Mechanism of Xiaoying Daotan decoction in treating Hashimoto's thyroiditis based on the Notch/Treg/Th17 pathway

Yingjia Zhou[1,2], Hongmei Shen[2], Wanning Lan[3], Yuzhen Shi[3], Qian Yao[2], Weibo Wen[4]

[1]Nanjing University of Traditional Chinese Medicine, Nanjing, China; [2]Integrated Traditional Chinese and Western Medicine Department, Yunnan Cancer Hospital/the Third Affiliated Hospital of Kunming Medical University, Kunming, China; [3]Guangzhou University of Chinese Medicine, Guangzhou, China; [4]Academic Affairs Department, Yunnan Hospital of Traditional Chinese Medicine, Kunming, China

Contributions: (I) Conception and design: Y Zhou; (II) Administrative support: W Wen; (III) Provision of study materials or patients: Y Zhou, H Shen, Q Yao; (IV) Collection and assembly of data: Y Zhou, H Shen; (V) Data analysis and interpretation: Y Zhou, H Shen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Weibo Wen. Academic Affairs Department, Yunnan Hospital of Traditional Chinese Medicine, No. 120, Guanghua Street, Wuhua District, Kunming 650021, China. Email: zxbzyjzmy@163.com; Yingjia Zhou. Integrated Traditional Chinese and Western Medicine Department, Yunnan Cancer Hospital/the Third Affiliated Hospital of Kunming Medical University, No. 519, Kunzhou Road, Xishan District, Kunming 650032, China. Email: zhouyingjia1105@163.com.

Background: The study created mice model of Hashimoto's thyroiditis (HT) and induced thyroid inflammatory cell lines, exploring the mechanism of Xiaoying Daotan decoction on HT.

Methods: Divided HT mice models into model group (0.2 mL saline), Western medicine group (0.2 mL levothyroxine sodium tablets), traditional Chinese medicine group (0.2 mL Xiaoyin Daotan prescription), and Notch protein inhibition group (0.2 mL Xiaoyin Daotan prescription). After treatment, serum Notch protein expression and T cell (Treg)/T helper cell 17 (Th17) cytokines levels were detected through Enzyme linked immunosorbent assay (ELISA). Use real-time qualitative polymerase chain reaction detected Notch protein expression. Thyroid inflammatory cell lines were induced and divided into 5 groups: blank group, iNotch group (knocking down the Notch protein gene of thyroid inflammatory cells), NC group (Notch protein carrier negative control group), iNotch + DS group and DS group (knocking down the Notch protein gene of thyroid inflammatory cells). The cells were treated with serum containing Xiaoying Daotan decoction. After culture, detected Notch protein expression level and Treg/Th17 cytokine level in each group.

Results: For the animal experiment, the serum Notch protein expression, the serum levels of key activating proteins Signal Transducer and Activator of Transcription 3 (STAT3), RAR-related orphan receptor gamma T (RORγt), and interleukin (IL)-22 of Th17 cells of mice in the model group was significantly higher than that of the other groups. Compared with the model group and Western medicine group, the serum transforming growth factor-β (TGF-β) level of the mice in the traditional Chinese medicine group and the Notch protein inhibition group was significantly higher. All the differences were statistically significant ($P<0.05$). For the cell experiment, the β-actin value of Notch protein in thyroid inflammatory cell genes was significantly downregulated and the key activation protein of Treg was significantly upregulated in iNotch + DS group and DS group compared with the other 3 groups. Levels of Th17 key activating proteins STAT3, IL-17, and IL-22 in the iNotch group, iNotch + DS group, and DS group were lower than those of the blank group and NC group, both with statistically significant difference ($P<0.001$).

Conclusions: The mechanism of Xiaoying Daotan decoction on HT could be related to the immune inflammatory response of the Treg/Th17 cell axis mediated by the Notch protein pathway.

Keywords: Xiaoying Daotan decoction; Hashimoto's thyroiditis (HT); Notch protein pathway; regulatory T cell (Treg)/T helper cell 17 (Th17) cytokine; immune inflammatory response

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Introduction

Hashimoto's thyroiditis (HT), also known as chronic lymphocytic thyroiditis (1), is a classic representative of autoimmune thyroiditis (AIT). Therefore, the study of HT has a certain degree of reference significance for the study of AIT. The prevalence of HT is about 3–4% (2), which accounts for about 22.5% of all thyroid diseases. With changes in lifestyle and improvements in detection technology, the incidence of this disease has been increasing, and the age of onset is mainly 30–50 years of age, with significantly more women than men impacted by HT (men *vs.* women: 1:4–20) (3,4). Studies have shown that the prevalence of papillary thyroid cancer in HT patients is higher than that of the normal population, suggesting that HT may be a risk factor for thyroid cancer (5). Some scholars have even proposed that HT is a precancerous lesion of papillary thyroid cancer (6). Additionally, HT during pregnancy and the perinatal period increases the risk of miscarriage, premature delivery, and fetal mental retardation (4). Therefore, the early detection and intervention of HT has important clinical significance.

HT is considered to be the first autoimmune disease, and the Notch signaling pathway plays a key role in regulating the immune system of cells. The Notch signaling pathway is composed of the Notch receptor, Notch ligand [Delta/Serrate/lag-2 (DSL) protein], C-repeat binding transcription factor-1, Suppressor of Hairless, Lag-1 (CSL), DNA binding protein, other effectors, and Notch regulatory molecules. It is a highly conserved intercellular signaling pathway that regulates cell proliferation and differentiation, determines cell fate, and participates in cellular processes in embryonic and adult tissues. Many studies have found that Notch signaling may be involved in regulatory T cells (Treg) function (7,8), and others have shown that the Notch signaling pathway can participate in a variety of autoimmune diseases by regulating the levels of Treg/T helper cell 17 (Th17) cytokines to mediate the immune inflammatory response (9-12).

