

Anti-inflammatory effects of luteolin on experimental autoimmune thyroiditis in mice

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Abstract. Hashimoto's thyroiditis (HT) is the most common organ-specific autoimmune disease and is believed to be a predominately T cell-mediated autoimmunity. Signal transducer and activator of transcription (STAT)3 is a crucial transcription factor of T cell-mediated immunity, with key roles in the proliferation and migration of T helper (Th) cells, differentiation of Th cells into Th17 cells, and the balance between Treg cells and Th17 cells. Flavonoid luteolin has been shown to markedly inhibit Tyr705 activation/phosphorylation of STAT3 and exert anti-inflammatory effects in multiple sclerosis. In the present study, the effect of luteolin on experimental autoimmune thyroiditis (EAT) was analyzed in C57BL/6 mice. Hematoxylin and eosin examination showed that luteolin attenuated lymphocytic infiltration and follicle destruction in thyroid glands. Immunohistochemistry results demonstrated that luteolin significantly reduced the phosphorylation of STAT3 within the thyroid. An *in vitro* study was carried out in a RAW264.7 macrophage cell line. Western blot findings demonstrated that luteolin significantly inhibited interferon- γ -induced increases in cyclooxygenase 2, phosphorylated STAT1 and phosphorylated STAT3 expression levels and the secretion

of the proinflammatory cytokine tumor necrosis factor- α in supernatants. The present findings indicated that luteolin may exert potent anti-inflammatory effects on murine EAT, which may provide a novel therapeutic medication strategy for the early intervention of HT.

Introduction

Hashimoto's thyroiditis (HT) was first discovered by Hakaru Hashimoto in 1912. It is now recognized as the most common autoimmune disease (1), and the most frequent cause of hypothyroidism (2). Diagnosis of HT is based on thyroid dysfunction, an enlarged thyroid gland with a diffusely hypoechogenic pattern by ultrasound examination, and detection of serum thyroid peroxidase (TPO) and thyroglobulin (TG) antibodies, of which TPO is more important (3). It is now believed that HT is a predominately T cell-mediated autoimmunity (4,5). TPO-specific T cells alone are able to induce thyroid destruction, leading to hypothyroidism (6). It has been reported that CD4⁺CD25⁺ T cell depletion increases the incidence of autoimmune thyroid disease (AITD) (7), indicating that the imbalance between effector T cells and regulatory T cells (Treg) may be a key factor in the pathogenesis of AITD. Clinical statistics have also demonstrated that intrathyroidal Treg cells were decreased in patients with AITD, contributing to the incomplete regulation of autoreactive T cells and immune tolerance in AITD (8,9). In addition, T helper 17 (Th17) cells is also one subset of CD4⁺ T cells that may be associated with the pathogenesis of AITD (10,11). A newly identified subset of T cells, known as follicular helper T (Tfh) cells, has also been reported to have an important role in autoimmune disease (12). As a crucial transcription factor for the pathogenesis of autoimmunity, signal transducer and activator of transcription (STAT)3 has a key role in Th17 differentiation (13). Pathogenic Th17 responses in mice are restrained by Tregs and this function is dependent on the role of STAT3 (14). Balance and homeostasis between these two subsets are modulated by STAT3 (15), indicating the essential role of STAT3 in the immunological mechanisms of autoimmune diseases, including AITD. In addition to STAT3, STAT1

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also has a critical role in the signal transduction pathway of interferon-gamma (IFN- γ) and may be a novel target for anti-inflammatory treatment.

Flavonoids, a plant-derived food, are considered to exert anti-inflammatory effects (16). As one of the most common flavonoids, luteolin is present in numerous edible plants and plants used in traditional Chinese medicine. Luteolin has been shown to possess anti-inflammatory activity both *in vitro* and *in vivo* (17-20). Previous studies have demonstrated that Tyr705 activation/phosphorylation of STAT3 is markedly inhibited by luteolin (21,22), and luteolin has also been shown to inhibit the phosphorylation of STAT1 (23). In addition, as an anti-inflammatory medication, luteolin has been proven to be effective against other autoimmune diseases, including multiple sclerosis (24,25) and experimental autoimmune encephalomyelitis (26). Therefore, the present study focused on the effects of luteolin on experimental autoimmune thyroiditis (EAT) and the possible mechanisms associated with STAT1 and STAT3 were discussed.

