

Antibodies against thyroid-stimulating hormone receptor cause maternal-neonatal transmission of Graves' Disease

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Abstract. The present study aimed to investigate whether the thyroid-stimulating hormone receptor (TSHR) autoantibodies (Ab) from mothers with Graves' disease (GD) could cause neonatal thyroid disease and the underlying mechanisms of this. An adenovirus expressing the TSHR A-subunit and a control adenovirus expressing β -galactosidase was constructed by Beijing Sino Geno Max Co., Ltd. The sequences were subsequently verified and amplified via PCR. A GD model was established in female BALB/c mice (n=90) by three intramuscular injections of a TSHR-expressing adenovirus (Ad-TSHR). Mice injected with Ad- β -galactosidase served as a sham immunization group. The immunized females were paired with unimmunized males to generate offspring. The serum levels of TSHR-Ab and thyroxine (T4) of mothers and neonates were measured after delivery. Breast milk was collected from the stomachs of neonatal mice to determine the TSHR-Ab levels. The positive rate of serum TSHR-Ab (>0.3 IU/l) in the TSHR group was 99% (89/90) and 0% in the sham group. The mother mice in the TSHR group had elevated serum T4 levels and the thyroid pathological features of Graves' hyperthyroidism. GD mice gave birth to smaller newborns with thyroid pathological changes and higher serum levels of TSHR-Ab and T4,

compared to the offspring in the sham group. The TSHR-Ab levels in breast milk from the GD mice declined with time. Mice immunized with Ad-TSHR exhibited the clinicopathological features of human GD and give birth to neonates with thyroid disease at birth.

Introduction

Graves' disease (GD) is an autoimmune disorder of the thyroid gland. The annual incidence of GD is 20-50 cases per 100,000 persons (1). Approximately 3% of women and 0.5% of men develop GD during their lifetime (2). Most patients with GD present with overt hyperthyroidism along with a variety of symptoms, including palpitations, tremulousness, heat intolerance, weight loss, and anxiety (3). The main pathogenic mechanisms responsible for GD are the stimulation and secretion of thyroid hormone [thyroxine (T4) and triiodothyronine (T3)] and the stimulation of thyrocyte growth by thyroid-stimulating hormone receptor (TSHR) autoantibodies (TSHR-Ab) (4).

Maternal GD has several effects on the fetus during pregnancy, including low birth weight and intrauterine growth retardation (5). Moreover, elevated TSHR-Ab levels in pregnant women with GD may affect the fetal thyroid gland through the placenta, resulting in fetal or neonatal thyroid dysfunction and other adverse effects, either temporarily or long-term (3). In addition, it has been reported that neonates with transient thyroid disease from their euthyroid TSHR-Ab positive mother may suffer from worsening of their condition during breastfeeding, potentially due to the presence of TSHR-Ab in breast milk that enters the blood through the immature neonatal intestinal mucosa (6). However, the relevance of this mode of transmission and condition aggravation is still controversial, as only small amounts of TSHR-Ab are thought to be present in breast milk (7-11).

In the present study, a GD model was established in mice by repeated injection of TSHR-expressing adenoviruses to confirm whether TSHR-Ab in mothers can cause neonatal thyroid disease and the mechanisms underlying this. Excessive serum T4 was considered as the criterion for hyperthyroidism diagnosis. The serum T4 levels of both mothers and neonates were measured when the mice with GD gave birth. Breast milk

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Abbreviations: GD, Graves' disease; IgG, immunoglobulin G; PBS, phosphate-buffered saline; PFU, plaque formation unit; T4, thyroxine; TSHR-Ab, thyroid-stimulating hormone receptor autoantibodies; TSH, thyroid-stimulating hormone; TSHR, thyroid-stimulating hormone receptor

Key words: Graves' disease, thyroid-stimulating hormone receptor, antibody against TSHR, thyroxine, maternal-neonatal transmission

was collected from the stomachs of neonatal mice to determine the milk TSHR-Ab levels.

Materials and methods

Animal and grouping. All experimental procedures were carried out according to the guidelines of the Medicine Laboratory Animal Center of Anhui Medical University. Male and female BALB/c mice (age, 6 weeks; weight, 16.6 ± 0.028 g) were obtained from the Medicine Laboratory Animal Center and maintained in a pathogen-free environment under a 12 h light/dark cycle, a temperature of $22 \pm 1^\circ\text{C}$ and a humidity of 45-55%. Animals received free access to food and drink. The study was approved by the institutional animal care and use committee of Anhui Medical University (approval reference no. PJ-YX2019-038).

