TSH suppression was induced in rat model after total thyroidectomy

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Abstract

Background: This study aimed to induce an animal model for TSH suppression therapy after total thyroidectomy in rats.

Methods: A total of 60 Wistar rats were randomly divided into 6 groups, including sham-operated group (SO group), total thyroidectomy group (TD group), L-T4 treatment I group (TS-I group), II group (TS-II group), III group (TS-III group), and IV group (TS-IV group), in which the rats were accordingly treated with 1.4, 1.6, 1.8, and 2.0 μg/100g body weight after total thyroidectomy.

Results: HE staining in the TD group and all L-T4 treated rats showed that the resected tissue was normal thyroid gland in rats, and no residual thyroid tissue was found in the neck tissue of the cross-section of thyroid gland. The serum levels of T3 in the TS-II group were not significantly different from those in the SO group, whereas the serum level of T4 was slightly higher than that in the SO group, and the serum level of TSH was slightly lower than that in the SO group.

Conclusions: Rats subcutaneously injected with L-T4 at a dose of 1.6 μg/100g body weight for 15 days after total thyroidectomy could induce an animal model for TSH suppression therapy. It may be used as an animal model for TSH suppression therapy.

Key words: Animal model, Thyroidectomy, TSH suppression therapy
Introduction

Thyroid cancer is a common malignant tumor of endocrine system, and its incidence is annually increasing \(^1\). Oral administration of L-thyroxine (L-T4) in order to inhibit thyroid stimulating hormone (TSH) is a standard procedure for postoperative treatment of differentiated thyroid cancer (DTC)\(^2\). Postoperative TSH suppression therapy for DTC patients refers to the use of thyroid hormone tablets to suppress TSH at or below the lower limit of normal value after surgery, which not only can supplement the deficiency of thyroid hormone after surgery, but also inhibit the growth of cancer cells \(^3\). TSH inhibitory level is closely associated with recurrence, metastasis, and cancer-related death of DTC, especially in high-risk DTC patients. The increase of serum level of TSH might promote DTC postoperative progress \(^4\). The overall prognosis of low-risk DTC patients is significantly improved when the TSH level is suppressed at 0.1-0.5 mU/L after surgery; when the TSH level in high-risk DTC patients is suppressed below 0.1 mU/L, the recurrence and metastasis of tumor are significantly reduced \(^5\). However, long-term TSH suppression therapy in DTC patients can lead to drug-induced subclinical hyperthyroidism. Subclinical hyperthyroidism significantly increases the incidence of cardiovascular events and mortality risk, and enhances the incidence of osteoporosis and fracture risk in postmenopausal women as well \(^6,7\), and even cause cognitive impairment in patients \(^8\). In order to further elucidate the effects of TSH suppression therapy on thyroid-cancer patients after thyroidectomy, it is very important to create animal models and study the relevant mechanisms. The purpose of this study was to induce an animal model for TSH suppression therapy after total thyroidectomy in Wistar rats by resection of thyroid tissues and administration of L-T4 to apply TSH suppression therapy in DTC patients.

Materials and methods

Ethics statement

All research animals were treated in accordance with the Principles of Laboratory Animal Care formulated by the U.S. National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the U.S. This study
was approved by the biomedical ethics committee of Inner Mongolia Medical University (No. YKD2014063).

**Experimental animal**

Here, clean-grade female Wistar rats aged 8 weeks with an average body weight (BW) of (200±30) g were purchased from the Laboratory Animal Center of Inner Mongolia Medical University (Inner Mongolia, China; Production License No. SCXK (Mongolia) 2015-0001). Feeding conditions were as follows: illumination time: 12/12h, relative humidity: 45%-50%, ambient temperature: 22-23℃; all rats were free to drink and eat.