Materials and methods

Animals. A total of 30 female 8-week-old C57BL/6 mice weighing 20.35 ± 0.86 mg were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). Prior to the study, the mice were housed in a clean-grade animal breeding center with an indoor temperature of 20-24°C and humidity of 50-70%, under alternate dark/light cycles. Tap water and laboratory feed were available *ad libitum*. All procedures were performed in accordance with the guidelines outlined by the Animal Research Ethics Committee of Jinling Hospital (Nanjing, China).

Chemicals and reagents. Luteolin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Jilin, China). Luteolin (20 mg/ml) was dissolved in DMSO and stored at -20°C. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Mouse T4 and TNF- α enzyme-linked immunosorbent assay (ELISA) kits were purchased from ExCell (Shanghai, China). Antibodies used in western blot and immunohistochemistry were as follows: Rabbit monoclonal phospho-STAT3 antibodies (Tyr705; #9145), rabbit monoclonal phospho-STAT1 antibodies (Tyr701; #9167), rabbit monoclonal STAT3 antibodies (#9139), rabbit monoclonal STAT1 antibodies (#14994) and rabbit COX2 antibodies (#12282), and were all purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit GAPDH antibodies (#BS60630) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (#BS10043) were purchased from Bioworld Technology, Inc. (Nanjing, China).

In vivo study

Establishment of an EAT model and treatment with luteolin. Mice were divided into four groups: Luteolin (n=10), dexamethasone (Dex; n=5; positive control), Tg (n=10), and control (n=5). For the induction of autoimmune thyroiditis, 100 μ g porcine Tg (pTg; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was emulsified in 100 μ l Freund's complete adjuvant

(CFA; Sigma-Aldrich; Merck Millipore) and was subcutaneously injected into each mouse (except the control) on day 0. A second subcutaneous injection was administered on day 14 using the same amount of pTg in incomplete Freund's adjuvant (IFA; Sigma-Aldrich; Merck Millipore). Following the second immunization, Luteolin and Dex-treated mice were given daily intraperitoneal injections of luteolin (10 mg/kg/day) and dexamethasone (5 mg/kg/day; both Sigma-Aldrich; Merck Millipore), respectively, whereas TG mice were administered PBS instead. After 7 days of treatment, all mice were sacrificed by cervical dislocation following pentobarbital anesthesia (50 mg/kg, i.p.). Blood samples and thyroid tissues were obtained. Sera were stored at -80°C. Thyroid tissues were fixed in 4% paraformaldehyde solution, sectioned, and hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) were performed for histopathological examination. Mononuclear cell infiltration index was scored as follows: 0, no infiltration; 1, interstitial accumulation of cells between two or three follicles; 2, one or two foci of cells at least the size of one follicle; 3, extensive infiltration, 10-40% of total area; 4, extensive infiltration, 40-80% of total area; and 5, extensive infiltration >80% of total area.

Detection of serum T4 and antibodies against pTg. Serum T4 was assayed using ELISA according to manufacturer's instructions. Antibodies against pTg were detected by ELISA. Briefly, flat-bottomed 96-well plates (Costar 3590; Corning, Inc., Corning NY, USA) were coated overnight at 4°C with 100 μ l pTg (#T1126; Sigma-Aldrich; Merck Millipore) diluted to 100 μ g/ml in PBS, and then washed twice with PBS with 0.05% Tween 20 (PBST). Free protein binding sites were blocked by adding 1% bovine serum albumin (BSA) for 2 h at 37°C. Following washing with PBST, the sera from individual mice were diluted 1:1,600 in PBS with 1% BSA and incubated overnight at 4°C. Following extensive washing of the plates, HRP-conjugated goat anti-mouse IgG (1030-05; Southern Biotech, Birmingham, AL, USA), diluted 1:5,000 in PBS with 1% BSA, was added and the plates were incubated for 1 h at 37°C and subsequently washed. The substrate, 50 μ l/well tetramethylbenzidine, was added for 20 min and the reaction was terminated with 50 μ l/well 2 NH₂SO₄, after which the optical density was measured at 450 nm.