An adenovirus expressing TSHR A-subunit (Ad-TSHR-289) and a control adenovirus expressing β -galactosidase (Ad- β -gal) were obtained from Dr Chun-Rong Chen (Cedars-Sinai Medical Center and the University of California Los Angeles, USA). Beijing Sino Geno Max Co., Ltd. verified the sequences and amplified these viruses. The TSHR immunization group included 90 female mice that were intramuscularly injected with Ad-TSHR-289 [3.3×10^8 plaque formation unit (PFU)/ $50 \mu\text{l}$ of phosphate-buffered saline (PBS)]. All mice were injected three times, once every three weeks. Female mice (90) were injected intramuscularly with Ad- β -gal (3.3×10^8 PFU/ $50 \mu\text{l}$ of PBS) and served as the sham immunization group (12). After mice were anesthetized with sodium pentobarbital (1%; 50 mg/kg intraperitoneally), blood samples were taken from angular vein of the eyes by capillary after the third injection and the serum TSHR-Ab levels were measured to determine the success rate of modeling.

Female mice (15-week-old) in each group were bred with males (two females and one male per cage). Vaginal plugs were checked the morning following breeding. The females displaying a vaginal plug were considered pregnant and this time point was considered to be pregnancy day 0.5. Pregnant female mice were caged individually and fed until delivery with free access.

Mother mice, 30 in each group were sacrificed after breast feeding on days 1, 7 or 21 after delivery by cervical dislocation. The offspring of the mother mice were sacrificed at the same time by cervical dislocation. Prior to cervical dislocation all mice were anesthetized by sodium pentobarbital (1% and 50 mg/kg intraperitoneally) before blood collection by retrobulbar plexus.

The milk masses from the gastric sac of the offspring of the same mother were pooled in a single tube. 100 μl of the milk was added to cold PBS, which was mixed and centrifuged at $1,006.29 \times g$ (4°C) for 20 min. The supernatant was taken and diluted with PBS 3 times in series for testing.

Blood samples were collected for detecting TSHR-Ab and T4 and the thyroids were harvested for histological examination. Milk from the gastric sac of neonatal mice from the same mother was collected to determine milk TSHR-Ab levels (Fig. 1).

Preparation of adenoviruses. Both Ad-TSHR-289 and Ad- β -gal were propagated in 293T cells by researchers from Beijing Sino Geno Max Co., Ltd. They were purified using

an ion-exchange chromatographic column (13), aliquoted, and stored at -80°C . The concentration of viral particles was determined using a standard PFU protocol (14).

Measurement of TSHR-Ab and T4 levels. The levels of TSHR-Ab and T4 in $50 \mu\text{l}$ of undiluted sample (serum or milk) were measured. The TSHR-Ab levels were determined using an Elecsys[®] Anti-TSHR kit [cat. no. YZB/GEM 1362-2009; Roche Diagnostics (Shanghai) Co., Ltd.] according to the manufacturer's instructions and a Cobas e analyzer (measurement range 0.3-40 IU/l, functional sensitivity 0.9 IU/l; both supplied by Roche Diagnostics (Shanghai) Co., Ltd.) using a competitive electrochemiluminescence immunoassay, based on the inhibitory effect on the binding of the thyroid-stimulating human monoclonal antibody M22 (labeled with ruthenium) to porcine TSHR (15).

T4 levels were determined using the iodine thyroxine radioimmunoassay kit (cat. no. S10930056; Tianjing Jiuding Medical Bioengineering Co., Ltd.) according to the manufacturer's instructions and a γ -ray counter [sensitivity of $0.3 \mu\text{g}/\text{dl}$ ($1 \mu\text{g}/\text{dl} = 12.87 \text{ nmol}/\text{l}$); intragroup variability of 7.0% and intergroup variability of 11.2%; Tianjin Jiuding Medical Bioengineering Co., Ltd.] in a liquid-phase equilibrium competitive radioimmunoassay, according to its inhibitory effect on the binding of ^{125}I -labeled T4 to goat anti-T4 antibody. The normal range of T4 was determined to be the mean T4 level in the sham group \pm three standard deviations. T4 levels higher than the highest normal level were defined as hyperthyroidism (16).