Helper T cells (Th) and Treg, are both T cells, which are important cells involved in the body's autoimmune response. The main surface marker of Th is cluster of differentiation 4 (CD4), and its cell subpopulations Th1, Th2, Th17 and follicular helper T cells (Tfh) were reported to be closely related to the occurrence and development of HT. Treg, which are mainly composed of cluster of differentiation 4^+ ($CD4^+$), cluster of differentiation 25^+ ($CD25^+$), and Forkhead box $P3^+$ ($FOX\text{-}P3^+$) cells, account for 5–10% of $CD4^+$ T cells in the peripheral blood and spleen of healthy people and mice, and play an important role in regulating the immune response, maintaining the body's immune autostability and preventing the occurrence of autoimmune diseases. CD4 and CD25 is a class of immune cells, while FOX-P3 is an autoantigen, and all of them play an important role in immune response.

Since HT is an autoimmune disease, we considered that the Notch protein may also promote the occurrence and development of HT by affecting the levels of Treg/Th17 cytokines. Presently, it has been clinically observed that Xiaoying Daotan decoction can effectively improve the clinical symptoms of HT patients. Therefore, in the present study, we explored the mechanism of Xiaoying Daotan decoction in animal and cell experiments to verify this.

Chinese medicine Xiaoying Daotan decoction consists of *Prunella vulgaris* (*Xiakucao*), which has the functions of clearing heat and draining the liver fire, dissipating masses and swelling, *Fritillaria* (*Tubeimu*), with the additional function of detoxification, and *Bupleurum* (*Chaihu*), which has the functions of soothing the liver, relieving depression, and clearing the ministerial fire. These are the chief medicine, which mainly has the functions of soothing the liver and clearing heat, dispersing masses and swelling. O*ysters* (*Muli*) with the functions of calming the liver and suppressing yang, softening firmness and dispelling masses, and *Panshanlong* (*Chuanshanlong*), which has the functions of promoting blood circulation, removing blood stasis, softening hardness and dissolving phlegm, *Antler cream* (*Lujiaoshuang*) with the function of invigorating blood, reducing swelling, supplement deficiency and warming yang, *Turmeric* with the effects of promoting qi and resolving constraint, cooling the blood and promoting blood circulation, and *roasted Xiangfu* (*Zhixiangfu*) with the functions of soothing liver and relieving depression, rectifying qi and Menses, make up the deputy medicines, which together perform the functions of promoting qi,

relieving depression and promoting blood circulation, at the same time assisting the chief medicine to strengthen the effects of soothing the liver and relieving stagnation, dissipating masses and swelling. *Angelica* (*Danggui*), which has the functions of replenishing and invigorating blood, regulating Menses and relieving pain, *white peony* (*Hangshao*), which has the functions of softening liver and relieving pain, nourishing blood and regulating Menses, and *Chuanxiong*, which has the effects of promoting blood circulation and dispeling blood stasis, promoting qi and resolving constraint, as the assistant medicine, nourish the blood, promote blood circulation and qi, and relieve depression. Combining the pathogenesis of qi stagnation, phlegm coagulation, and blood stasis, the assistant medicine strengthens the power of chief medicine and deputy medicine in the aspect of invigorating and nourishing the blood. *Licorice* (*Gancao*), which has the effects of replenishing qi and fortifying the spleen, harmonizing the center and relieving acute pain, is used as envoy medicine to reconcile various medicine properties. The whole prescription has the effects of soothing the liver and regulating qi, resolving phlegm and dissipating masses.

Thyroglobulin (thyroglobulin, Tg) and immune adjuvant susceptible strains of mouse-induced experimental AIT (experimental AIT, EAT) is an ideal animal model to study the pathogenesis of AIT. In the establishment of this model, there are 2 types of Tg used for immune induction: porcine Tg (pTg) and mouse Tg (murineTg, mTg). CBA/J mice are recognized as EAT-susceptible mice. It has been reported that the EAT model, which was established by immunizing CBA/J mice with Tg plus Freund's adjuvant, has a high success rate and reliability, and the experimental method is simple and feasible. At present, pTg is mostly used to immunize mice with Tg and adjuvant to induce EAT in China, and only a few studies use mTg. Moreover, according to the literature, this modeling method is suitable for simulating the target disease (13). In addition, the incidence rate of HT in females is much higher than that in males, so the female mice immunized with PTg plus Freund's adjuvant to CBA/J were used as models in animal experiments in the present study.

The purpose of the present study was to investigate the mechanism of Notch/Treg/Th17 pathway in the treatment of HT. Current research results indicate that the mechanism of Xiaoying Daotan decoction in treating HT may be related to the immune inflammatory response of the Treg/Th17 cell axis mediated by Notch protein pathway. The mechanism of action was verified by animal experiments and cell experiments. This may provide a scientific basis for the clinical application of Xiaoying Daotan decoction in the treatment of HT.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-6253).

Methods

Experimental animals

Basic information of the experimental animals

The animal was from The Department of Zoology, Kunming Medical University, healthy mice without gene modification.

Because HT is more common in female patients, therefore, according to the experimental needs, all the experimental animals were female mice, without reproductive plan. The mice were fed in the specific pathogen free (SPF) animal laboratory, and the light/dark cycle was 12 hours each, the temperature was 24 ℃, and the food type was SPF rat chow.

