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The Inhibitory Effects of *Ahnjeonbaekho-tang* on FRTL-5 Cell Proliferation and Thyroxine Synthesis

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ABSTRACT

Objective : Graves' disease, the most common cause of hyperthyroidism, is an autoimmune disorder associated with autoantibodies to the TSH receptor. The clinical features of Graves' disease are goiter and hypermetabolic symptoms induced by excessive hormones. Antithyroid drug therapy is the first-line treatment for Graves' disease in Korea, Japan and European countries. Yet in spite of a long period and high-dose of treatment, it is hard to achieve remission because of adverse effects, frequent recurrence and resistance to antithyroid drugs. Recently, it has been reported that the abnormal thyroid hormone and clinical symptoms of Graves' disease were reduced by *Ahnjeonbaekho-tang* (AJBHT).

Methods : To investigate the effectiveness and action mechanism of AJBHT, we studied the influence of AJBHT on FRTL-5 thyroid cell proliferation, DNA synthesis and expression of T4, TSH, cAMP, Tg and TPO mRNA.

Results : AJBHT significantly inhibited the FRTL-5 cell proliferation, DNA synthesis, T4 synthesis, cAMP production and the expression of Tg mRNA in comparison with control and MMI.

Conclusions : These results suggest that AJBHT may inhibit the cell proliferation and DNA synthesis by regulating the cAMP, and suppress the T4 synthesis by modulating Tg mRNA expression and cAMP synthesis, and that it may be useful agent for treating the goiter and hormone abnormality of Graves' disease.

Key words: Ahnjeonbaekho-tang (AJBHT), FRTL-5, Cell proliferation, DNA synthesis, cAMP, Thyroxine, TSH, Thyroglobulin mRNA, TPO mRNA

I. INTRODUCTION

The hyperthyroidism is a clinical disorder characterized as increasing free thyroxine (T4) or triiodothyronine (T3)[1,2,3]. The most common cause of hyperthyroidism is Graves' disease, which is an autoimmune disorder

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associated with circulating immunoglobulins that stimulate the thyrotropin (TSH) receptor, resulting in sustained thyroid overactivity[1,4,5]. The clinical features of Graves' disease are summarized by hypermetabolic symptoms, enlarged thyroid, ophthalmopathy and dermopathy.

There are three treatment options for Graves' disease: radioactive iodine, surgery and antithyroid drugs[1,2,6]. It has been reported that antithyroid drugs are the major treatment in Korea, Japan and European countries[1,7]. However, only 40-60 % of patients with antithyroid drugs can achieve remission because of adverse effects, frequent recurrence and resistance to antithyroid drugs[1,3]. The patients having failures of antithyroid drug often have been led to select the alternative therapy including Oriental Medicine.

Although there is no exact medical category to Graves' disease in Oriental medicine, several medical categories such as Yangmyung-disease, Youngryu, Toan, Sogal or Jungchung, and several herbal medicines have been applied to its treatment. However, only few clinical studies of herbal medicines to evaluate their clinical effects have been reported in spite of their therapeutic effects. Recently, it has been reported that Ahnjeonbaekho-tang (AJBHT) improved the abnormal thyroid hormone and clinical symptoms of Graves' disease patients with methimazole (MMI) resistance[8]. This result means that AJBHT may have a different mechanism with MMI which reduce the synthesis of thyroid hormone by inhibiting thyroid peroxidase (TPO) activity.

Thus to understand the action mechanism of AJBHT, we evaluated the influence of AJBHT on cell proliferation, DNA synthesis and expression of T4, TSH, adenosine 3,'5'-cyclic monophosphate (cAMP), thyroglobulin (Tg) and TPO in TSH-activated FRTL-5 cells.