Histological examination of the thyroid gland. The dissected thyroids were fixed in 10% buffered formalin for at least 24 h at 37°C , dehydrated and embedded in paraffin. Sections ($5 \mu\text{m}$) were stained with hematoxylin-eosin (17). The analysis was performed using the Olympus Cue-2 image analysis system connected to a fluorescence microscope (Olympus Corporation; magnification, $\times 200$ and $\times 400$). The area of each follicle in five random fields was evaluated from each section. Samples were taken from hyperthyroid ($n=9$) and euthyroid mice ($n=8$).

Statistical analysis. Statistical analyses were performed using SPSS 19.0 (SPSS, Inc.). The TSHR-Ab levels were compared between the two groups using the Mann-Whitney U rank-sum test. The T4 levels are presented as the mean \pm the standard deviation and were analyzed using the Student's t-test. $P < 0.05$ was considered to be statistically significant.

Results

Serum TSHR-Ab in mice after TSHR immunization. Serum TSHR-Ab was not detectable ($< 0.3 \text{ IU}/\text{l}$) in the sham group ($n=90$) after three injections of adenovirus. The positive rate of serum TSHR-Ab ($22.5 \pm 1.12 \text{ IU}/\text{l}$) in the TSHR group ($n=90$) was 99% (89/90), indicating that only one mouse did not develop autoimmunity to TSHR. After delivery, the serum TSHR-Ab levels were higher in the TSHR group on days 1, 7 and 21 ($n=30$ per time point) compared with the sham group (all $P < 0.01$). The serum TSHR-Ab levels of the mother mice in the TSHR group were $20.59 \pm 1.63 \text{ IU}/\text{l}$

The technology roadmap

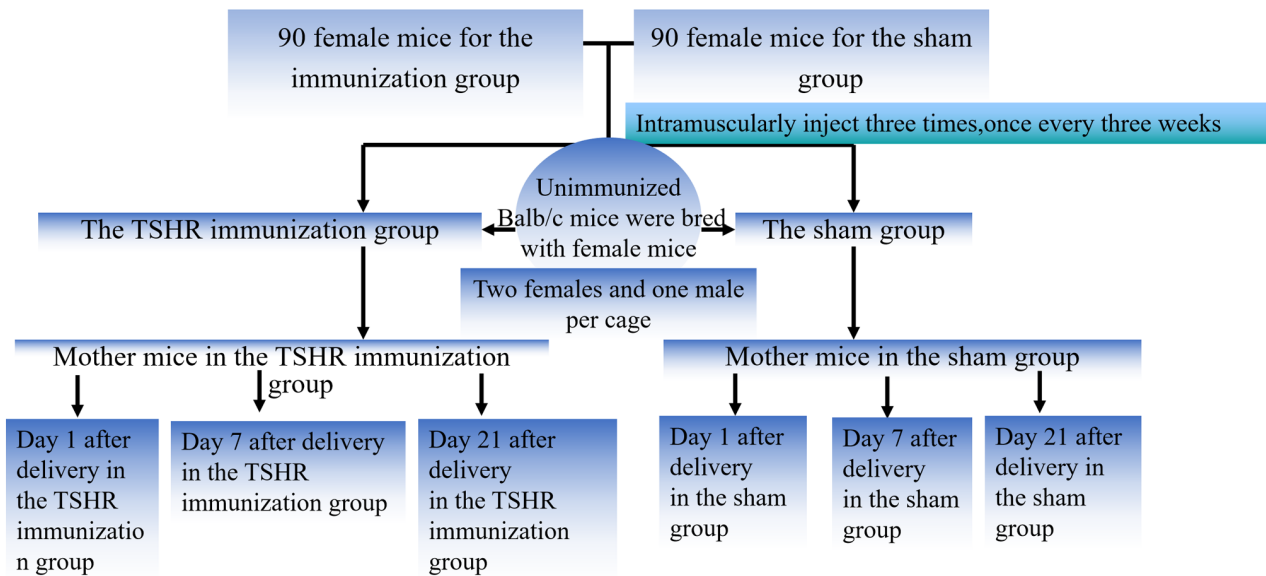


Figure 1. Schematic of experimental procedures. TSHR, thyroid-stimulating hormone receptor.