The developmental stage of experimental animals is 4–6 weeks: The age of model group was 4.90±0.74 W, Western medicine group was 4.90±0.88 W, traditional Chinese Medicine group was 4.70±0.82 W, Notch protein inhibition group was 4.90±0.88 W.

The weight of experimental animals: The weight of model group was 19.32±0.46 g, Western medicine group was 19.26±0.41 g, traditional Chinese medicine group was 19.38±0.50 g, Notch protein inhibition group weight was 19.45±0.39 g.

Before the experiment, the relevant characteristics and health status of the mice (body weight, microbiological status and experimental infancy) were shown in *Table 1*.

Experimental animals and groups

Forty CBA/J female mice, weighing 18–20 g were selected for the experiment. Using SPSS version 23.0 (IBM, Armonk, NY, USA), each mouse was first numbered, and then the mice were randomly divided into the following 4 groups: model group, Western medicine group, traditional Chinese medicine group, and Notch protein inhibition group, with 10 mice in each group.

Experimental drugs

The Chinese medicine, Xiaoying Daotan decoction, comprises 30 g *Prunella vulgaris*, 15 g *Fritillaria*, 10 g

Table1 Relevant characteristics and health status of the mice before the experiment

Body weight in mice	Microbiological status	Experimental growth period
18–20 g	SPF level	Early adolescence

SPF, specific pathogen free.

Bupleurum, 30 g *oyster*, 30 g *Panshanlong*, 30 g *antler cream*, 15 g *turmeric*, 15 g *roasted Xiangfu*, *Angelica* 15g, 30 g *white peony*, 15 g *Chuanxiong*, and 10 g *licorice*. For levothyroxine sodium tablets, the specification is 50 μg/tablet.

Modeling method

The 40 mice were given water and feed. Taking human medication as a reference, the patients were given levothyroxine sodium tablets half an hour before breakfast, so the mice were also given levothyroxine sodium intragastric half an hour before feeding. Chinese medicine is usually administered half an hour after meals for the patients, so mice were also given Chinese medicine intragastric administration half an hour after feeding. Mice in model group were given normal saline intragastrically half an hour before feeding. After 1 week of adaptive feeding, the model was built in the second week. For the first immunization, powdered pTg was dissolved in phosphate-buffered saline, mixed with the same volume of Freund's complete adjuvant, and fully emulsified; 100 μg pTg was subcutaneously injected into the foot pads and back of each mouse once a week for 2 consecutive weeks. There was no treatment in the third week. The boost immunization commenced in the 4th week: pTg was mixed with the same volume of incomplete Freund's adjuvant, and after fully emulsifying, the same method as above was used to inject 100 μg pTg subcutaneously into the foot pads and back of each mouse once a week for 4 consecutive weeks. At the same time, the mice were given 0.64 g/L sodium iodide high iodine water (14).

Medication method

After 4 weeks of modeling, 2 mice from each group were randomly selected for the pre-experiment, and the remaining mice were administered the drug after the successful modeling was confirmed. The dosage was calculated according to the maximum dose taken by humans and the body surface area of the mice. The conversion relationship between 20 g mice and 70 kg humans is 0.0026:1. For 1 dose of Xiaoying Daotan decoction, add 500 mL of water, decoct 300 mL of medicinal solution, then heat and concentrate the medicinal solution to 100 mL, and cool to 25 ℃ naturally. In the traditional Chinese medicine group, the mice were intragastrically administered Xiaoying Daotan decoction 0.1 mL/10 g per day. In the Notch protein inhibition group, 100 μg of the Notch protein inhibitor was injected into the abdominal cavity of mice after successful modeling, and 0.1 mL/10 g·per day of Xiaoying Daotan decoction was used to gavage mice. The Western medicine control group mice were given Levothyroxine sodium tablets. Specifically referred to Levothyroxine sodium tablets were crushed, and 500 μg was dissolved in 50 mL saline and prepared into a 10 μg/mL solution. And then the Western medicine control group mice were gavaged at a dose of levothyroxine sodium tablet solution 0.1 mL/10 g. At the same time, mice in the blank control group and model group were given 0.1 mL/10 g saline by gavage. Each group was treated with drugs once a day for 6 weeks.

Detection indicator

Enzyme-linked immunosorbent assay (ELISA) was used to detect the serum Notch protein expression of mice in each group. ELISA was used to detect the levels of key Treg activation proteins FOX-P3, transforming growth-factor-β (TGF-β), interleukin (IL)-10, and Th17 cell activation proteins Signal Transducer and Activator of Transcription 3 (STAT3), RAR-related orphan receptor gamma T (RORγt), IL-17, IL-22 levels in each group of mice. STAT3 is a transcription factor involved in some cytokines as well as growth factor-mediated signaling transduction, which also plays a role in multiple autoimmune diseases (15). RORγt, an immune cell-specific isoform of RORγ, is a key transcription factor for the development of Th17 cells both in human and mouse.

Ethical statement

Experiments were performed under a project license (IRB number: KMMU2019433) granted by Ethics Committee of Kunming Medical University, in compliance with Chinese national or institutional guidelines for the care and use of animals.

Cell experiment

Induced thyroid inflammatory cell line

Nthy-ori 3-1 human thyroid epithelial cell line (purchased from Shanghai Cell Bank) was purchased, and lipopolysaccharide (LPS) was added to RPMI-1640 medium to create an inflammatory environment for HT. Real-time qualitative polymerase chain reaction method was used to detect whether the expression of Notch protein in the cells increased. After confirming that the expression of Notch protein increased, the thyroid inflammatory cell line was successfully induced; β-actin expression was used as an internal control to standardize the expression of targeted genes.