In addition, rats were randomly divided into 6 groups, Figure 1 shows the research design and experimental process. ① Sham-operated group (SO group): 10 rats were fed with standard diet and distilled water. After the rats were anesthetized with 10% chloral hydrate solution, the neck was incised to expose the thyroid gland without thyroid ablation, and then suturing was performed. ②Total thyroidectomy group (TD group): After 10 rats were adaptively fed for 1 week, total thyroidectomy was undertaken. Rats were fed with standard diet and high-calcium water (1% calcium gluconate). On the 1st day after surgery, the abdominal subcutaneous injection of placebo was given daily. ③L-T4 treatment I group I (TS-I group): After 10 rats were adaptively fed for 1 week, total thyroidectomy was carried out. Rats were fed with standard diet and high-calcium water (1% calcium gluconate). On the 1st day after surgery, the abdominal subcutaneous injection of L-T4 at a dose of 1.4 μg/100g BW was given daily. ④L-T4 treatment II group (TS-II group): After 10 rats were adaptively fed for 1 week, total thyroidectomy was undertaken. Rats were fed with standard diet and high-calcium water (1% calcium gluconate). On the 1st day after surgery, the abdominal subcutaneous injection of L-T4 at a dose of 1.6 μg/100g BW was given daily. ⑤L-T4 treatment III group (TS-III group): After 10 rats were adaptively fed for 1 week, and total thyroidectomy was performed. Rats were fed with standard diet and high-calcium water (1% calcium gluconate). On the 1st day after surgery, the abdominal subcutaneous injection of L-T4 at a dose of 1.8μg/100 g BW was given daily. ⑥L-T4 treatment IV group (TS-IV group): After 10 rats were adaptively fed for 1 week, total thyroidectomy was carried out. Rats were fed with standard diet and high-calcium...
water (1% calcium gluconate). On the 1\textsuperscript{st} day after surgery, the abdominal subcutaneous injection of L-T4 at a dose of 2.0\(\mu\)g/100g BW was given daily.

**Establishment of reference range of thyroid function in normal rats**

In a previous study, normal reference values for serum thyroid hormone in adult Wistar rats in the local area were successfully established, including 0.73-0.98 ng/ml for T3, 4.2-8.4 ng/ml for T4, and 0.76-1.29\(\mu\)IU/ml for TSH\(^9\).

**Procedure of total thyroidectomy in rats**

Referring to our previous study\(^10\), a longitudinal incision was made in the middle of the neck, which was about 2.0-2.5 cm in length. Neck skin and subcutaneous connective tissue were incised. The sternohyoid muscle of the trachea was bluntly separated along the middle line. After being opened with a distractor, white trachea and a pair of oval thyroid glands attached to the thyroid cartilage on both sides of the trachea ring could be seen. The bilateral thyroid glands were connected across the trachea by the isthmus, and were in deep red-brown (Figure 2 A). Then, the superior thyroid arteries on both sides were ligated, the thyroid isthmus was pinched with tweezers, the thyroid isthmus was cut with ophthalmic scissors (Figure 2 B), one side of the broken end was lifted, and the thyroid gland was bluntly separated close to the surface of trachea, thus, the recurrent laryngeal nerve walking between the thyroid gland and trachea could be observed. After the recurrent laryngeal nerve was carefully stripped, unilateral thyroid gland and isthmus were completely resected. The other side of the thyroid gland was then removed with the same technique until there was no thyroid tissue below the thyroid cartilage (Figure 2 C-E). Intraoperative care should be taken to avoid damaging blood vessels, such as intraoperative bleeding, and sterile cotton swab or gauze needs to be timely used to stop bleeding. After the operation, the resected thyroid tissue was fixed in the 10\% neutral solution of formaldehyde (Figure 2 F). After confirming no bleeding, distractor in the neck was removed, the sternohyoid muscle of the trachea was reset, the muscle membrane and skin were sutured layer-by-layer, the incision was covered with gauze after iodophor disinfection, and warm light was used until recovery.

**TSH suppression was induced in rat model after total thyroidectomy**

Since the 1\textsuperscript{st} day after surgery, rats in the SO group and TD group were subcutaneously
injected with placebo (saline) daily. Rats in the I, II, III, and IV groups were treated with L-T4. Since the 1\textsuperscript{st} day after surgery, L-T4 was subcutaneously injected into the abdomen daily at doses of 1.4, 1.6, 1.8 and 2.0 \(\mu\text{g}/100\ \text{g BW}\), respectively. After 15 days of injection, rats were sacrificed, and serum samples were collected. Simultaneously, the neck was cut off at the upper and lower 1 cm of the thyroid location, and neck tissue in the cross-section of thyroid gland was obtained as well.