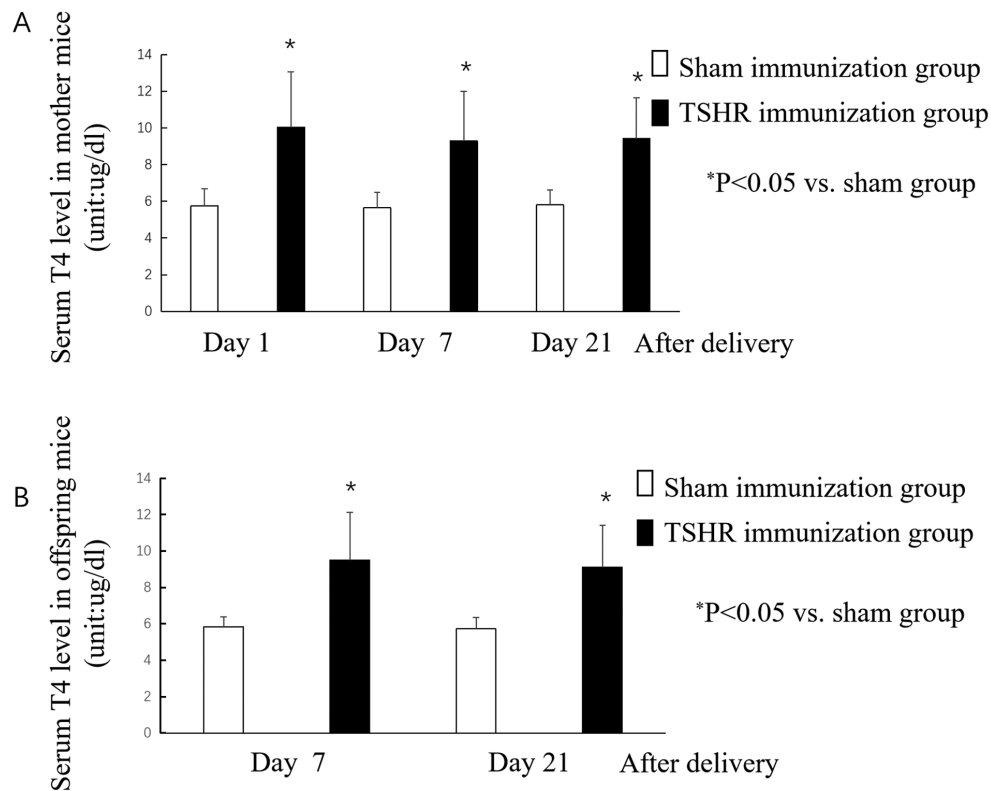


Figure 2. T4 levels in mothers and their offspring. (A) Mother mice and (B) their offspring in each group were sacrificed at different time points after delivery and blood samples collected to measure the T4 levels. n=30 per time point. Results are presented as the mean \pm SD for each group. T4, thyroxine; TSHR, thyroid-stimulating hormone receptor. *P<0.05 vs. sham group.

on day 1, 22.49 ± 1.13 IU/l on day 7 and 23.43 ± 1.10 IU/l on day 21 (n=30 per time point), respectively in the TSHR group. After delivery, the serum T4 levels of the mother mice in the sham group were 5.7 ± 0.955 , 5.6 ± 0.857 and 5.8 ± 0.826 μ g/dl on days 1, 7 and 21 (n=30 per time point), respectively. The

serum T4 levels of mother mice in the TSHR group were higher than in the sham group (all P<0.05; Fig. 2A). The normal range of T4 was determined to be the mean T4 level in the sham group \pm three standard deviations. T4 levels higher than the highest normal level were defined as

Table I. T4 and TSHR-Ab levels in the mother mice.

Characteristic	Day 1 after delivery in the sham group	Day 1 after delivery in the TSHR immunization group	Day 7 after delivery in the sham group	Day 7 after delivery in the TSHR immunization group	Day 21 after delivery in the sham group	Day 21 after delivery in the TSHR immunization group
T4 $\mu\text{g}/\text{dl}$	5.700 \pm 0.955	10.000 \pm 3.011 ^a	5.600 \pm 0.857	9.305 \pm 2.702 ^a	5.800 \pm 0.826	9.400 \pm 2.243 ^a
TSHR-Ab IU/l	<0.30	20.59 \pm 1.63 ^b	<0.30	22.49 \pm 1.13 ^b	<0.30	23.43 \pm 1.10 ^b

^aP<0.05 and ^bP<0.01 vs. the sham group. Mice were bled periodically, and sera were tested for T4 and TSHR-Ab levels. N=30 mice per group. Results are presented as the mean \pm SD for each group. T4, thyroxine; TSHR, thyroid stimulating hormone receptor; TSHR-Ab, thyroid stimulating hormone autoantibodies.

Table II. Serum T4, TSHR-Ab levels and weight of offspring mice.