In vitro study

Cell culture and treatment. RAW264.7 mouse macrophage cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin). In brief, cells were grown in 6-well plates and stimulated by human IFN- γ (10 ng/ml) overnight. Cells were treated with luteolin (20 μ mol/l) for 6 h the next day.

Western blot analysis. Western blotting was performed to detect the expression of COX2, an anti-inflammatory marker, and STAT1 and STAT3 transcription factors, which are downstream of the interleukin (IL)-6 signaling pathway. Cells were washed with PBS, harvested and lysed using radioimmunoprecipitation assay buffer. Protein concentrations were determined using a bicinchoninic protein kit according to the

manufacturer's instructions. Protein (50 μg) of each sample was resolved using 10% SDS-PAGE, then transferred to a PVDF membrane. The membrane was blocked with 5% BSA for 2 h at room temperature, then washed with TBST (1:1,000) three times. Phospho-STAT3 (Tyr705; #9145), phospho-STAT1 (Tyr701; #9167), total STAT3 (#9139), total STAT1 (#14994), COX2 (#12282) and GAPDH antibodies (#BS60630) were used at a dilution of 1:1,000 and incubated at 4°C overnight, followed by HRP-conjugated goat anti-rabbit antibodies (#BS10043) at a dilution of 1:20,000 for 80 min at room temperature. Detection of HRP-conjugated antibodies was performed using an ECL Plus Blotting Reagent and a Quality One documentation system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cytokine assay. TNF- α concentrations were measured in the supernatants of cultured RAW264.7 cells using a sandwich ELISA kit (#EM008-48; ExCell, Shanghai, China) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software, version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data was analyzed using the t-test and one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Luteolin inhibits lymphocytic infiltration in thyroids of EAT mice. Anatomical observation demonstrated that 4/10 mice manifested with a goiter (Fig. 1A), indicating the occurrence of EAT. H&E examination of thyroid glands demonstrated that $43 \pm 5.7\%$ TG mice exhibited infiltration of immune cells between follicles, whereas the incidences of Luteolin and Dex-treated mice are 23 ± 5.7 and $17 \pm 11.5\%$, respectively. The infiltration index of the thyroid sections also indicated significantly decreased infiltration of lymphocytes in the luteolin and Dex-treated mice, compared with the TG-treated mice ($P < 0.05$, Fig. 1B and C).

Luteolin reduces plasma T4 levels, while increases plasma anti-pTG levels. T4 concentrations and anti-pTG antibodies were evaluated via ELISA assays. The results demonstrated that all three groups with EAT (Dex, Luteolin and TG) exhibited increased anti-TG antibodies compared with control mice, and luteolin and Dex treatment both significantly increased antibody levels compared with the TG group ($P < 0.05$; Fig. 2A). Serum T4 concentrations in TG mice were mildly elevated, compared with the other groups, but the difference was not significant ($P > 0.05$; Fig. 2B).

Luteolin inhibits STAT3 phosphorylation in thyroid glands. The effect of luteolin on the phosphorylation of STAT3 (Y705) in thyroid sections was evaluated by IHC. Phosphorylated STAT3 expression was significantly increased in TG mice compared with the control ($P < 0.05$), whereas luteolin and Dex treatment markedly inhibited this alteration (Fig. 2C and D).