II. MATERIALS AND METHODS

1. Preparation of aqueous extracts

The air-dried Ahnjeonbaekho-tang (Table 1. All of them were purchased from the Division of Pharmacy, Kyung Hee Oriental Medical Center, Seoul, Korea) 200 g were added to 1,500 ml of distilled water and boiled for 4 hours at 100 ℃. The sieve filtrated solvents were concentrated by Rotary evaporator (Model NE-1, EYELA Co., Japan) and dried with Freeze Dryer (Model FD-1, EYELA Co., Japan). And those extracts were added to distilled water (1 g/10 ml) again and boiled for 2 hours at 95 ℃. The boiled solution was centrifuged at 14,000 rpm for 20 minutes and supernatant was obtained. The collecting rate of AJBHT was 11.8 %.

2. Fisher rat thyroid cells (FRTL-5) culture

The Fisher rat thyroid cells (FRTL-5) were provided by Dr. Minho Song (Department of Internal Medicine, Chungnam National Univ.). Cells were grown in Coon's modified Ham's F-12 medium (K.C. Biological, Louis, MO, USA), supplemented with calf serum (Gibco; Invitrogen Corp., CA, USA), and a six-hormone mixture containing 10 ㎍/ml insulin (Sigma Chemical Co., St. Louis, MO, USA), 5 ㎍/ml transferrin (Sigma), 10 ng/ml

lycyl-L-histidyl-L-lysine acetate (Sigma), 10 ng/㎖ somatostatin (Sigma), 10 uM cortisol (Sigma), 1 mU/㎖ TSH (Sigma), 100 U/㎖ penicillin (Sigma), 100 ㎍/㎖ streptomycin (Sigma) (6H medium). 5H medium without TSH was also used. Cells were cultured in a humidified atmosphere of 5 % CO_2 at 37 ℃. Cells used in these experiments were between the 5th and 20th passage.

3. MTT assay in FRTL-5 cell

Cell viability of AJBHT was measured by a modified 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay. FRTL-5 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and incubated in serum-free media for 24 hours. The cells were added with different concentrations of AJBHT in TSH-free or TSH-containing media and incubated for 2 days. And MTT solution were added to each well and incubated for 2-3 hours. The medium then was removed and 100 ㎕ of dimethylsulfoxide (DMSO) was added. Absorbance at 570 nm was read for each well using spectrophotometer (Dynatech Inc., Alexandria, VA, USA).

4. Measurement of cell proliferation

FRTL-5 cells were cultured in Coon's modified Ham's F-12 medium with 1 mU/㎖ TSH (6H medium) for 1 or 2 days. Cells were harvested by treatment with 0.05 % Trypsin-0.53 mM EDTA and washed twice with phosphate buffered saline (PBS) containing 5 % calf serum. Cells were seeded at a density of 1×10^5 cells/well and cultured in Coon's modified Ham's F-12 medium without 1 mU/㎖ TSH (5H medium) for 72 hours. Cells were incubated in 6H medium containing the different concentrations of AJBHT (15, 30 ug/ml) which were determined by MTT assay, and 1 mM MMI for 48 hours. Finally, cells were harvested by treatment with 0.05 % Trypsin-0.53 mM EDTA and counted directly with microscope.

5. Measurement of DNA synthesis

DNA synthesis of FRTL-5 cell was measured as the incorporation of ^{3}H-thymidine into trichloroacetic acid (TCA)-insoluble material. After incubation in 6H medium for 1 or 2 days, cells were seeded into 96-well dish at a density of 1×10^5 cells/well, and incubated in 5H medium for 72 hours. The medium was changed to 6H medium with 15, 30 ug/ml AJBHT and 1 mM MMI, and incubated for 48 hours. During the last 4 hours, cells were added with ^{3}H-thymidine (1 uCi/㎖, Amersham, Uppsala, Sweden). And then the radioactivity was counted in a scintillation counter to measure the DNA synthesis.