Characteristic	Day 7 after delivery in the sham group	Day 7 after delivery in the TSHR immunization group	Day 21 after delivery in the sham group	Day 21 after delivery in the TSHR immunization group
N	192	180	187	180
T4 $\mu\text{g}/\text{dl}$	5.834 \pm 0.561	9.500 \pm 2.61 ^a	5.719 \pm 0.638	9.100 \pm 2.287 ^a
TSHR-Ab IU/l	<0.30	19.62 \pm 1.59 ^b	<0.30	18.69 \pm 2.09 ^b
Weight g	6.10 \pm 1.82	3.90 \pm 1.34	11.20 \pm 2.03	8.90 \pm 1.51

^aP<0.05 and ^bP<0.01 vs. the sham group. Mice were bled periodically, and sera were tested for T4 and TSHR-Ab levels. Results are presented as the mean \pm SD for each group. T4, thyroxine; TSHR, thyroid stimulating hormone receptor; TSHR-Ab, thyroid stimulating hormone autoantibodies.

hyperthyroidism (16). Thus, 22 (73%), 18 (60%) and 23 (77%) mother mice in the TSHR immunization group were diagnosed with hyperthyroidism on days 1, 7 and 21, respectively (Table I). These data suggest that TSHR immunization using Ad-TSHR-289 was an effective way to obtain a GD model in mice.

Higher serum levels of TSHR-Ab and T4 were observed in the offspring of the TSHR group. Serum TSHR-Ab levels were higher in the offspring of the TSHR group on day 7 (P<0.01) and day 21 (P<0.01) after delivery, as compared with the offspring in the sham group (TSHR-Ab <0.3 IU/l at all time points). The serum TSHR-Ab levels of the offspring of the TSHR group were 19.62 \pm 1.59 and 18.69 \pm 2.09 IU/l on days 7 and 21, respectively. The serum T4 levels of the offspring in the sham group were 5.8 \pm 0.561 and 5.7 \pm 0.638 $\mu\text{g}/\text{dl}$ on days 7 and 21, respectively. The offspring in the TSHR group had higher serum T4 levels (both P<0.05; Fig. 2B). Considering the mean T4 levels \pm three standard deviations of the sham group as the normal range, the incidence of hyperthyroidism in the offspring of the TSHR group was 63% (114/180) and 72% (129/180) on days 7 and 21 after delivery, respectively. The serum T4 levels of the offspring in the TSHR group were 9.503 \pm 2.61 and 9.123 \pm 2.287 $\mu\text{g}/\text{dl}$ on days 7 and 21, respectively (Table II).

Thyroid appeared larger in the TSHR mother mice than in the sham mice. The mother mice with hyperthyroidism in the TSHR immunization group had larger thyroids than sham mice (Fig. 3A). Histological examination revealed that the thyroids exhibited diffuse enlargement with hypertrophy and

hypercellularity of follicular epithelia, with occasional protrusion into the follicular lumen, compared with normal thyroids from the sham group. These changes were consistent with the clinicopathological features of Graves' hyperthyroidism in humans (Fig. 3B) (12).

TSHR-immunized mothers gave birth to smaller newborns with thyroid dysplasia. The average weights of the newborns in the TSHR group were 3.9 \pm 1.34 g on day 7 and 8.9 \pm 1.51 g on day 21 after delivery, which was lower than in the sham group (6.1 \pm 1.82 and 11.2 \pm 2.03 g, respectively). The newborns born in the TSHR group were thinner and smaller than those born in the sham group (Fig. 4A; Table II).

The follicular epithelial cells from the thyroids were studied in each group. Thyroids were diffusely enlarged, without interstitial lymphocytic infiltration and nodular progression in the offspring in the TSHR group compared with those in the sham group. Thyroid follicles in the goiters also had cuboidal thyroid epithelial cells with marked intracellular vacillation, indicating higher secretory activity (18). This showed a difference in the pathology of follicular epithelial cells of the thyroid gland in the offspring of the TSHR group in comparison with the sham group (Fig. 4B).