Grouping and administration method

(I) Blank group (thyroid inflammatory cell line); (II) iNotch group (knocking down the Notch protein gene of thyroid inflammatory cells); (III) NC group (Notch protein carrier negative control group); (IV) iNotch + DS group (after knocking down the Notch protein gene of thyroid inflammatory cells, took drug-containing serum); (V) DS group (thyroid inflammatory cells that did not knock down the Notch protein gene, took medicated serum for administration).

Detection indicator

Detection of Notch protein expression in thyroid inflammatory cell genes: After culturing, cells were collected and the expression of Notch protein in each group was detected by Real-time qPCR and Western blot.

Detection of Treg/Th17 cell-related transcription factors in the culture supernatant of thyroid inflammatory cells: ELISA method was used to detect the levels of key Treg activation proteins FOX-P3, TGF-β, IL-10, and Th17 key activation proteins STAT3, RORγt, IL-17, IL-22 levels.

Experimental observation process

We took pictures under a microscope to record the cell growth. The cell growth of each group would be observed 48 h after transfection. The cell experiment was done only once. The experimental flowchart is shown in *Figure 1*.

Statistical analysis

For the descriptive statistical analysis, qualitative indicators are described by percentage or composition ratio, and quantitative indicators are described by mean ± standard deviation. For the comparative analysis and qualitative data of the 2 groups, χ^2-test, Fisher's exact probability method, and Wilcoxon rank sum test were used. The quantitative data conformed to the normal distribution with *t*-test, and did not conform to the normal distribution with Wilcoxon rank sum test. The hypothesis test used a 2-sided test uniformly, and the test statistics and the corresponding P value are given. $P \leq 0.05$ was considered statistically significant, and $P \leq 0.01$ was considered more significant. The animal experiment was done only once, and the experimental flowchart is shown in *Figure 2*.

Results

All experiment data were used without shedding. There was no abnormal death of mice in the process of modeling and experiment, and all data were used without shedding.

Animal experiment

Comparison of serum Notch protein expression in mice of each group

As seen in *Table 2*, compared with the model group, the expression of Notch protein in the other groups was significantly decreased, and the difference was significant ($P<0.001$). In addition, the other groups were compared in pairs, and the difference was not statistically significant ($P>0.05$).

Comparison of key activating protein levels of Treg in mouse serum in each group

As seen in *Table 3*, the 4 groups of mouse serum Treg key activation protein FOX-P3 levels were compared in pairs, and the difference was not statistically significant ($P>0.05$). Serum TGF-β levels of the traditional Chinese medicine group and the Notch protein inhibition group were significantly higher than those of the model group and the Western medicine group, and the difference was statistically significant ($P<0.05$). There was no statistically significant difference in serum IL-10 levels between the 4 groups of mice ($P>0.05$).

Comparison of key activated protein levels of Th17 cells in mouse serum in each group

As seen in *Table 4*, compared with the model group, the serum STAT3, RORγt, and IL-22 levels of mice in the other groups decreased significantly, and there was a statistically significant difference ($P<0.05$). There was no significant