Luteolin inhibits the expression of COX2 and phosphorylation of STAT1 and STAT3. Western blot analysis of RAW264.7 macrophage cell line demonstrated that luteolin markedly

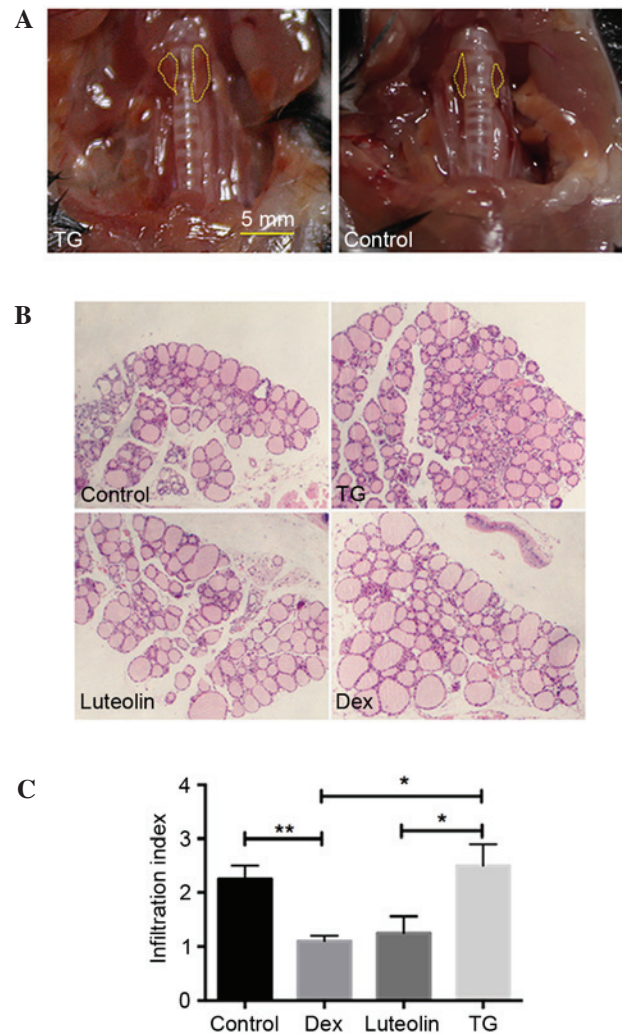


Figure 1. Luteolin significantly inhibited the infiltration of lymphocytes caused by autoimmune thyroiditis. (A) 4/10 mice exhibited enlargement of the thyroid glands. (B) Hematoxylin and eosin staining were performed in all mice; 40% of the section from TG-treated mice demonstrated a large amount of lymphocytic infiltration between the follicles compared with the control (magnification, $\times 40$). Luteolin and Dex mice-treated exhibited markedly decreased levels of infiltration of immune cells. (C) Infiltration index of monocytes in the thyroid sections in each group, $P < 0.05$; $^{**}P < 0.01$. TG thyroglobulin; Dex, dexamethasone.

inhibited the increased expression of COX2, phosphorylated (p)-STAT1 (Y701) and p-STAT3 (Y705) induced by IFN- γ treatment, whereas total STAT1 and STAT3 remained unchanged. These findings demonstrated the anti-inflammatory effect of luteolin by inhibiting the STAT1 and STAT3 signaling pathway *in vitro* (Fig. 3A).

Luteolin reduces TNF- α secretion in the RAW264.7 cell line. TNF- α concentrations were measured in the supernatants of RAW264.7 cells using ELISA kits. TNF- α concentration levels were significantly increased when treated with IFN- γ , whereas they were markedly decreased after treatment with luteolin ($P < 0.05$; Fig. 3B).

Discussion

STAT3 has an important role in T cell-mediated immunity, including the proliferation (27) and migration (28) of T cells,