TSHR-Ab levels in breast milk from the TSHR group were not significantly different from that of the sham group. Offspring mice were sacrificed and breast milk was collected from their stomachs for the determination of TSHR-Ab level. The levels of TSHR-Ab in breast milk were lower than 0.3 IU/l in all mice of the sham group. In the TSHR group, 9 out

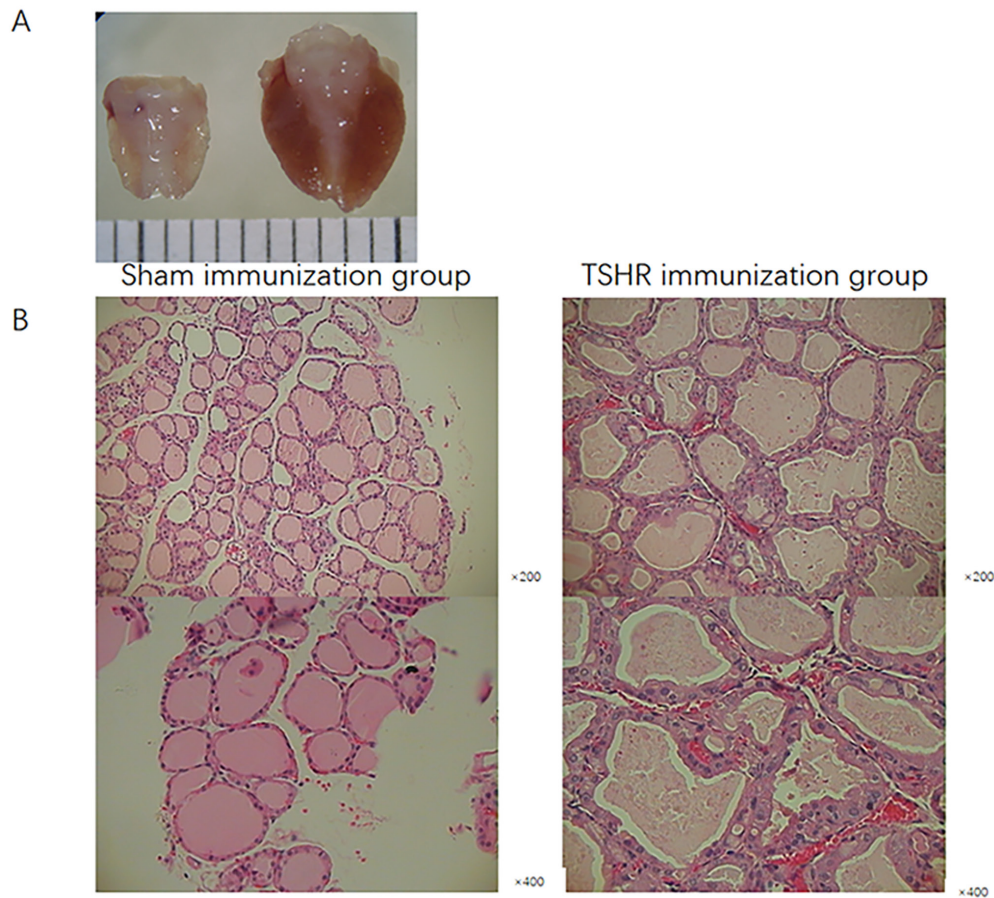


Figure 3. Morphological changes in the thyroids of the TSHR immunized mice. The mother mice in each group were sacrificed on day 1 after delivery and the thyroid glands were harvested. (A) Representative images of the thyroid glands from a mother mouse in the sham group (left) and a hyperthyroid mother mouse in the TSHR group (right). (B) Histological examination of the thyroids with hematoxylin-eosin staining. Upper/lower left: from a mother mouse in the sham group; upper/lower right: from a hyperthyroid mother mouse in the TSHR group. Magnification: upper panel: x200; lower panel: x400. TSHR, thyroid-stimulating hormone receptor.

of 30 mother mice had detectable TSHR-Ab levels in their breast milk on day 1 after delivery (1.1, 1.2, 1.4, 2.1, 2.4, 2.9, 3.5, 5.3 and 6.5 IU/l). The proportion of mother mice with breast milk TSHR-Ab levels >0.3 IU/l declined to 4/30 on day 7 after delivery (1.5, 1.8, 2.3 and 3.4 IU/l). However, no significant difference was observed between the two groups on days 1 ($P=0.055$) or 7 ($P=0.230$). The levels of TSHR-Ab in breast milk were all lower than 0.3 IU/l in the TSHR group on day 21.

Discussion

Maternal autoimmune thyroid disorders may affect fetal and neonatal thyroid function through placental transfer of TSHR-Ab (15). Additionally, anti-thyroid drugs can affect fetuses and neonates through both the placenta and breast milk. However, few reports focus on TSHR-Ab in breast milk (19). The hypothesis that transmission and aggravation of thyroid conditions can occur through breast milk is controversial, as only small amounts of TSHR-Ab are thought to be present in breast milk (1,8-11). Whether TSHR-Ab can affect neonates through breast milk was unclear, therefore, a GD animal model of pregnancy needed to be established to investigate the dynamics of TSHR-Ab in both the mother and the fetus/neonate.