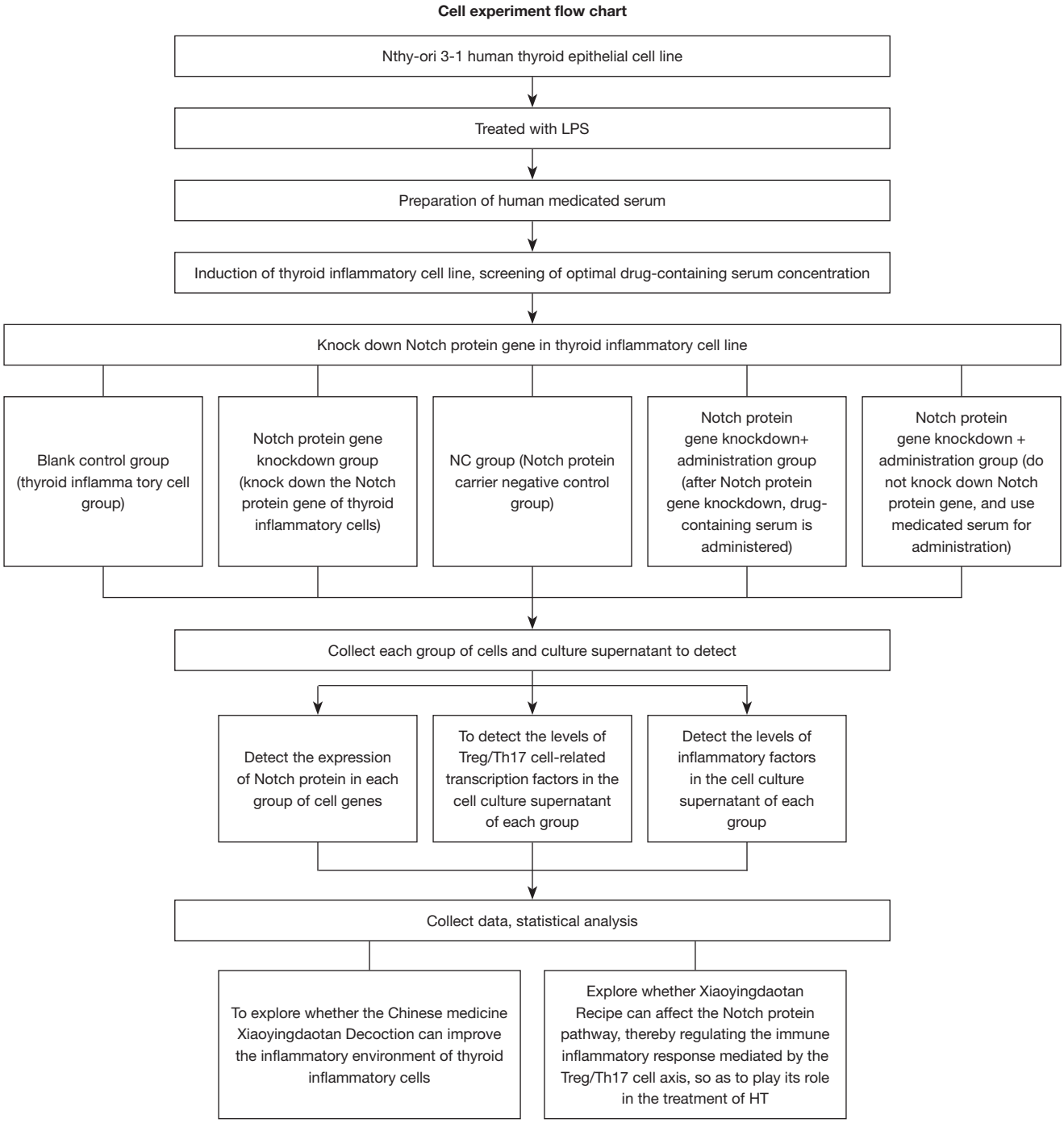

Figure 1 Cell experiment flowchart.

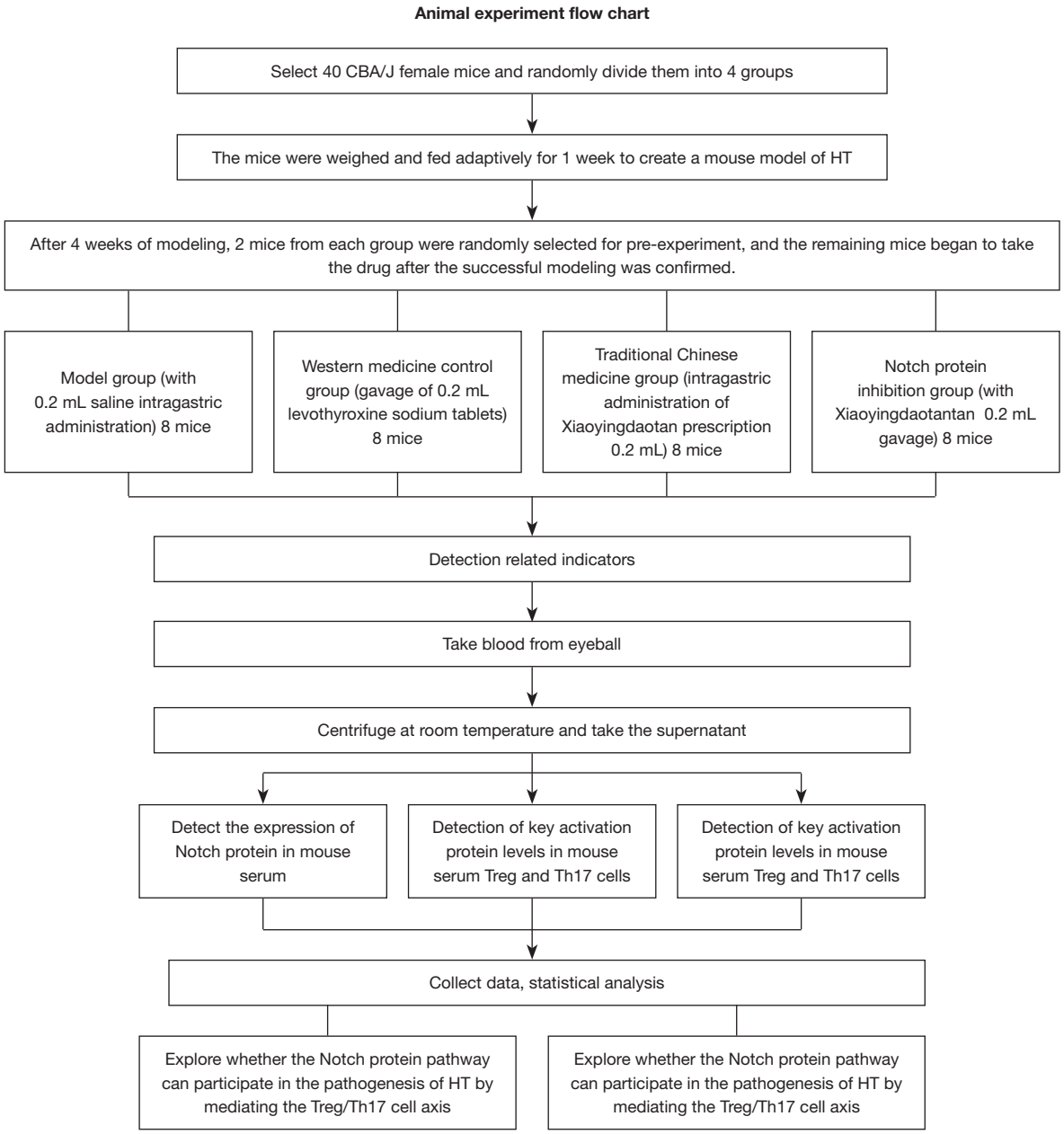

Figure 2 Animal experiment flowchart.

Table 2 Comparison of serum Notch protein expression of mice in each group

Indicator	Model group	Western medicine group	Traditional Chinese medicine group	Notch protein inhibition group
Notch (nmol/L)	9.54±1.31	5.34±0.59	5.56±1.27	5.31±0.11

There is no falling data.

