An Alternative Therapy for Graves’ Disease: Clinical Effects and Mechanisms of an Herbal Remedy

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Graves’ disease, the most common cause of hyperthyroidism, is an autoimmune disorder. Antithyroid drugs have been selected as the first-line treatment of Graves’ disease in Korea, Japan, and European countries. However, antithyroid drugs such as methimazole (MMI) and propylthiouracil (PTU) have limitations in clinical applications because of their side effects. In this study, we performed a clinical trial and in vitro study to investigate the clinical effects and action mechanism of Ahnjeonbaekho-tang (AJBHT), an herbal remedy for Graves’ disease. In a clinical study of Graves’ disease patients who had side effects from antithyroid drugs, we found that treatment by AJBHT resulted in a reduction of serum triiodothyronine (T\textsubscript{3}) and free thyroxine (FT\textsubscript{4}) levels and an increase in thyroid stimulating hormone (TSH) levels (T\textsubscript{3}; \( p<0.0001 \), FT\textsubscript{4}; \( p=0.0012 \), TSH; \( p=0.0370 \), respectively). In vitro, AJBHT significantly inhibits FRTL-5 cell proliferation, DNA synthesis, cyclic AMP production, T\textsubscript{3} synthesis, and the expression of thyroglobulin (Tg) mRNA in comparison with the control. These results suggest that AJBHT might suppress T\textsubscript{3} synthesis by modulating adenosine 3',5'-cyclic monophosphate (cAMP) and Tg expression, and therefore, AJBHT could be an alternative therapy for Graves’ disease patients who have side effects from antithyroid drugs.

Key words herbal remedy; Graves’ disease; antithyroid effect; clinical trial; FRTL-5 cell

Graves’ disease, an autoimmune disorder caused by autoantibodies against the thyrotropin receptor, is the most common cause of hyperthyroidism.\textsuperscript{1,2} The three main treatments of Graves’ disease are surgery, radioiodine, and antithyroid drugs.\textsuperscript{2,3} The selection of treatment depends on many factors, including the preference of clinicians and patients, availability of a skilled surgeon, and the cost and local restriction on the therapeutic use of radioisotopes.\textsuperscript{2} Radioiodine is generally preferred by U.S. clinicians,\textsuperscript{3} whereas antithyroid drugs, such as methimazole (MMI) and propylthiouracil (PTU), are the first-line treatments in Korea, Japan, and European countries.\textsuperscript{4} However, a major problem with antithyroid drugs therapy is that it is associated with a variety of minor side effects, as well as potentially life-threatening or even lethal complications.\textsuperscript{3} Most patients with side effects can be switched to another antithyroid drug or radioiodine therapy,\textsuperscript{3} whereas some patients in Korea select an alternative therapy, such as herbal medicines.\textsuperscript{4} However, there have been very few clinical trials and pharmaceutical studies to provide strong evidence of the efficacy of herbal medicine in the treatment of hyperthyroidism, although herbal medicines alone or added to other routine treatments have therapeutic potential for patients with hyperthyroidism.\textsuperscript{5,6,7}

Ahnjeonbaekho-tang (AJBHT), an herbal remedy for Graves’ disease, consists of eight medicinal herbs, including Pueraria thunbergiana and Scutellaria baicalensis, which are the main herbs regulating thyroid hormone.\textsuperscript{8} Pueraria thunbergiana contains isoflavones, such as daidzein, daidzein, puerarin, and puerarin xyloside.\textsuperscript{8} The main components of Scutellaria baicalensis are baicalin, baicalein, chrysin, oxorlin, wogonin, and wogonoside.\textsuperscript{9} Among these components, daidzin and baicalin are known to have antithyroid effects.\textsuperscript{10,11} Recently, we reported that AJBHT treatment can improve clinical symptons and decrease levels of thyroid hormone in Graves’ disease patients who have side effects from antithyroid drugs.\textsuperscript{6} Moreover, AJBHT also has therapeutic effects on patients with Graves’ disease who are MMI resistant or respond poorly to conventional doses of MMI due to impairment of thyroid uptake of MMI.\textsuperscript{12} This result suggests the possibility that AJBHT may act through a different mechanism than MMI to reduce the synthesis of thyroid hormone by inhibiting thyroid peroxidase (TPO) activity.\textsuperscript{5} However, there have been no studies to understand the mechanism by which AJBHT acts. Moreover, the case size of our previous clinical study was not large enough to confirm any clinical effects in the treatment of Graves’ disease.

