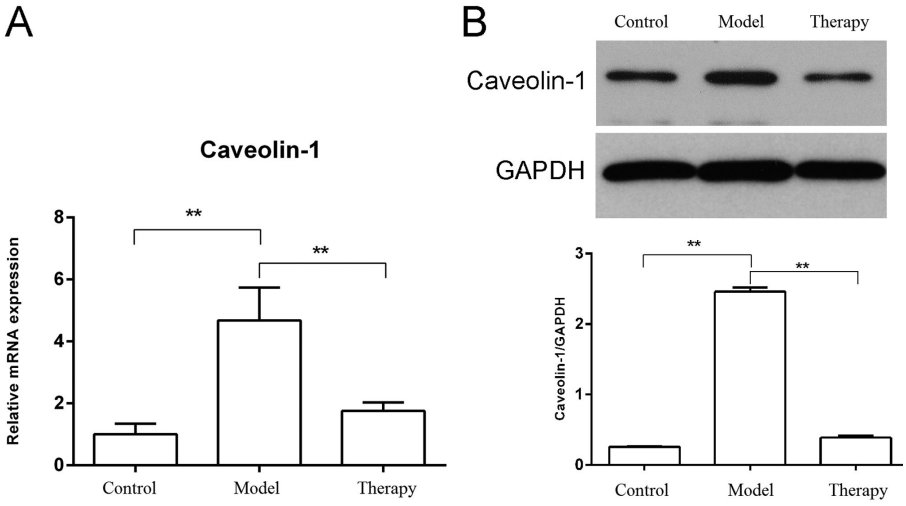

Figure 1. — Effect of thyroid hormone on the expression of caveolin-1 in rats with hypothyroidism. A) mRNA level measured using RT-PCR results. B) protein levels measured using Western blot analysis.

group was significantly higher than that in the control group ($p < 0.01$) and lower, but not significantly ($p > 0.05$) than in the therapy group. Analysis of protein level revealed similar trends (Figure 1B). Quantification with Quantity One showed that the relative caveolin-1 amounts were 0.26 ± 0.01, 2.46 ± 0.06, and 0.39 ± 0.03 in control, model, and therapy groups, respectively.

Discussion

Thyroid is a very important endocrine organ in animals. Thyroid dysfunction can cause infertility, spontaneous abortion, pregnancy hypertensive disease, premature birth, placental abruption, fetal distress, and low infant weight. It also affects the brain and bone development, resulting in mental disorder and stunt body development [1, 2]. The clinical implications of hypothyroidism has been investigated intensively and well documented. However, the molecular mechanisms underlying the disease is still unclear. A better understanding of mechanism will provide new clues for diagnosis and treatment of the disease.

In this study, the authors successfully constructed hypothyroidism models by feeding the rats with PTU-containing water. They observed that the rats fed with PTU showed typical hypothyroidism symptoms, such as behavioral retardation, limb weakness, and dull eyes. The hypothyroidism was further confirmed by TSH, FT_4, and TPO-Ab tests. Because thyroid hormone is very important for the nervous system, deficiency in the hormone disturbs the normal development and maturation of neural cells, seriously hinders the growth and development of the fetal brain, and subsequently affects the pregnancy outcome. The present results further showed that intervention of hypothyroidism models with L-T_4 can restore the levels of TSH, FT_4, and TPO-Ab to normal levels in the models, suggesting that abnormal levels of these parameters are likely due to thyroid hormone deficiency in the hypothyroidism models.

Pregnancy outcomes can be used to assess the health of pregnant rats and development of infants [13]. The present assessments showed that the hypothyroidism has negative impacts on pregnancy outcomes, such as the litter size, infant weight and length, and number of live infants. On the other hand, significantly improved pregnancy outcomes were obtained when the hypothyroidism rats were supplemented with the thyroid hormone, suggesting that thyroid hormone deficiency is fatal to fetal development. Since brain development occurs in the early fetal stage, thyroid hormone deficiency likely affects brain development, leading to increased infant mortality and reduced infant size.

Caveolin-1 is involved in the regulation of cell membrane composition and cell surface expansion during cell migration. It also affects the polarization of signal molecules and the remodeling of the cytoskeleton, and is very important for the development of the nervous system [13, 14]. Therefore, the authors investigated the expression of caveolin-1 in the pla-

centa of pregnant rats. These results showed that caveolin-1 was significantly upregulated in model group as compared to the control at both mRNA and protein levels. On the other hand, caveolin-1 level was significantly reduced when the models were supplemented with the thyroid hormone. This is consistent with results obtained the early work [15]. Since caveolin-1 is involved in various membrane-related processes and regulates signal transduction [16, 17], it plays a very important role in the development of nervous system. Earlier studies found that thyroid dysfunction may result in disruption of cell migration where caveolin-1 is involved in transmembrane signal transduction and lipid transportation [18, 19]. Downregulation of caveolin-1 after the thyroid hormone intervention suggests that the gene is likely associated with thyroid function. Early study showed that caveolin-1 may play an important role in the synthesis of thyroid hormone and the maintenance of thyroid cell number [20]. Considering these results together with the present data, the authors believe that there may be a compensatory or feedback mechanism that balances caveolin-1 expression and thyroid hormone level, that is, upregulation of caveolin-1 is to compensate the reduced thyroid hormone level, while supplement of thyroid hormone may suppress the upregulation to certain extents. A similar hypothesis was proposed early [21]. Therefore, it can be concluded that the caveolin-1 gene has an indirect impact on the fetus development and pregnancy outcomes through the regulation of thyroid hormone. It will be the present authors' further interest to deliberate more detailed molecular mechanism related to the process.