Table 3 Comparison of key activation protein levels of serum regulatory T cells in each group of mice

Indicator	Model group	Western medicine group	Traditional Chinese medicine group	Notch protein inhibition group
FOX-P3 (pg/mL)	87.09±12.75	70.51±10.21	99.62±26.59	116.26±50.54
TGF-β (pg/mL)	3,376.47±350.86	2,758.24±901.61	4,906.81±285.96	4,580.69±421.81
IL-10 (pg/mL)	57.88±33.13	34.42±30.66	41.01±31.46	43.64±25.13

FOX-P3, Forkhead box P3; IL, interleukin; TGF-β, transforming growth factor-β. There is no falling data.

Table 4 Comparison of key activating protein levels of T helper cell 17 cells in mouse serum in each group

Indicator	Model group	Western medicine group	Traditional Chinese medicine group	Notch protein inhibition group
STAT3 (pg/mL)	89.65±25.09	63.08±3.67	57.51±4.71	59.54±11.77
RORγt (ng/mL)	262.25±131.97	158.78±20.32	151.01±5.65	121.98±7.44
IL-17 (pg/mL)	1,159.00±919.60	656.36±114.00	663.32±233.69	671.83±84.12
IL-22 (pg/mL)	332.31±47.66	102.26±27.95	44.51±38.28	80.05±53.74

IL, interleukin; RORγt, Retinoic acidrelated orphan recertor gamma T; STAT3, Signal Transducer and Activatorof Transcription 3. There is no falling data.

difference in serum IL-17 levels among the 4 groups of mice (P>0.05).

Cell experiment

Experimental observation process

The pictures of cell growth in each group 48 h after transfection are shown in the *Figure 3*.

Comparison of the expression levels of Notch protein in thyroid inflammatory cell genes in each group

Western blotting was used to detect the β-actin value of the Notch protein in the 5 groups of thyroid inflammatory cell genes. As shown in *Figure 4*, after pairwise comparison, the iNotch + DS group and the DS group showed that the β-actin value of Notch protein in the thyroid inflammatory cell gene was significantly downregulated, with statistical difference (P<0.05), compared with the other three groups

Comparison of the levels of key activation proteins FOX-P3, TGF-β, and IL-10 in Treg culture supernatants of each group

As seen in *Table 5*, the levels of key Treg activation proteins FOX-P3, TGF-β, and IL-10 in the cell supernatant of the 5 groups were compared in pairs. Compared with the other 3 groups in the iNotch + DS group and DS group, the levels of FOX-P3 and TGF-β in the cell supernatant were significantly increased, and the difference was significant (P<0.001). However, there was no statistically significant difference between the iNotch + DS group and the DS group (P>0.05).

Comparing the iNotch + DS group and DS group with the other 3 groups, IL-10 levels in the cell supernatant also increased significantly, with significant statistical differences (P<0.001). Compared with the iNotch + DS group and the DS group, the increase in IL-10 in the DS group was more than that in the iNotch + DS group, with a significant statistical difference (P<0.001).

Comparison of the levels of key activating proteins STAT3, RORγt, IL-17, and IL-22 in Th17 cell culture supernatants of each group

As seen in *Table 6*, the levels of Th17 key activation proteins STAT3, IL-17, and IL-22 in the cell supernatant of the iNotch group, iNotch + DS group, and DS group were significantly lower than those in the blank group and NC group, and the difference was statistically significant (P<0.001). When comparing the RORγt levels in the cell supernatant of each group, although the iNotch + DS group had a greater decrease in RORγt than the other groups, the difference was not statistically significant (P>0.05).

Discussion

The literature analysis indicated that Treg is a type of $CD4^+$ T-cell subgroup with unique functions discovered