Criteria of successful induced model

Referring to the above-mentioned standard value range of thyroid function in rats, when the serum T3 and T4 in each LT-4 treatment groups were within the normal value or T4 was slightly elevated, and TSH was below the lower limit of normal value, the animal model of TSH suppression therapy after total thyroidectomy in rats was induced successfully.

Assessment of indexes and methods

Determination of serum T3, T4, and TSH

On the 15\textsuperscript{th} day after surgery, serum samples were collected from rats in the TD group and all L-T4 treated rats, and then, serum T3, T4, and TSH were determined by Cobas E601 automatic electrochemiluminescence immunoassay system (Roche).

Hematoxylin and eosin (HE) staining of resected tissues and neck tissue

Resected neck tissue of rats in the TD group and all L-T4 treated rats were fixed with 10\% formaldehyde neutral solution, dehydrated, and embedded in paraffin. After performing a routine 4 \(\mu\text{m}\) section, HE staining was undertaken. At the same time, the neck tissue of the cross-section of thyroid gland in rats of the SO group and thyroidectomized rats were removed and stained with HE.

Statistical analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for performing statistical analysis. W-test was used to analyze the normality of each index. Data conforming to normal distribution were described by mean \pm standard deviation (\(X\pmS\)). T-test was used for comparing those two groups, and analysis of variance (ANOVA) was employed for making comparison among multiple groups. P < 0.05 was statistically considered as
Results

HE staining of resected neck tissue

HE staining was performed in the resected neck tissue of rats in the TD group and all L-T4 treated rats. Under light microscopy, a great number of round or irregular follicular structures were observed, the follicles were rich in glia, and blood capillaries and scattered parafollicular cells could be seen among the follicles, which proved that the excised tissue was normal thyroid gland in rats (Figure 2 G).

HE staining of the cross-section of neck after total thyroidectomy

Follicular structure, tracheal cartilage, connective tissue, and muscle tissue were observed in neck tissues of the cross-section of thyroid gland of rats in the SO group after HE staining (Figure 3 A). However, no follicular structure was found in the thyroidectomized rats, while tracheal cartilage, connective tissue, muscle, and inflammatory cells infiltrated in the resected area could be seen, indicating that the thyroid gland of rats was fully resected (Figure 3 B).

Serum levels of thyroid hormone

The serum levels of T3 and T4 in the TD group were significantly lower than those in the SO group, whereas the serum level of TSH increased significantly (P < 0.01); the serum levels of T3 and TSH in the TS-I group were not significantly different from those in the SO group (P > 0.05), while the serum level of T4 was slightly higher than that in the SO group (P < 0.05); the serum levels of T3 in the TS-II group were not significantly different from those in the SO group (P > 0.05), while the serum level of T4 was slightly higher than that in the SO group, and the serum level of TSH was slightly lower than that in the SO group (P < 0.01); the serum levels of T3 and T4 in the TS-III group were remarkably higher than those in the SO group, whereas the serum level of TSH was significantly lower than that in the SO group (P < 0.01); the serum levels of T3 and T4 in the TS-IV group were significantly higher than those in the SO group, however, the serum level of TSH was significantly lower than that in the SO group (P < 0.01) (Figure 4). To sum up, after thyroidectomy, rats in the TD group were
in the hypothyroidism state; rats in the TS-I group were in the replacement state of thyroid function; rats in TS-II group were in the TSH suppression state; and rats in the TS-III group and TS-IV group were in the hyperthyroidism state. Therefore, injection of LT-4 into rats at a dose of 1.6 μg/100g BW led to form a successful model for TSH suppression therapy after total thyroidectomy in rats.