In this study, we performed a clinical trial to investigate the clinical effects of an aqueous extract of AJBHT in Graves’ disease patients who have side effects from antithyroid drugs. In addition, to understand its action mechanism, we analyzed the effects of AJBHT on cell proliferation, DNA synthesis, and the expression of adenosine 3',5'-cyclic monophosphate (cAMP), thyroxine (T\textsubscript{4}), thyroid stimulating hormone (TSH), thyroglobulin (Tg), and TPO in TSH-activated FRTL-5 cells.

MATERIALS AND METHODS

Subjects of Clinical Study This study was carried out between February 2004 and June 2006 in the Oriental Medical Hospital, Kyung Hee University. In this study, we included 22 consecutive patients who were diagnosed with Graves’ disease and had previously experienced side effects from antithyroid drugs. A diagnosis of Graves’ disease was based on clinical symptoms and laboratory findings, including suppressed thyroid-stimulating hormone (TSH) levels, elevated serum thyroid hormone levels, and detectable levels of TSH receptor autoantibodies (TRAb). Exclusion criteria were cardiovascular, renal, pulmonary, hepatic, gastrointestinal, endocrine, psychiatric or neoplastic disease, previous radioiodine or thyroidectomy treatment, moderate or severe ophthalmopathy, impending thyrotoxicosis, multinodular goiter, and pregnancy. Patients previously treated with antithyroid...
roid drugs (MMI, PTU) whose treatment had been interrupted for 2 months before the study were included.

Clinical Study Design Patients were treated with AJBHT (aqueous extracts 6 g) three times a day for 3 months. Twelve patients started without a washout period because they had not taken antithyroid drugs for over 2 months and had abnormal thyroid hormone levels, and ten patients started after a washout period. A clinical and laboratory assessment was performed at baseline and then monthly for 3 months. Serum levels of free thyroxine (FT₄), triiodothyronine (T₃), TSH, and TRAb were measured in the morning at 100 U/ml penicillin and 100 μg/ml streptomycin in free media for 24 h. The cells were added with different concentrations of AJBHT in TSH-free or TSH-containing media and incubated for 48 h. The absorbance at 570 nm was read for each well using a spectrophotometer (Dyntech Inc., Alexandria, VA, U.S.A.).

Measurement of DNA Synthesis and Cell Proliferation in FRTL-5 Cells FRTL-5 cells were cultured in Coon's modified Ham's F-12 medium with 1 μM/μl TSH (6H medium) for 48 h. Cells were seeded at a density of 1×10⁵ cells/well and cultured in Coon's modified Ham's F-12 medium without 1 μM/μl TSH (5H medium) for 72 h. Then cells were incubated in 6H medium containing the different concentrations of AJBHT (15 and 30 μg/ml), which were determined by MTT assay, and 1 mM MMI for 48 h. During the first 4 h, ³H-thymidine (1 μCi/ml, Amersham, Uppsala, Sweden) was added to the cells. The incorporation of ³H-thymidine was counted in scintillation fluid using a β-counter. In parallel, cell proliferation was measured by direct cell counting with a microscope.

Measurement of Cyclic AMP Level in FRTL-5 Cells To measure cyclic AMP (cAMP) levels in FRTL-5 cells, a cAMP RIA kit (New England Nuclear, Chicago, Ill., U.S.A.) was used. After incubation in 6H medium for 48 h, cells were seeded at a density of 1×10⁵ cells/well and incubated in 5H medium for 72 h. The medium was then changed to 6H medium with 15 or 30 μg/ml AJBHT and 1 mM MMI and incubated for 4 h. Cells were incubated with 250 μl Hank’s Balanced Salt Solution (HBSS) containing 100 μl/ml TSH, 0.4 % BSA, 10 mM HEPES (pH 7.4) and 0.5 mM 3-isobutyl-1-methylxanthine (IMX) for 2 h, and HBSS was removed. To extract the cAMP from the cells, 300 μl ethanol was added. After 12 h incubation at ~20 °C, the ethanol extract was collected, lyophilized, and reconstituted with the assay buffer from the cAMP RIA kit. A diphenylamine solution was added to the wells to measure DNA.