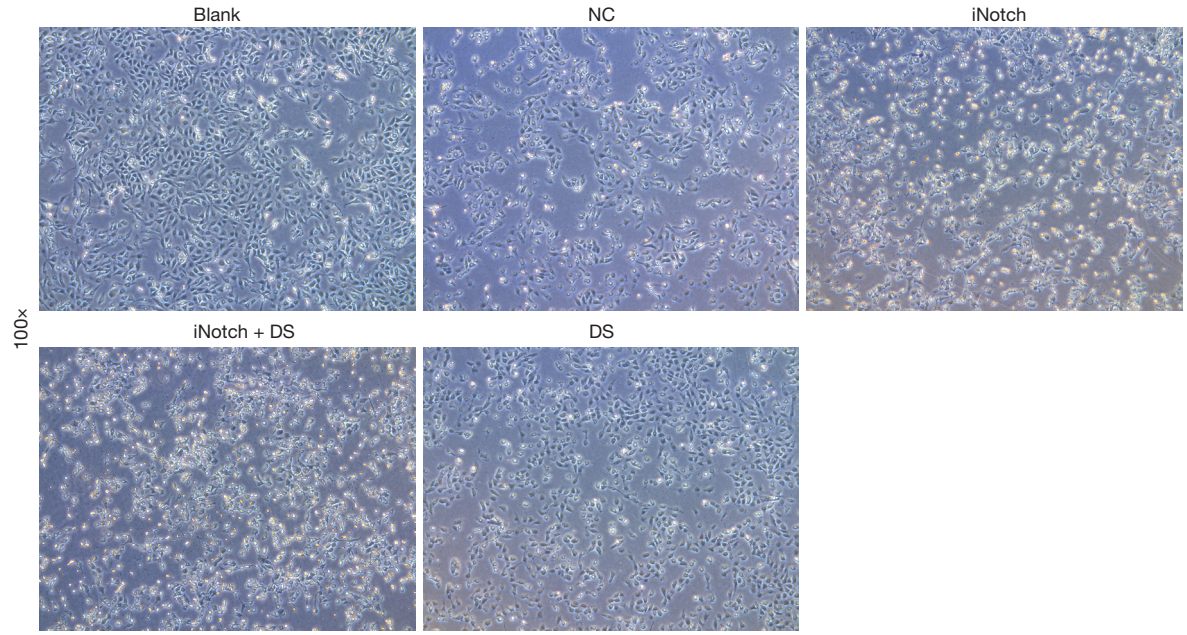

Figure 3 Cell growth in each group 48 h after transfection. Blank group (×100 magnification); NC group, Notch protein carrier negative control group (×100 magnification); iNotch group (×100 magnification), iNotch, knocking down the Notch protein gene of thyroid inflammatory cells; iNotch + DS group (×100 magnification), after knocking down the Notch protein gene of thyroid inflammatory cells, took drug-containing serum; DS group (×100 magnification), thyroid inflammatory cells that did not knock down the Notch protein gene, took medicated serum for administration.

in recent years. It exists in the peripheral blood and spleen and other organs of healthy people. It is a full-time suppressor cell, which can play a good immunomodulatory role (16). Treg can selectively inhibit effector T cells and autoreactive T cells in the body. This effect is an important mechanism for the formation and maintenance of immune homeostasis. The decrease in the number of Treg or the dysfunction results in the immune system not being able to produce good immune tolerance to the stimulation of self-antigens, therefore causing the occurrence of autoimmune diseases (17). FOX-P3 is a key regulator that affects the differentiation and function of Treg (18). TGF-β can induce the expression of FOX-P3 and the secretion of IL-10, which is closely related to the production of peripheral Treg. Th17 mainly secretes IL-17 and plays an indispensable role in autoimmune diseases. Its main function is to gather inflammatory cells, such as neutrophils, causing cell infiltration and tissue destruction (19). Th17 cells are characterized by signal transduction, STAT3, and RORγt, and mainly secrete IL-17 and IL-22.

The immune balance of the Treg/Th17 cell axis is of significance for maintaining the body's normal immune response and preventing the occurrence of autoimmune diseases. Under normal circumstances, Treg and Th17 cells are in a state of immune balance. However, when the number of Treg is insufficient or their function is defective, their immunosuppressive ability decreases, leading to the excessive proliferation of Th17 cells, which can cause a variety of autoimmune diseases. Through the detection of Treg and Th17 cytokines in peripheral blood and thyroid tissue, it was found that Th17 cell response in thyroid tissue and peripheral blood of HT patients increased, while Treg immunosuppression decreased, indicating that the immune imbalance of the Treg/Th17 cell axis is involved in the development of HT.

The Notch signaling pathway plays an important role in the exchange of information between cells. In the immune system, the Notch signaling pathway does not only regulate the generation of T/B cell lineages but also participates in the regulation of the differentiation and function of peripheral mature T cells and their subpopulations. For example, Notch signaling can be widely expressed on the surface of $CD4^+$ T cells, which has an important impact on the proliferation, differentiation, apoptosis of Treg

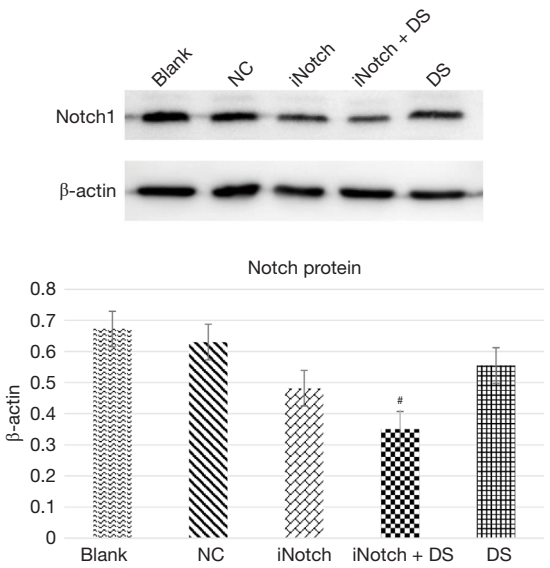

Figure 4 Comparison of Notch protein expression in thyroid inflammatory cell genes in each group. DS, thyroid inflammatory cells that did not knock down the Notch protein gene, took medicated serum for administration; iNotch, knocking down the Notch protein gene of thyroid inflammatory cells; NC, Notch protein carrier negative control group. #, P<0.05 significantly different from the control group.

and Th17 cells and the immune function of cells. The expression of Notch signal may promote the occurrence and progression of autoimmune encephalomyelitis (20), rheumatoid arthritis (21), aplastic anemia (22), and other autoimmune diseases. The occurrence and development of these diseases are considered to be caused by the regulation of Treg/Th17 cytokine levels by the Notch signaling pathway. Notch signaling is widely involved in a variety of immune system diseases by regulating the Treg/Th17 cell axis. The activation of the Notch signaling pathway may be the key to regulating the relationship between both (23-25).