6. Measurement of T4 and TSH level

To measure T4 and TSH level, Rodent T4 ELISA (Enzyme-Linked Immunosorbent Assay) test kit (Endocrine technologies Inc., USA) and Rodent TSH ELISA test kit (Endocrine technologies Inc.) were used. After incubation in 6H medium for 1 or 2 days, cells were seeded into 24-well dish at a density of 1×10^5 cells/well and incubated in 5H medium for 72 hours. And then the medium was changed to 6H medium with 15, 30 ug/ml

AJBHT and 1 mM MMI. After incubation for 48 hours, supernatant was harvested. To measure the concentration of T4, microtiter wells, coated with antibody, were prepared, and 50 ㎕ of samples and standard T4 solution were applied, then followed by 100 ㎕ of T4 HRP-conjugate, 100 ㎕ of TBM color solution, 50 ㎕ of 2N HCl stop solution, respectively. And to measure the concentration of TSH, microtiter wells, coated with antibody, were prepared, and 100 ㎕ of samples and standard T4 solution were applied, then followed by 100 ㎕ of TSH enzyme conjugate, 100 ㎕ of TBM color solution, 50 ㎕ of 2N HCl stop solution, respectively. Absorbency was measured by ELISA reader at 450 nm.

7. Measurement of cAMP level

To measure the cAMP level of FRTL-5 cells, the cAMP RIA kit (New England Nuclear, Chicago, IL, USA) was used. After incubation in 6H medium for 1 or 2 days, cells were seeded at a density of 1×10^5 cells/well and incubated in 5H medium for 72 hours. And then the medium was changed to 6H medium with 15, 30 ug/ml AJBHT and 1 mM MMI, and incubated for 4 hours. Cells were incubated with 250 ㎕ Hank's Balanced Salt Solution (HBSS) containing 100 ㎕/㎖ TSH, 0.4 % BSA, 10 mM HEPES (pH 7.4) and 0.5 mM 3-isobutyl-L-methylxanthine (IMX) for 2 hours and HBSS was removed. To extract the cAMP from the cells, 300 ㎕ ethanol was added. After 12 hour incubation at -20 ℃, the ethanol extract was collected, lyophilized, and reconstituted with the assay buffer from cAMP RIA kit. Diphenylamine solution was added to the wells to measure DNA. Results were expressed as picomoles per well.

8. RNA isolation

After incubation in 6H medium for 1 or 2 days, cells were plated in 10 cm^2 dish at a density of 1×10^6 cells/dish and incubated in 5H medium for 72 hours. And then cells were incubated in 6H medium with 15, 30 ug/ml AJBHT and 1 mM MMI for 48 hours, and supernatant was removed. Total RNA was isolated by RNA Zol B (TELTEST: Friendswood, TX, USA).

9. Reverse transcription polymerase chain reaction (RT-PCR) of Tg and TPO mRNA

Reverse transcription polymerase chain reaction (RT-PCR) of Tg and TPO mRNA

To evaluate the expression level of Tg and TPO mRNA, we performed semi-quantitative RT-PCR. The reaction mixture containing 1 ㎍ RNA, PCR buffer, 5 mM $MgCl_2$, 1 mM dNTP, 20 U of RNasin, 2.5 μM of Oligo (dT) and 100 U of Moloney murine leukemia virus reverse transcriptase was incubated at 42 ℃ for 50 minutes, then heated at 70 ℃ for 15 minutes. PCR was carried out in Eppendorf's Mastercycler Gradient PCR device (Eppendorf, Hamburg, Germany). Each sample mixture contained PCR buffer, dNTP, *Taq* polymerase and each primers: *Tg*, 5'-ACTCCACAGATGACTATGCC-3' and 5'-GCATGACTTTCAGAGAGAGG-3', *TPO*, 5'-GCCTCCTGTCTGTAAAGATG-3' and 5'-GTAGCCTGCCAGAATCTATG-3', *β-actin*, 5'-CCTCTATGCCAACACAGT-3' and 5'-AGCCACCAATCCACACAG-3'. The

expected PCR product size was 267 bp (for Tg), 164 bp (for TPO) and 155 bp (for β -actin).