Measurement of Thyroxine (T₄) and Thyroid Stimulating Hormone (TSH) Level in FRTL-5 Cells To measure T₄ and TSH levels, a rodent T₄ enzyme-linked immunosorbent assay (ELISA) test kit and rodent TSH ELISA test kit (Endocrine technologies Inc., U.S.A.) were used. After incubation in 6H medium for 48 h, cells were seeded into a 24-well dish at a density of 1×10⁵ cells/well and incubated in 5H medium for 72 h. Then the medium was changed to 6H medium with 15 or 30 μg/ml AJBHT and 1 mM MMI. After incubation for 48 h, the supernatant was harvested. To measure the concentration of T₄ or TSH, microtiter wells coated with antibody, were prepared and 50 μl of samples and standard T₄ or TSH solution were applied, then followed by 100 μl of T₄ HRP-conjugate or TSH enzyme conjugate, 100 μl of TBM color solution, and 50 μl of 2 N HCl stop solution. Absorbency was measured by an ELISA reader at 450 nm.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR) of Thyroglobulin (Tg) and Thyroid Peroxidase (TPO) mRNA in FRTL-5 Cells After incubation in 6H medium for 48 h, cells were plated in a 10 cm² dish at a density of 1×10⁵ cells/dish and incubated in 5H medium for 72 h. Cells were then incubated in 6H medium with 15 or 30 μg/ml AJBHT and 1 mM MMI for 48 h. Total RNA was isolated by RNA Zol B (TELTEST; Friendswood, TX, U.S.A.). To evaluate the expression level of Tg and TPO mRNA, we performed a semi-quantitative re-
verse transcription polymerase chain reaction (RT-PCR). The reaction mixture containing 1 μg RNA, PCR buffer, 5 mM MgCl₂, 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 °C for 50 min, then heated at 70 °C for 15 min. All experimental samples were reverse transcribed in the same set of experiments, and the efficiency of the reaction was controlled by β-actin amplification. PCR was carried out in a gradient PCR device (Eppendorf, Hamburg, Germany). Each sample mixture contained PCR buffer, 2.5 mM dNTP, 2 U Taq polymerase, and 5 μM of each primer: Tg, 5' - ACTCCACAGATGACTATGCC-3', 5' - GCATGACTCCAGAGAGG-3', TPO, 5' - GCCTCTGTCTG- TAAAGATG-3' and 5' - GTAGCTGCCCAGAATCTATG-3', β-actin, 5' - CCTCTATGCCCAACAGT-3' and 5' - AGCCCACCATCACCACAG-3'. The PCR consisted of 30 cycles (for Tg, TPO) at 60 °C and 30 cycles (for β-actin) at 55 °C. The expected PCR product size was 267 bp (for Tg), 164 bp (for TPO), and 155 bp (for β-actin). The reaction products were subjected to densitometry after electrophoresis on 2% agarose gel and staining with ethidium bromide.

Statistical Analysis Statistical comparisons were performed using the paired t-test for clinical trials and one-way analysis of variance (ANOVA) followed by Tuckey's post hoc test for cell study. All data are presented as the mean±standard deviation (S.D.). All p values are two-tailed, and significance was taken at p<0.05.

RESULTS

Patients We enrolled 22 Graves' disease patients who had experienced side effects from antithyroid drugs. The types of side effects were rash (n=8), urticaria (n=5), arthralgia (n=3), gastrointestinal upset (n=3), agranulocytosis (n=2) and hepatotoxicity (n=1). Four patients dropped out due to their inability to comply with study requirements (n=3) or because they had moved away (n=1). The remaining eighteen patients were followed for 3 months. The characteristics of the 18 remaining Graves' disease patients were as follows: the mean age was 32.06±6.71 (years), sex 7/11 (M/F), and BMI 20.81±2.91. There were no serious drug reactions or side effects associated with AJBHT treatment.