Numerous experiments have shown that the Notch signaling pathway can mediate immune inflammatory response by regulating the levels of Treg/Th17 cytokines, thereby participating in a variety of autoimmune diseases. However, HT is considered to be the first disease of autoimmune origin. Therefore, the Notch protein may also promote the occurrence and development of HT by affecting the levels of Treg/Th17 cytokines. The Nthy-ori 3-1 human thyroid epithelial cell line was purchased and treated with LPS to create an inflammatory environment for HT. Cell experiments were designed to verify the expression of Notch protein as one of the detection

Table 5 Comparison of key activation protein levels of Treg in the cell culture supernatant of each group

Indicator	Blank	NC	iNotch	iNotch + DS	DS
FOX-P3 (pg/mL)	145.35±8.46	95.64±7.75	108.37±31.98	452.33±57.77	501.11±105.24
TGF-β (pg/mL)	1,368.11±86.33	1,164.04±574.71	1,242.13±893.17	15,857.97±547.26	16,127.11±722.36
IL-10 (pg/mL)	102.83±30.06	79.70±11.39	81.08±19.14	474.47±6.32	641.27±58.13

DS, knocking down the Notch protein gene of thyroid inflammatory cells; FOX-P3, Forkhead box P3; IL, interleukin; iNotch, knocking down the Notch protein gene of thyroid inflammatory cells; TGF-β, transforming growth factor-β; NC, Notch protein carrier negative control group. There is no falling data.

Table 6 Comparison of T helper cell 17 key activation protein levels in the cell culture supernatant of each group

Indicator	Blank	NC	iNotch	iNotch + DS	DS
STAT3 (pg/mL)	262.20±61.47	234.26±51.53	52.67±11.26	44.74±8.55	48.63±2.15
RORγt (pg/mL)	82.42±14.08	73.57±12.10	73.04±35.37	64.01±2.94	66.66±20.85
IL-17 (pg/mL)	18.71±1.29	18.58±0.55	5.73±0.20	5.57±0.79	5.72±0.92
IL-22 (pg/mL)	59.84±4.65	56.33±4.00	7.18±1.63	5.74±0.46	6.46±1.73

DS, knocking down the Notch protein gene of thyroid inflammatory cells; IL, interleukin; iNotch, knocking down the Notch protein gene of thyroid inflammatory cells; NC, Notch protein carrier negative control group; RORγt, RAR-related orphan receptor gamma T; STAT3, signal transducer and activator of transcription 3. There is no falling data.

indicators, and the pathogenesis of HT was further studied at the molecular protein level.

The results of the animal experiments showed that both the Chinese medicine, Xiaoying Daotan decoction, and the Western medicine, levothyroxine sodium tablets, can effectively downregulate the expression level of the Notch protein, which is equivalent to the use of Notch protein inhibitors to downregulate the Notch protein. By analyzing and comparing the key activation protein levels of serum Treg in each group of mice, we found that Xiaoying Daotan decoction, a traditional Chinese medicine, can effectively upregulate serum TGF-β levels in HT mouse models, even for HT mouse models in which Notch protein is inhibited. After treatment with Xiaoying Daotan decoction, the serum TGF-β level in mice can still be effectively increased, but FOX-P3 and IL-10 cannot be increased. Through the analysis and comparison of the key activation protein levels of Th17 cells in each group of mice, it was found that Xiaoying Daotan decoction can effectively downregulate serum STAT3, RORγt, and IL-22 levels in the HT mouse model, even for HT mouse models in which Notch protein is inhibited; however, the downregulating effect on IL-17 was not obvious.

The cell experiment results showed that Xiaoying Daotan decoction can downregulate the expression of the Notch protein in thyroid inflammatory cells, and its downregulation effect is equivalent to that of the Notch gene knockdown group. Through the analysis and comparison of the key activation protein levels of Treg in the cell culture supernatant of each group, it was found that Xiaoying Daotan decoction can upregulate the levels of FOX-P3, TGF-β, and IL-10 in the cell supernatant, regardless of whether the Notch gene is knocked down or not. Xiaoying Daotan decoction can exert this upregulation effect. Through the analysis and comparison of the key activation protein levels of Th17 cells in the cell culture supernatant of each group, it was found that Xiaoying Daotan decoction can downregulate the levels of STAT3, IL-17, and IL-22 in the cell supernatant, even if the Notch protein is blocked. After knocking down Notch protein and then giving medicated serum therapy, it can still downregulating the levels of STAT3, IL-22 and I L-17, but it cannot effectively downregulate the level of RORγt in the cell supernatant.

Studies have confirmed that Xiaoying Daotan decoction can effectively downregulate the expression of Notch protein in the HT mouse model and thyroid inflammatory cells, and can effectively upregulate Treg cytokines and downregulate Th17 cytokines. Therefore, the mechanism of Xiaoying Daotan decoction in the treatment of HT may be related to the immune inflammatory response of the Treg/Th17 cell axis mediated by the Notch protein pathway. The findings of the present study verified the efficacy of Xiaoying Daotan decoction in treating HT through animal and cell experiments, and clarified the possible mechanism of HT, and had certain reference value for clinical treatment and laboratory research of HT.

The present study has some limitations. First, the pathogenesis of HT is complex. Although we found that the Treg/Th17 cell axis mediated by the Notch protein pathway can promote the occurrence and development of HT, the possibility that other signaling pathways dominate or influence the role of the Notch/Treg/Th17 pathway cannot be ruled out. Therefore, upstream signaling pathways and other mechanisms must be explored in detail in future studies. The safety of Xiaoying Daotan decoction was not evaluated in the present study. Xiaoying Daotan decoction is rich in drug composition, so it is difficult to define the core drugs and compounds that inhibit Notch protein expression in thyroid inflammatory cells. More detailed studies are needed in the future to clarify the compounds that inhibit the Notch/Treg/Th17 pathway to guide the development of new drugs to treat HT.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (IRB number: KMMU2019433) granted by Ethics Committee of Kunming Medical University, in compliance with Chinese national or institutional guidelines for the care and use of animals.

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