In a previous study, a GD mouse model was successfully established in syngeneic AKR/N mice by repeated injections with fibroblasts stably transfected with cDNAs of the human TSHR and major histocompatibility complex class II (20). Elevated TSHR-Ab and serum T4 levels and diffuse goiter and thyrocyte hyperplasia were induced in $\sim 20\%$ of mice using this method (20). Subsequently, other models were successfully established using the following approaches: i) TSHR cDNA vaccination using a eukaryotic expression plasmid (the DNA-TSHR model); ii) immunization with a recombinant adenovirus coding TSHR (the Ad-TSHR model) (21,22); iii) immunization with dendritic cells (DCs) infected with Ad-TSHR (the DC-TSHR model) (23); and iv) a model involving combined DNA-TSHR and *in vivo* electroporation (24). According to a study by Chen *et al* (12), the Ad-TSHR A-subunit is more effective at inducing GD than both the Ad-TSHR and Ad-TSHR-D1NET (12). Therefore, GD animal models were established in the present study via immunization with Ad-TSHR-289. TSHR-Ab levels were found to be the highest in the early stage of the study, i.e. day 1 after virus delivery, and could be detected in a majority of Ad-TSHR-289-treated mice (89/90), which was significantly different from the sham mice (0/90). These results suggested that the Ad-TSHR-289 induced model of GD was successfully established. In the study by Chen *et al* (12), the incidence

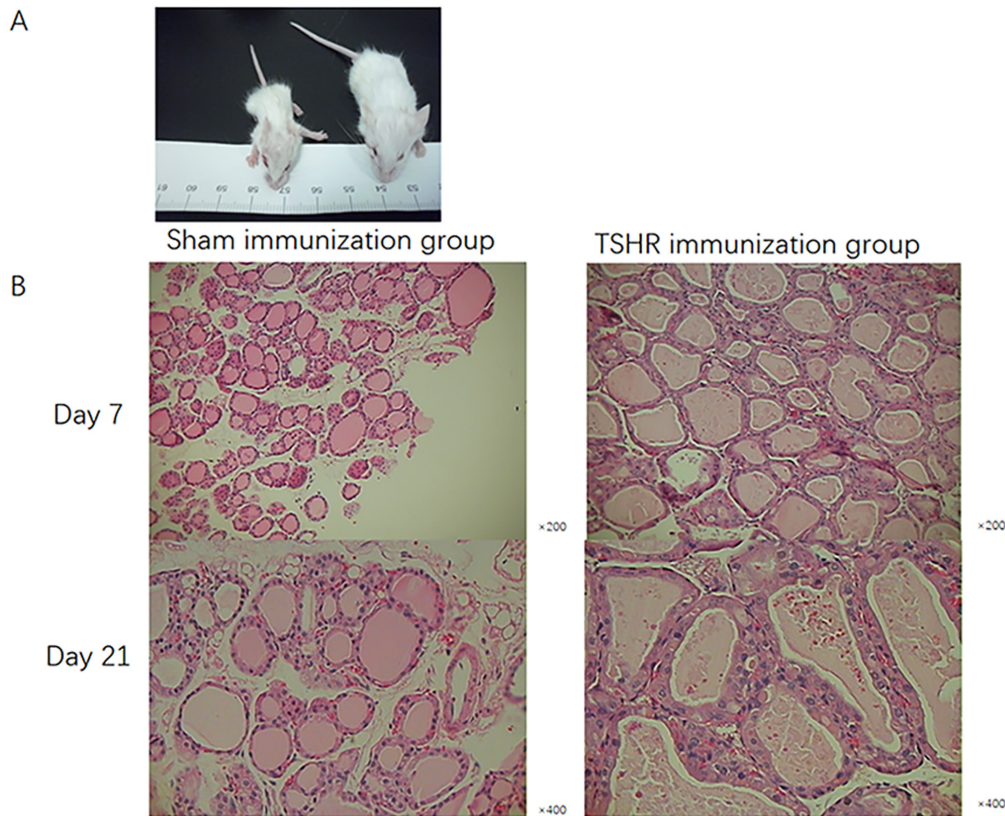


Figure 4. Thyroid morphology in the offspring. (A) Photos of the offspring on day 21 after birth. Left: offspring with hyperthyroidism in the TSHR group; right: offspring in the sham group. (B) Histological examination of the thyroids from neonatal mice with hematoxylin-eosin staining. Upper/lower left: from offspring in the sham group; upper/lower right: from hyperthyroid offspring in the TSHR group. Upper panel: day 7 after birth. Lower panel: day 21 after birth. Magnification, x400. TSHR, thyroid-stimulating hormone receptor.