10. Statistical analysis

Statistical comparisons were performed by using the one-way analysis of variance (ANOVA) followed by Tuckey's post-hoc test (GraphPad PRISM statistical package ver 2.00, Graphpad software Inc., San Diego, USA). All data are presented as the mean ± standard deviation (S.D.). All P values are two tailed, and $P<0.05$ was considered the limit for statistical significance.

III. RESULTS

1. Effect of AJBHT on FRTL-5 cell viability

In order to exclude the cytotoxic effect of AJBHT and to determine the experimental concentration, MTT activity was evaluated with medium containing the different concentrations of AJBHT. Cell viability in the presence of AJBHT (15, 30 ug/ml) was similar to that of untreated control cells, however the cell viability of 60 ug/ml AJBHT was 49.75 %. Thus 15 and 30 ug/ml of AJBHT were determined for the experimental concentration (Fig. 1).

2. Effect of AJBHT on cell proliferation

To evaluate the anti-proliferative effect of AJBHT, cells were incubated in 6H medium containing the different concentrations of AJBHT (15, 30 ug/ml) which were determined by MTT assay, and 1 mM MMI as active-control. And cell proliferation was measured by direct cell counting. 30 ug/ml AJBHT significantly inhibited the FRTL-5 cell proliferation ($P<0.001$). But 15 ug/ml AJBHT and 1 mM MMI had no significant effect on cell proliferation (Fig. 2).

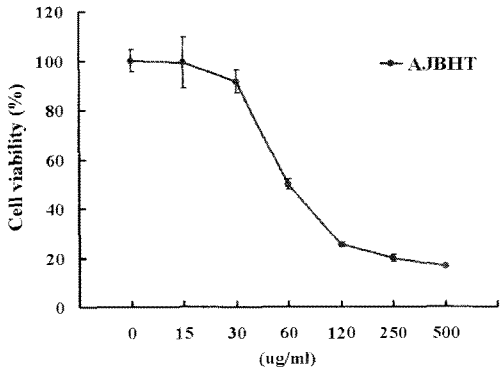

Fig. 1. Cell Viability of AJBHT on FRTL-5. AJBHT, Ahnjeonbaekho-tang.

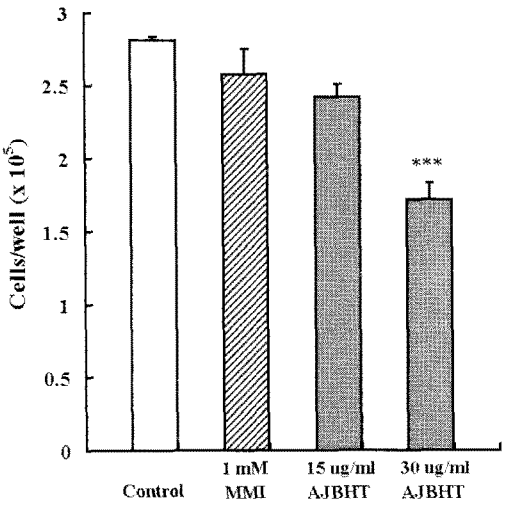

Fig. 2. Effect of AJBHT on FRTL-5 Cell Proliferation. ***$P<0.001$ compared with control. MMI, methimazole; AJBHT, Ahnjeonbaekho-tang.

3. Effect of AJBHT on DNA synthesis

To study the DNA synthesis of FRTL-5 cell, incorporation of ^{3}H-thymidine into DNA was measured after incubation with 15, 30 ug/ml AJBHT and 1 mM MMI. AJBHT significantly inhibited ^{3}H-thymidine incorporation into DNA in a dose dependent manner (15 ug/ml, 2207.889 ± 183.429, P<0.001; 30 ug/ml, 514.889 ± 15.752, P<0.001). And the inhibition of DNA synthesis by AJBHT was corresponded with the results shown in cell proliferation. In contrast, 1 mM MMI markedly increased incorporation of ^{3}H-thymidine into DNA (P<0.001) (Table 1) (Fig. 3).

Table 1. Prescription of AJBHT.