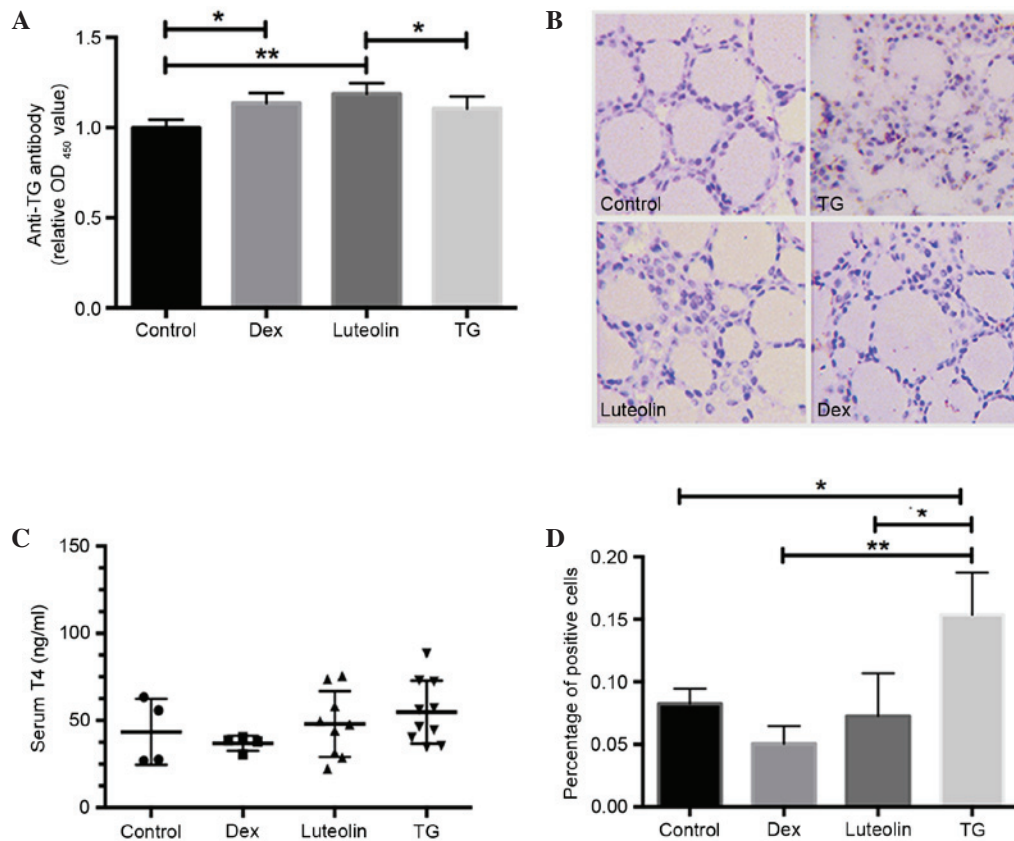


Figure 2. Luteolin inhibited the phosphorylation of STAT3 in thyroid glands. (A) Serum TG antibodies were detected by ELISA. All three groups with EAT (Dex, Luteolin and TG) exhibited increased anti-TG antibody levels compared with the control. (B) Serum T4 concentrations were detected by ELISA. TG-treated mice demonstrated mildly elevated T4 levels compared with the other groups, without significance ($P>0.05$). (C) Thyroid sections of TG mice stained positive for p-STAT3 (Y705) during immunohistochemical analysis (magnification, x400), whereas the other groups stained negative. (D) Quantification analysis based on the percentage of positively stained cells in one visual field. Four visual fields were selected randomly in every section. All values are expressed as mean \pm standard deviation. * $P<0.05$; ** $P<0.01$. TG thyroglobulin; Dex, dexamethasone.

differentiation into Th17 cells (29), and balance between Treg cells and Th17 cells (30,31). Moreover, STAT3 and its downstream SOCS3 gene polymorphism are associated with AITD susceptibility and IL-6 secretion (32-34). Cytokines, such as IL-6, are important in the pathogenesis of AITD due to their functions in recruiting inflammatory cells in the thyroid, upregulating some inflammatory molecules and interfering in the production of thyroid hormones (35). It has been demonstrated that IL-6-STAT3 signaling has a crucial role in dendritic cell differentiation during T cell-mediated immune responses *in vivo* (36). Thyroid follicular epithelial cells are able to synthesize and secrete large quantities of IL-6 (37), which further promotes the development of autoimmune responses. Therefore, it is theoretically reasonable to target IL-6/STAT3 to intervene in the early stage of autoimmune thyroiditis in order to explore novel therapeutic strategies for HT. Previous studies have shown that luteolin has potent anti-inflammatory effects *in vitro* and *in vivo* (38,39) and the mechanisms involved include the activation of NF- κ B, which leads to the expression of IL-6 and COX-2 (18,40). Activator protein-1 (AP-1) is also an important transcription factor associated with immune responses. Expression of IL-6 is induced by AP-1 and NF- κ B (41). Jang *et al* (41) found that luteolin was able to reduce LPS-induced IL-6 expression by inhibiting JNK and AP-1 pathways both *in vitro* and *in vivo*, and the mice treated with luteolin exhibited decreased plasma and hippocampal IL-6 levels.