Clinical Effects of AJBHT on T₄, FT₄, TSH, and TRAb Levels in Graves' Disease Patients Who Suffered Side Effects from Antithyroid Drugs At the time of baseline, the mean serum T₃, FT₄, and TSH levels were 373.00±101.70 ng/dl, 3.13±1.22 ng/dl and 0.08±0.04 U/ml, respectively. At 3 months after AJBHT administration, the mean serum T₃ and FT₄ levels had significantly decreased to 214.89±68.03 ng/dl (p<0.0001) and 1.83±0.75 ng/dl (p=0.0012), respectively, and TSH levels significantly increased to 0.16±0.12 U/ml (p=0.0370) (Fig. 1). AJBHT improved thyroid hormone based on T₃ measurements for 17 patients (94.4%), FT₄ in 15 patients (83.3%), and TSH in 14 patients (77.8%). Biochemical euthyroidism was achieved with AJBHT treatment based on levels of T₃ in 10 patients (55.5%), FT₄ in 12 patients (66.7%), and TSH in 4 patients (22.2%). However, there were no significant changes in TRAb levels between the baseline and 3-month measurements (47.94±21.01% vs. 40.56±25.50%, respectively).

Effect of AJBHT on FRTL-5 Cell Viability In order to exclude cytotoxic effects from AJBHT treatment and to determine the experimental concentration, MTT activity was evaluated with medium containing different concentrations of AJBHT. There was no variation in the optical density (O.D.) for MTT activity in the absence and presence of AJBHT (0 μg/ml AJBHT, 2.514±0.077; 15 μg/ml AJBHT, 2.493±0.115; 30 μg/ml AJBHT, 2.299±0.263), but the cell viability at 60 μg/ml AJBHT was 1.251±0.116 O.D. Thus, 15 and 30 μg/ml of AJBHT were chosen as the experimental concentrations.

Effect of AJBHT on Cell Proliferation and DNA Synthesis in FRTL-5 Cells The anti-proliferative effect of AJBHT was evaluated on TSH-activated FRTL-5 cells. After incubation with 6H medium, cells proliferated to 281500±2100 cells/well, and 30 μg/ml AJBHT markedly inhibited cell proliferation (171500±12000 cells/well, p<0.001). In contrast, 15 μg/ml AJBHT and 1 mM MMI had no significant effect on cell proliferation (Table 1). In the analysis of DNA synthesis of FRTL-5 cell, AJBHT significantly inhibited ³H-thymidine incorporation into DNA in a dose-dependent manner (15 μg/ml, 2207.889±183.429 cpm/well, p<0.001; 30 μg/ml, 514.889±15.752 cpm/well, p<0.001). In addition, the inhibition of DNA synthesis by AJBHT corresponded with the results shown for cell proliferation. In contrast, 1 mM MMI markedly increased incorporation of ³H-thymidine into DNA (p<0.001) (Table 1).

Effect of AJBHT on FRTL-5 Cell cAMP Synthesis in FRTL-5 Cells To study the effect of AJBHT on the TSH-induced cAMP concentration, cAMP levels were measured by RIA in media with 15 or 30 μg/ml AJBHT and 1 mM MMI. Treatment with 30 μg/ml AJBHT significantly inhibited the TSH-induced cAMP production (p<0.05), but treatment with 15 μg/ml AJBHT and 1 mM MMI had no significant effect on cAMP production (Table 1).

Effect of AJBHT on FRTL-5 Cell T₃ and TSH Synthesis in FRTL-5 Cells The quantitative measurements of T₃ and TSH levels were performed using T₃ and TSH ELISA test kits after incubation with 15 or 30 μg/ml AJBHT and
Table 1. The Effect of AJBHT on Cell Proliferation, DNA Synthesis, and cAMP Expression in FRTL-5 Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell proliferation (Cells/well)</th>
<th>DNA synthesis (cpm/well)</th>
<th>cAMP (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.815±0.021</td>
<td>23985.330±246.875</td>
<td>0.030±0.005</td>
</tr>
<tr>
<td>1 mM MMI</td>
<td>2.575±0.177</td>
<td>27464.560±1128.345***</td>
<td>1.810±0.185</td>
</tr>
<tr>
<td>15 µg/ml AJBHT</td>
<td>2.420±0.085</td>
<td>2207.889±183.429***</td>
<td>2.060±0.113</td>
</tr>
<tr>
<td>30 µg/ml AJBHT</td>
<td>1.715±0.120***</td>
<td>514.889±15.752***</td>
<td>1.500±0.099*</td>
</tr>
</tbody>
</table>