Medicinal herb	Botanical origin	Dose(g)
Puerariae Radix	*Pueraria thunbergiana*	40
Scutellariae Radix	*Scutellaria baicalensis*	16
Gypsum Fibrosum	$CaSO_4 \cdot 2H_2O$	8
Platycodi Radix	*Platycodon grandiflorum (Jacq.) A. DC.*	8
Angelicae Tenuissimae Radix	*Angelica tenuissima Nakai*	8
Cimicifugae Rhizoma	*Cimicifuga foetida L.*	8
Angelicae Dahuricae Radix	*Angelica dahurica Benth. Et Hook*	8
Glycyrrhizae Radix	*Glycyrrhiza uralensis Fisch.*	8
Total amount		104

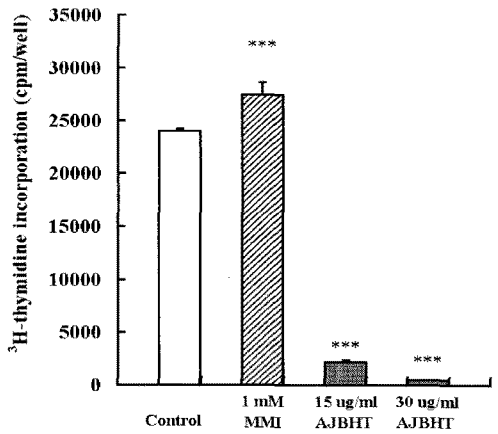

Fig. 3. Effect of AJBHT on DNA Synthesis of FRTL-5 cell. ***P<0.001 compared with control. MMI, methimazole; AJBHT, Ahnjeonbaekho-tang.

4. Effect of AJBHT on FRTL-5 cell T4 and TSH synthesis

The quantitative measurements of T4 and TSH were performed using T4 and TSH ELISA test kit after incubation with 15, 30 ug/ml AJBHT and 1 mM MMI. AJBHT significantly inhibited T4 level in a dose dependent manner (15 ug/ml, 4.131 ± 0.996, P<0.05; 30 ug/ml, 3.081 ± 0.620, P<0.001). 1 mM MMI also inhibited T4 level, but the level was higher than AJBHT (4.912 ± 1.279, P<0.05). In contrast, both AJBHT and MMI had no significant inhibitory effect on TSH level (Table 4) (Fig. 4).

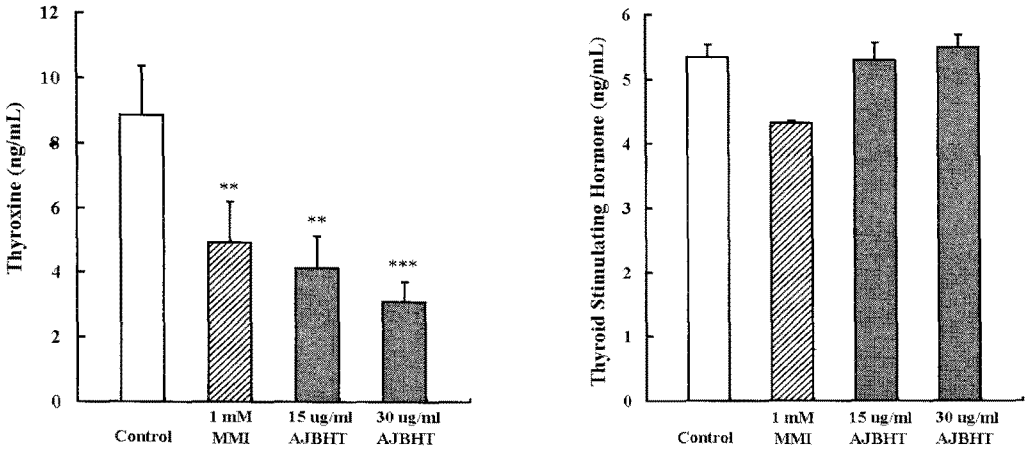

Fig. 4. Effect of AJBHT on FRTL-5 Cell T4 TSH Synthesis. $^{**}P<0.01$, $^{***}P<0.001$ compared with control. MMI, methimazole; AJBHT, Ahnjeonbaekho-tang.