**Discussion**

In this study, we induced an animal model of TSH suppression therapy after total thyroidectomy in Wistar rats for the first time to simulate TSH suppression therapy after surgery for DTC. In order to treat DTC patients with TSH suppression therapy after thyroidectomy, complete resection of thyroid tissue should be firstly carried out to reduce the effect of residual thyroid tissue on the subsequent experiments. The thyroid gland is located in the ventrolateral part of the first four or five tracheal rings below the thyroid cartilage, and the left and right lateral lobes are connected by the isthmus across the ventral surface of the trachea. The glands are brown-red, which is about 3.9-5.5 mm in length and 2.0-3.0 mm in width, covering about 4-5 tracheal rings, the thyroid gland and parathyroid gland cannot be distinguished by naked eyes. The blood supply of thyroid was very rich, there were extensive anastomosis in arteries, from the capsule with connective tissue into the gland, fenestrated capillary network was formed around the follicles, and then the capillary network reintegrated into the venous network, resulting in more blood loss during surgical resection. In the neck of rats, both bilateral recurrent laryngeal nerves are in the tracheoesophageal groove. The recurrent laryngeal nerve innervates the movement of the vocal cords and distributes in the most of the larynx, which is responsible for the sensation of the laryngeal muscles and the mucosa below the vocal cords. Unilateral recurrent laryngeal nerve injury was manifested as vocal cord paralysis, bilateral recurrent laryngeal nerve was manifested as aphonia, dyspnea, and even asphyxia death. In this study, the superior thyroid artery was initially ligated and then, the thyroid isthmus was cut off, and the thyroid gland was fully removed by blunt dissection. This not only reduced the residual thyroid tissue caused by sharp resection, but also well controlled intraoperative bleeding and
protected the recurrent laryngeal nerve. In addition, do not force too much during the operation, so as to avoid crushing thyroid tissue, causing bleeding, as well as affecting the clarity of the surgical field of vision, which not only can increase the probability of recurrent laryngeal nerve injury, but also intensify the crush of the residual thyroid tissue in the surgical area. HE staining was undertaken in the resected tissue of the experimental rats, and the excised tissues were determined to the normal thyroid gland. At the same time, HE staining was carried out in the neck tissue of the cross-section of thyroid gland, in which no follicular structure was found in the histology of the thyroid gland, while tracheal cartilage, smooth muscle, connective tissue, and inflammatory cells infiltrated in the resected area were observed, indicating that thyroidectomy was successfully performed and all resected thyroid tissues.

Race, region, nutrition, iodine intake, and measurement methods all affected thyroid hormone levels, and TSH reference values varied significantly with age, gender, hourly and race. Therefore, it is necessary to establish the reference value of thyroid function in Wistar rats before forming a model for TSH suppression therapy after total thyroidectomy in rats. In a previous study, we successfully established normal reference values for serum thyroid hormone in adult Wistar rats in the local area. This not only provided a normal reference value range for this study, but also provided basic data for the study on thyroid-related diseases in this region. In rat experiments, the exogenous administration of L-T4 included intragastric perfusion, subcutaneous injection, intraperitoneal injection, and subcutaneous mini-pump implantation. The advantage of oral administration is that it is easy to operate and does not cause irritation to rats, however, it cannot properly control the dose, and that is easily affected by the food intake (or water intake) and digestive function of rats. Intraperitoneal injection can accurately control the dose of drug given each time, however, because the absorption rate of drug is very fast, it would lead to drastic fluctuations in the levels of T3 and T4 in plasma or tissue. Compared with intraperitoneal injection, if subcutaneous injection of L-T4 into the back of rats is adopted, the mini-pump does not cause drastic fluctuation of thyroid hormone level in rats, however, the dose cannot be adjusted according to the change in the body weight of rats. Because hypothyroidism can lead
to increase of body weight in rats after thyroidectomy, this change in body weight might lead to a mismatch between dose and body weight. For this reason, we selected a method for subcutaneous injection of LT-4. This method could accurately control the dose and adjust it at any time according to the change of body weight in rats. Although it can still cause fluctuations in thyroid hormone levels in rats, the subcutaneous drug absorption is slower than that of abdominal cavity, and the fluctuations of thyroid hormone levels in rats are more moderate.