Conclusion

PTU can successfully induce hypothyroidism in rat and hypothyroidism can seriously hinder the pregnancy process, resulting in developmental disorders in fetal rat. To investigate the effect of thyroid hormone L-T_4 on the pregnancy outcomes and expression of caveolin-1 in rat models of thyroid dysfunction.

Acknowledgment

This work was supported by the Guangzhou Medical Science Projects (grant no. 20151A010098).

References

[1] Ke L.Q., Hu Y., Yang K., Tong N.: "Chinese herbal medicines for hypothyroidism". *Cochrane Database Syst. Rev.*, 2015, *2*, CD008779.

[2] Saki F., Dabbaghmanesh M.H., Ghaemi S.Z., Forouhari S., Ranjbar Omrani G., Bakhshayeshkaram M.: "Thyroid function in pregnancy and its influences on maternal and fetal outcomes". *Int. J. Endocrinol. Metab.*, 2014, *12*, e19378.

[3] Ma L., Qi H., Chai X., Jiang, F., Mao S., Liu J., *et al.*: "The effects of screening and intervention of subclinical hypothyroidism on pregnancy outcomes: a prospective multicenter single-blind, randomized, controlled study of thyroid function screening test during pregnancy". *J. Matern. Fetal Neonatal Med.,* 2015, 1.

[4] Foeller, M.E., Silver R.M.: "Combination levothyroxine + liothyronine treatment in pregnancy". *Obstet. Gynecol. Surv.*, 2015, *70*, 584.

[5] Catli G., Abaci A., Buyukgebiz A., Bober E.: "Subclinical hypothyroidism in childhood and adolescense". *J. Pediatr. Endocrinol. Metab.*, 2014, *27*, 1049.

[6] Abdalla S.M., Bianco A.C.: "Defending plasma T3 is a biological priority". *Clin. Endocrinol. (Oxf.)*, 2014, *81*, 633.

[7] Dan Z., Tong W., Li Y., Wang Z., Jing N., Gai H., *et al.*: *China J. Endocrinol. Metab.*, 2001, *17*, 712.

[8] Empson M., Flood V., Ma G., Eastman C.J., Mitchell P.: "Prevalence of thyroid disease in an older Australian population". *Intern. Med. J.,* 2007, *37*, 448.

[9] Andersen S.L., Olsen J., Wu C.S., Laurberg P.: 'Severity of birth defects after propylthiouracil exposure in early pregnancy". *Thyroid*, 2014, *24*, 1533.

[10] Zhu S.: "Establishment of subclinical hypoyroidism animal model". China Medical University, 2008.

[11] Mai J.: "A mechanism study on hypoyroidism-nduced reproductive insult in male rats". Lazhou, China: Lazhou University Press, 2014.

[12] Livak K.J., Schmittgen T.D.: "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method". *Methods*, 2001, *25*, 402.

[13] Miranda M.L., Anthopolos R., Wolkin A., Stapleton H.M.: "Associations of birth outcomes with maternal polybrominated diphenyl ethers and thyroid hormones during pregnancy". *Environ. Int.*, 2015, *85*, 244.

[14] Liu J., Wang X.B., Park D.S., Lisanti M.P.: "Caveolin-1 expression enhances endothelial capillary tubule formation". *J. Biol. Chem.*, 2002, *277*, 10661.

[15] Trushina E., Du Charme J., Parisi J., McMurray C.T.: "Neurological abnormalities in caveolin-1 knock out mice". *Behav. Brain Res.*, 2006, *172*, 24.

[16] Gaudreault S.B., Blain J.F., Gratton J.P., Poirier J.: "A role for caveolin-1 in post-injury reactive neuronal plasticity". *J. Neurochem.*, 2005, *92*, 831.

[17] Mauch D.H., Nagler K., Schumacher S., Goritz C., Muller E.C., Otto A., Pfrieger F.W.: "CNS synaptogenesis promoted by glia-derived cholesterol". *Science*, 2001, *294*, 1354.

[18] Auso E., Lavado-Autric R., Cuevas E., Del Rey F.E., Morreale De Escobar G., Berbel P.: "A moderate and transient deficiency of maternal thyroid function at the beginning of fetal neocorticogenesis alters neuronal migration". *Endocrinology*, 2004, *145*, 4037.

[19] de Escobar G.M., Obregon M.J., del Rey F.E.: "Iodine deficiency and brain development in the first half of pregnancy". *Public Health Nutr.,* 2007, *10*, 1554.

[20] Senou M., Costa M. J., Massart C., Thimmesch M., Khalifa C., Poncin, S., *et al.*: "Role of caveolin-1 in thyroid phenotype, cell homeostasis, and hormone synthesis: in vivo study of caveolin-1 knockout mice". *Am. J. Physiol-Endoc. M.*, 2009, *297*, E438.

[21] Royland J.E., Parker J.S., Gilbert M.E.: "A genomic analysis of subclinical hypothyroidism in hippocampus and neocortex of the developing rat brain". *J. Neuroendocrinol.*, 2008, *20*, 1319.

Corresponding Author:
C.H. WANG, M.D.
Shenzhen Maternal and Children Health Care Hospital of Southern Medical University
3012 Fuqiang Road
Shenzhen 518000 (China)
e-mail: wangchenhong09@sina.com