5. Effect of AJBHT on FRTL-5 cell cAMP synthesis

To study the effect of AJBHT on the TSH-induced cAMP concentration, cAMP level was measured in the medium with 15, 30 ug/ml AJBHT and 1 mM MMI using radioimmunoassay(RIA). 30 ug/ml AJBHT significantly inhibited the TSH-induced cAMP production ($P<0.05$). But 15 ug/ml AJBHT and 1 mM MMI had no significant effect on cAMP production (Table 5) (Fig. 5).

6. Effect of AJBHT on FRTL-5 cell Tg and TPO mRNA synthesis

To determine the possible action mechanism of AJBHT on FRTL-5 cells, the expressions of Tg and TPO mRNA level were evaluated using the semi-quantitative RT-PCR after RNA isolation by RNA Zol B. 30 ug/ml AJBHT significantly inhibited the expression of Tg mRNA level ($P<0.001$). But 1 mM MMI had no significant effect on Tg mRNA level. 1 mM MMI significantly inhibited the expression of TPO mRNA level ($P<0.001$). But 30 ug/ml AJBHT had no significant effect on TPO mRNA level (Table 6) (Fig. 6).

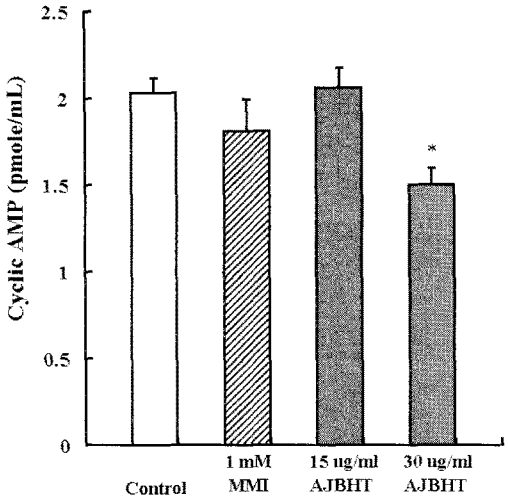

Fig. 5. Effect of AJBHT on FRTL-5 Cell cAMP Synthesis. $^{*}P<0.05$ compared with control. MMI, methimazole; AJBHT, Ahnjeonbaekho-tang.