In addition to choosing an appropriate method for administration, the dose and the duration of administration are important as well. In Samadi’s study, L-T4 was injected at a daily dose of 1.6 μg/100gBW, which could supplement the reduced thyroxine hormone and replace thyroid function in rats. Therefore, we herein, selected 4 different doses of LT-4, including 1.4, 1.6, 1.8, and 2.0 μg per 100 g BW after thyroidectomy. Van et al. confirmed that after rats were injected with radioisotope-labeled T4 for 6-8 days, the total amount of labeled metabolites excreted through feces and urine became stable and was equal to the daily injected dose of T4. However, in other previous studies, after total thyroidectomy, LT-4 was injected into rats for 12-13 days to achieve the replacement. Based on the above-mentioned findings, in this study, serum thyroid hormones were tested after 15 days of continuous injection of LT-4, in which the results confirmed that administration of L-T4 at a dose of 1.6 μg/100g BW to rats for 15 consecutive days after thyroidectomy could induce an animal model for TSH suppression therapy. The administered dose of LT-4 for replacement therapy was slightly different from the results of the above studies, and we believed that it might be related to the influence of region, iodine intake, and measurement methods on thyroid function. Inner Mongolia was a dry and cold area, normal reference values for serum thyroid hormone in adult Wistar rats were different from other areas. Similarly, the iodine intake was low compared with other regions as well. Our results showed the dose of LT-4 required for TSH suppression therapy in Wistar rats after thyroidectomy.

In conclusion, Rats subcutaneously injected with L-T4 at a dose of 1.6 μg/100g BW for 15 days after total thyroidectomy could be used as an animal model for TSH suppression therapy.
**Conflict of Interest:** The authors declare that they have no conflicts of interest.

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Figure legends

Figure 1. Overview of the research design and experimental process

Figure 2. Images of thyroidectomy and thyroid grand histology in rat

A: Anatomical appearance of the thyroid glands (ligated the superior thyroid arteries on both sides). B: Total thyroidectomy procedure for a rat. C: Anatomical appearance of the neck after total thyroidectomy. D: Exposed the right recurrent laryngeal nerve
after total thyroidectomy. E: Exposed the left recurrent laryngeal nerve after total thyroidectomy. F: Resected thyroid glands from a rat. G: Thyroid gland histology (EH, 10x10).

Figure 3. Histology of the cross-section of neck
A: Histology of the cross-section of thyroid gland in the normal rats (HE, 4x10).
B: Histology of the cross-section of thyroid gland after total thyroidectomy (HE, 4x10).

Figure 4. Serum levels of thyroid hormone
A: Serum levels of T3 in rats for each group (*P<0.01 vs. SO group;  b P<0.01 vs. SO group); B: Serum levels of T4 in rats for each group(*P<0.01 vs. SO group;  *P<0.05 vs. SO group;  #P<0.01 vs. SO group;  b P<0.01 vs. SO group); C: Serum levels of T3, T4, and TSH in rats for each group(*P<0.01 vs. SO group;  #P<0.01 vs. SO group;  ♭ P<0.01 vs. SO group;  b P<0.01 vs. SO group)
(Nota: Normal T3 level: 0.73-0.98 ng/ml; normal T4 level: 4.2-8.4 ng/ml; normal TSH level: 0.76-1.29 μIU/ml)
Fig. 1

Wistar rats

Randomly divided

Sham operated group (SO group)
Total thyroidectomy group (TD group)
L-T4 treatment I group (TS-I group)
L-T4 treatment II group (TS-II group)
L-T4 treatment III group (TS-III group)
L-T4 treatment IV group (TS-IV group)

Without thyroid ablation

Total thyroidectomy

HE staining of resected tissue

Injected placebo (saline)
Injected L-T4, 1.4μg/100g body weight
Injected L-T4, 1.6μg/100g body weight
Injected L-T4, 1.8μg/100g body weight
Injected L-T4, 2.0μg/100g body weight

HE staining of the neck tissue after total thyroidectomy

Determination of serum T3, T4, and TSH
Fig. 2
Fig. 4