FRTL-5 cells were cultured with 5H medium for 72 h, and then were incubated in 6H medium containing 15 or 30 µg/ml AJBHT and 1 mM MMI for 48 h. The cell proliferation was measured by direct cell counting, the amount of 3H-thymidine incorporation into TCA was measured using a β-counter, and cAMP levels were measured by cAMP RIA kit. MMI is methimazole, and AJBHT is Ahnjeonbaekho-tang. Results are presented as the mean±S.D. ∗p<0.05, ∗∗p<0.001 compared with control, from 3 independent experiments.

Table 2. The Effect of AJBHT on Synthesis of Thyroxine (T₄) and Thyroid Stimulating Hormone (TSH) in FRTL-5 Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>T₄ (ng/ml)</th>
<th>TSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.857±1.502</td>
<td>5.336±0.204</td>
</tr>
<tr>
<td>1 mM MMI</td>
<td>4.912±1.729**</td>
<td>4.327±0.033</td>
</tr>
<tr>
<td>15 µg/ml AJBHT</td>
<td>4.131±0.996**</td>
<td>5.288±0.272</td>
</tr>
<tr>
<td>30 µg/ml AJBHT</td>
<td>3.081±0.620***</td>
<td>5.481±0.206</td>
</tr>
</tbody>
</table>

FRTL-5 cells were cultured with 5H medium (without TSH) for 72 h and then incubated in 6H medium (with TSH) containing 15 or 30 µg/ml AJBHT and 1 mM MMI for 48 h. Supernatants were harvested for T₄ and TSH ELISA assay. MMI is methimazole, and AJBHT is Ahnjeonbaekho-tang. T₄ and TSH results are presented as the mean±S.D., from 3 independent experiments (**p<0.01, ***p<0.001).

DISCUSSION

Ahnjeonbaekho-tang (AJBHT) consists of eight medicinal herbs including Pueraria thunbergiana, Scutellaria baicalensis, Gypsum, Platycodon grandiflorum, Angelica tenuissima, Cimicifuga foetida, Angelica dahurica and Glycyrrhiza uralensis. Of these, Pueraria thunbergiana and Scutellaria baicalensis are known to regulate thyroid hormone, and the others contribute to relieving the clinical symptoms of hyperthyroidism. It has been reported that daidzein from Pueraria thunbergiana inhibits thyroid peroxidase (TPO)-cata- radical at the active site along with a radical form of lyzed iodination and coupling, and the baicalein from Scutellaria baicalensis inhibits TPO and thyroid type 1 deiodinase activity. In this study, AJBHT inhibited expression of both cAMP and Tg, in addition to having an anti-TPO effect. Thus, it was possible that daidzein and baicalein in AJBHT may be the primary antithyroid components.

In our clinical trial, AJBHT reduced the serum T₃ and FT₄ levels and increased TSH levels, without side effects. However, this study was conducted in a single group with a relatively short intervention period, which leads to several questions. First, compared with the improvement rates for T₃ and FT₄ levels, TSH levels had a relatively poor improvement rate (94.4% vs. 83.3% vs. 77.8%, respectively), with a corresponding poor achievement rate for biochemical euthyroidism (55.5% vs. 66.7% vs. 22.2%, respectively). It is possible that the intervention period for this clinical trial might have been too short to allow adequate recovery of the pituitary-thyroid axis. Thus, considering that there was an improvement in TSH levels, we expect that TSH would eventually improve to an euthyroid state with long-term treatment with AJBHT. Second, this clinical trial cannot predict remission/relapse after discontinuance of AJBHT. The main focus of this study was not to determine the period for disease remission/relapse but to identify the primary antithyroid effects of AJBHT. Therefore, the study period was not long enough to find significant changes in clinical parameters and TRAb levels, which are useful parameters for predicting the remission/relapse of Graves’ disease in combination with other clinical markers. Thus, a long term clinical trial is necessary to confirm the remission or relapse rate of Graves’ disease by AJBHT. Third, this clinical trial was performed with only non-typical Graves’ disease patients, who have side effects from antithyroid drugs, in the absence of a placebo control group. While this trial was limited because it was conducted with patients who had abnormal thyroid hormone levels and who had undergone a washout period of more than 2 months, we found antithyroid effects in both our clinical and in vitro studies. Thus, in the future, we need to perform a randomized controlled trial with general Graves’ disease patients.