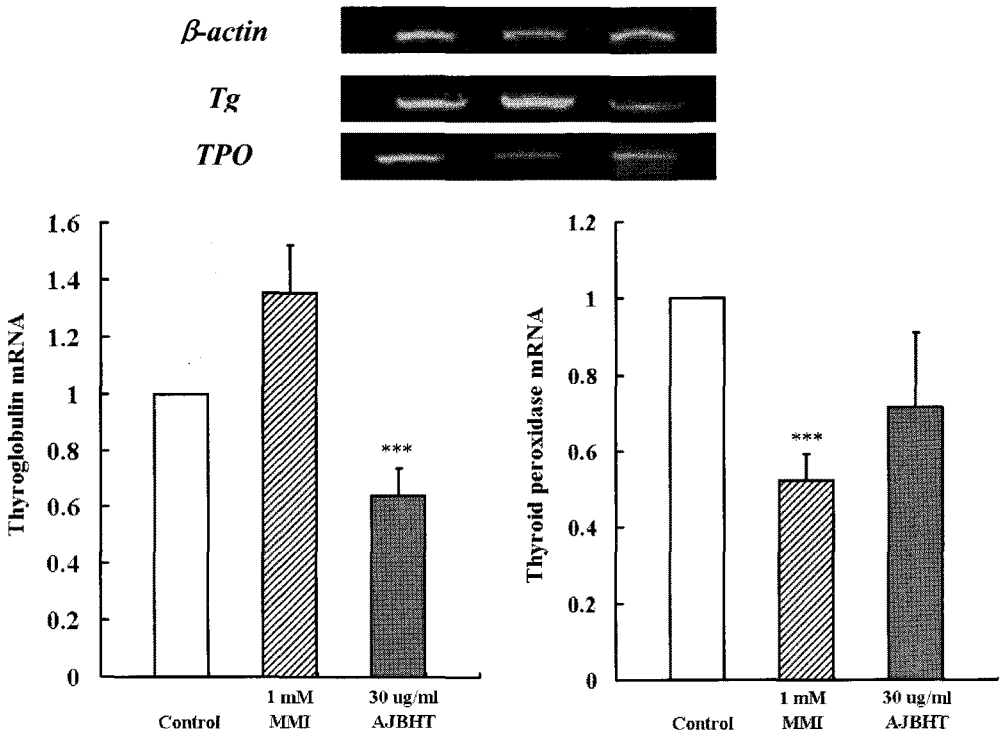

Fig. 6. Effect of AJBHT on FRTL-5 Cell Tg, TPO mRNA synthesis. ***P<0.001 compared with control. MMI, methimazole; AJBHT, Ahnjeonbaekho-tang.

IV. DISCUSSION

Hyperthyroidism is a pathological syndrome in which tissue is exposed to excessive amount of circulating thyroid hormones[1,2]. The most common cause of hyperthyroidism is Graves' disease, which is thought to be an autoimmune disorder in which antibodies formed act on the TSH receptor (TRAb)[1,2,5]. TRAb acts like TSH to stimulate the thyroid gland, but is not affected by the negative feedback control. Consequently, there is excessive production of thyroid hormone, resulted in hyperthyroidism. Patients usually have fatigue, weight loss despite good appetite, intolerance to heat, tachycardia, sweating, nervousness, anxiety, tremor, muscle weakness, menstrual irregularities, reduced libido and enlarged thyroid[1,2], and also have ophthalmopathy and dermopathy characteristically[1,2]. In most patients, T4 and T3 concentrations in serum are raised, serum TSH is suppressed and autoantibodies are detectable[7].

The three main treatments of Graves' disease are surgery, radioactive iodine and antithyroid drugs[1,2,6,9,10]. And antithyroid drugs are the first-line treatment in Korea, Japan and most European countries[7].

Antithyroid drugs, including propylthiouracil (PTU), methimazole (MMI) and carbimazole, inhibit synthesis of thyroid hormone within the thyroid gland by serving as substrates for TPO, which catalyzes the incorporation of oxidized iodide into tyrosine residues in Tg molecules and couples iodotyrosines (monoiodotyrosine and diiodotyrosine) into iodothyronines (T4 and T3)[6,9]. And they divert iodine from the synthesis of thyroid hormones. Antithyroid drugs do not interfere with the actions of exogenous thyroid hormone or inhibit the release of thyroid hormones. Antithyroid drugs may also have moderating effects on the underlying immunologic abnormalities in hyperthyroidism due to Graves' disease, but evidence on this point reported to date is inconclusive.

However, antithyroid drugs also have problems such as adverse effects, drug resistance and recurrence[1,2,4,6]. The adverse effects of antithyroid drugs occur in approximately 3-5% of patients and include agranulocytosis, hepatotoxicity, aplastic anemia, vasculitis, urticaria, rash, arthralgia, fever, sore throat, mouth ulcer, nausea, jaundice, etc. And it is reported that relapse rate after treatment is 30-50%[1,3,10]. Moreover, there have been reports of resistance to MMI, which is poorly responsive to conventional doses of MMI by impairment of thyroid uptake of MMI. Because of these problems, many patients have been led to find the alternative therapy including Oriental Medicine, and Oriental Medicine including herbal medicine has been applied to its treatment.

Recently, it has been reported that the abnormal thyroid hormone and clinical symptoms of Graves' disease were improved by AJBHT[8]. Especially, patients in this report had MMI resistance and adverse effects which had made them stopping the medication of antithyroid drugs.