of hyperthyroidism in Ad-TSHR-289-injected BALB/c mice ranged from 65 to 80% (12). In the present study, the TSHR-Ab-positive rate in the Ad-TSHR-289-treated group was 99%, which was higher than that in Chen's study. As the virus strains of Ad-TSHR-289 and Ad- β -gal are from Chen's laboratory, it is possible that pregnancy may directly affect the establishment of the GD model. During breeding and pregnancy, all BALB/c mice immunized with Ad-TSHR-289 conceived naturally and gave birth to offspring without any perceived difficulties. This may be related to the role of Th2 cytokines that predominate during pregnancy (25). Though the maternal immune system undergoes immune suppression to protect the fetus, hyperthyroidism may recur during the postpartum period as immune status reverts to a Th1 state. Similarly to other autoimmune diseases, GD generally, though not always (26), ameliorates during pregnancy (27) due to TSHR-Ab decline (28). Additionally, pregnancy and delivery can act as triggers for the onset/recurrence of hyperthyroidism in women with GD (29).

Fetal thyrotoxicosis is a rare disease resulting from the transfer of thyroid-stimulating immunoglobulins from the mother to the fetus through the placenta during the second half of pregnancy (20-30th week) (30). These autoantibodies bind to the TSHRs and increase the secretion of thyroid hormones (T4). An improvement in GD is always associated with a reduction in the levels of maternal serum TSHR-Ab during pregnancy. A mother may be euthyroid due to past treatment but still have persistent and active TSHR-Ab (31), affecting

fetal thyroid function through transplacental transfer. The results of the present study suggested that the serum T4 levels of mice with GD on days 1, 7 and 21 were higher than those in the Ad- β -gal injected mice. This was consistent with the serum T4 levels in the offspring of the mice with GD. TSHR-Ab could cross the placenta, like all immunoglobulin G (IgG) antibodies, to appear in the fetal circulation, at least during the time period investigated in this study. These data suggested that GD model was successfully established in the present study and can produce TSHR-Ab that entered the fetal mouse through the placenta.

Neonatal thyroid disease has been reported to develop due to TSHR-Ab in breast milk, possibly because neonates have an immature intestinal mucosa that allows for passage of macromolecules (6). Differing from humans, IgGs from breast milk in many animal species, including rodents, bovines, cats and ferrets, are transported across the intestinal epithelium into the neonatal circulation (32). In the present study TSHR-Ab levels were assessed in breast milk on day 1, 7 and 21. Antibody levels measured from breast milk gradually reduced from day 1 to day 21. The levels of thyroid autoantibodies in the mother with GD could affect the offspring's thyroid function through breast milk. Moreover, both maternal TSHR-Ab and T4 may cross the placental barrier and contribute to the development of GD in their offspring. There is a possibility that the timing of breast-feeding and sacrifice could affect the levels of TSHR-Ab in the stomach of the offspring. It was considered that the effect

of trans-placental diffusion of stimulating antibodies may be more prevalent than the effect of lactation in the development of GD in the offspring mice. However, additional studies are required to examine this hypothesis.

Compared to the control immunized group, GD mothers gave birth to smaller newborns with pathological thyroid changes typical of GD and higher serum levels of TSHR-Ab and T4. The TSHR-Ab levels in breast milk from the GD mice declined with time. The model mimics neonatal GD in humans. However, because the mouse mothers had stimulating TSHR-Ab in their sera when the pups were *in utero*, it is unclear whether any contribution was made to hyperthyroidism in the pups from breast milk TSHR-Ab.

In conclusion, an animal model of GD was successfully established by immunization with Ad-TSHR-289. Higher levels of thyroid auto-antibodies may result in neonatal thyroid disease. The levels of thyroid auto-antibodies in the mother with GD could affect the offspring's thyroid function through the placenta and breast milk.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YY, QQL and DDW contributed to the study design, data collection, statistical analysis, data interpretation and manuscript preparation. SMY contributed to the data collection and performed statistical analysis. LQY and DYL contributed to the data collection, statistical analysis and manuscript preparation. YY and SMY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the institutional animal care and use committee of Anhui Medical University (approval reference no. PJ-YX2019-038).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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