In our in vitro study, AJBHT inhibited the TSH-activated cell proliferation and DNA synthesis. However, MMI had no
inhibitory effect on cell growth, a finding similar to findings from previous studies.\textsuperscript{21} It has been reported that the growth of FRTL-5 cells is regulated by at least two different biological pathways. One is cAMP-dependent and activated by TSH, while the other is cAMP-independent and activated by insulin-like growth factor (IGF)-I.\textsuperscript{22} In this study, we induced cell proliferation using the TSH-cAMP pathway, and AJBHT inhibited the expression of cAMP. These findings suggest that the inhibitory effect of AJBHT on FRTL-5 cell growth might be mediated through cAMP signaling.

Thyroid hormone, especially T\textsubscript{4} biosynthesis, is initiated by TSH stimulating the TSH receptor, and cAMP then mediates the synthesis of Tg and TPO.\textsuperscript{23} Tg is a thyroid-specific protein that serves as a macromolecular precursor for thyroid hormone formation, and its biosynthesis is mainly increased by TSH via cAMP signaling.\textsuperscript{23} In the present study, the expression of TSH and TPO mRNAs was not suppressed by AJBHT, but the synthesis of T\textsubscript{4} and expression of cAMP and Tg mRNA was inhibited. These results suggest that AJBHT might have an inhibitory effect on T\textsubscript{4} synthesis through the suppression of cAMP production and Tg expression. Thus, AJBHT must have a different mechanism from that of antithyroid drugs like MMI, which interfere with TPO-mediated iodination of tyrosine residues in Tg.\textsuperscript{3} This would explain why AJBHT is effective in Graves’ disease patients who have MMI resistance. Thus, considering the inhibitory effect of AJBHT on T\textsubscript{4} synthesis via cAMP and Tg, AJBHT might be an effective treatment for Graves’ disease patients with MMI resistance, as well as for those with side effects from antithyroid drugs.

There are both minor and major side effects from antithyroid drugs. The minor side effects occur in approximately 5\% of patients and include cutaneous reactions (usually urticaria or macular rashes), arthralgia, and gastrointestinal upset.\textsuperscript{5,24} Major side effects occur only occasionally and include polyarthritis (1—2\%), agranulocytosis (0.1—0.5\%), hepatotoxicity (0.1—0.2\%) and vasculitis (rare).\textsuperscript{5,24,25} In this study, 19 patients had minor side effects and 3 had major side effects. Twelve patients had never taken antithyroid drugs, and ten patients were taking the minimum dose of antithyroid drugs at the time of enrollment. All patients had abnormal thyroid hormone levels, and most of them were recommended for radioiodine therapy. It is well known that radioiodine therapy is inexpensive, highly effective, easy to administer, and safe.\textsuperscript{26,27} However, radioiodine causes permanent hypothyroidism in virtually all patients, worsens pre-existing ophthalmopathy, and occasionally induces post-radioiodine exacerbation of hyperthyroidism due to radiation-related thyroiditis.\textsuperscript{28,29} Thus, AJBHT could be applied as an alternative therapy for Graves’ disease patients before radioiodine therapy.

In conclusion, the results from our clinical and in vitro studies suggest that AJBHT might suppress T\textsubscript{4} synthesis by modulating cyclic AMP and Tg levels, making it a possible alternative therapy for Graves’ disease patients who have side effects from antithyroid drugs.

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REFERENCES