AJBHT, herbal prescription, is a newly created by the Division of 6th Internal Medicine of Kyung Hee Oriental Medical Center on the basis of Yangmyung-disease[1]. In the respects of Oriental Medicine, Graves' disease could be linked to several medical terms like Yangmyung-disease, Youngryu, Toan, Sogal and Jungchung. The meanings of these terms are as follows: Youngryu, enlargement or nodule on neck: Toan, Rabbit-like eye: Sogal, severe thirst and weight loss: Jungchung, tachycardia and palpitation. Especially, Yangmyung-disease is a pattern of disease including fever, heat intolerance, sweating, tachycardia, thirst, eye pain, nervousness and insomnia. These signs and symptoms resemble hypermetabolic state of hyperthyroidism. Baekho-tang and Yeoldahanso-tang are well-known prescriptions for Yangmyung-disease in the Oriental Medicine[11]. In order to increase efficacy, these two prescriptions were combined together and named as Ahnjeonbaekho-tang (AJBHT).

Clinically, AJBHT has remarkable therapeutic effects on Graves' disease with MMI resistance and adverse effects[8]. This result induces the possibility that AJBHT has a different action mechanism with MMI. However, there has been no study for understanding the action mechanism of AJBHT.

In this study, we observed that AJBHT inhibited cell proliferation and DNA synthesis in a dose-dependent manner. However, MMI had no inhibitory effect on cell growth, which is compromised with the previous studies[12]. It has been reported that the growth of FRTL-5 cells is regulated by at least two different biological pathway. One is apparently cAMP dependent and activated by TSH, while the other is cAMP independent and activated by IGF-I [12,13]. In this study, we induced the cell proliferation using TSH-cAMP pathway, and AJBHT inhibited the expression of cAMP, in addition to suppression of cell proliferation and DNA synthesis. These findings suggest that the inhibitory effect of AJBHT on FRTL-5 cell growth may be mediated by regulating the cAMP expression. Clinically, goiter induced by cell growth is hardly resolved in spite of common sign of Graves' disease[1,2,4,9]. Taken these results, AJBHT could be applied to reduce the goiter of Graves' disease.

In this study, we evaluated the synthesis of T4 in FRTL-5 cells. As a result, AJBHT reduced the T4 synthesis. Thyroid hormone, especially T4 biosynthesis is initiated by TSH stimulating to TSH receptor, and cAMP then mediates the Tg and TPO synthesis. Tg is a thyroid-specific protein which serves as the macromolecular precursor for thyroid hormone formation. Its biosynthesis is mainly increased by TSH via its cAMP signal[14,15]. TPO is an enzyme which catalyzes the iodination and oxidative coupling of tyrosines in Tg to yield protein bound thyroid hormone.

Thus to find how AJBHT suppress the T4 synthesis, we evaluated the expression of TSH, cAMP, Tg and TPO mRNA related with T4 synthesis. In the present study, the expression of TSH and TPO mRNA level was not suppressed by AJBHT, but the expression of cAMP and Tg mRNA level was inhibited. These results suggest that AJBHT has inhibitory effect on T4 synthesis by suppressing cAMP production and Tg synthesis. And it suggest that AJBHT was effective in the Graves' disease patients with MMI resistance. Antithyroid drugs like as MMI, are absorbed in Tg and inhibit the synthesis of thyroid hormone by interfering with the incorporation of iodide into an organic form via inhibition of iodine oxidation, TPO and the coupling mechanism. In order to get a significant antithyroid effect, MMI must be absorbed in Tg sufficiently. However, patients with MMI resistance have obstacle of MMI absorption, and they therefore cannot achieve remission in spite of high-dose MMI treatment. Thus considering that AJBHT has inhibitory effect on T4 synthesis via cAMP and Tg, it may be a useful agent for treating Graves' disease, especially MMI resistance.

In conclusion, we suggest that AJBHT may be a useful agent for treating Graves' disease, considering AJBHT seems to inhibit cell proliferation by suppressing the DNA synthesis and cAMP expression, and to reduce the T4 synthesis by suppressing the synthesis of cAMP and Tg.

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