HT, which is also known as chronic lymphocytic thyroiditis, is the most common autoimmune disease. There is usually a long latency period before hypothyroidism occurs (42). Therefore, early intervention may theoretically prevent the development of the disease and maintain the normal structure and function of the thyroid glands. Thus, the present study aimed to explore the anti-inflammatory effects of luteolin on autoimmune thyroiditis and the mechanisms involved. A classical C57BL/6 mouse model of EAT was established. As a result, 4/10 mice exhibited goiter symptoms and infiltration of mononuclear cells into the thyroid glands. C57BL/6 mice are known to have a relative low incidence of EAT (43), which is consistent with the present findings. The effects of luteolin on EAT were subsequently evaluated. Mice treated with luteolin demonstrated significantly reduced infiltration of lymphocytes compared with TG mice. As an intracellular inhibitor of IL-6/STAT1 and STAT3 signaling pathway, luteolin significantly inhibited the phosphorylation of STAT1 and STAT3 in thyroid glands, as identified by H&E examination.

Anti-Tg antibodies were also elevated in the three EAT groups, as compared with the control; however, the treatment of luteolin and Dex appeared to further increase the antibodies. Although the mechanisms remain unknown, clinical data has shown that thyroid antibodies are elevated shortly after ¹³¹Iodine treatment for hyperthyroidism (44). The mechanisms involved require further investigation. In addition, serum T4

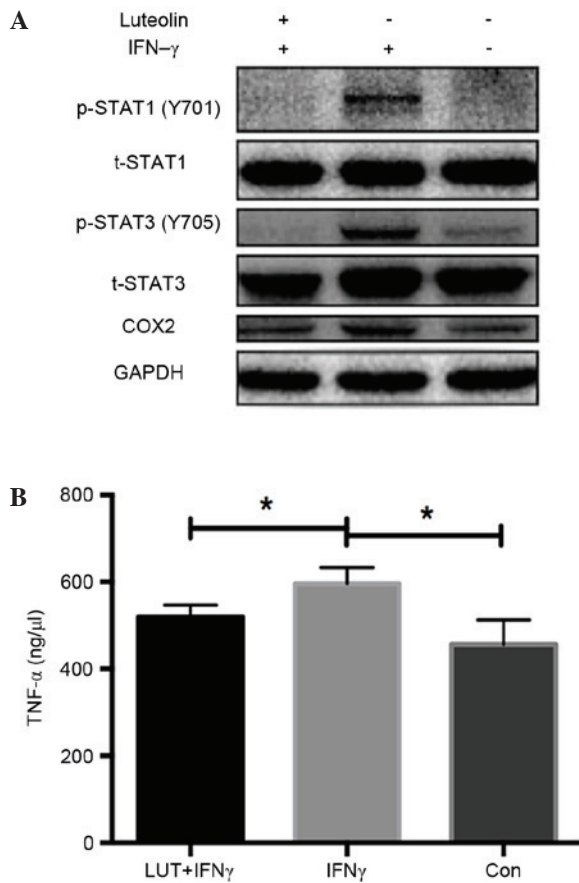


Figure 3. Luteolin inhibits the expression of COX2 and the phosphorylation of STAT1 and STAT3 and reduced TNF- α secretion in the RAW264.7 cell line. (A) RAW264.7 macrophages were stimulated by human IFN- γ (10 ng/ml) overnight, and treated with luteolin (20 μ mol/l) for 6 h the following day. Western blot results demonstrated that luteolin exerts an anti-inflammatory effect by inhibiting the increased expression of COX2, p-STAT1 (Y701) and p-STAT3 (Y705) induced by IFN- γ treatment, whereas total STAT1 and total STAT3 remain unchanged. (B) Concentrations of TNF- α were measured in the supernatants of cultured RAW264.7 cells using ELISA kits. TNF- α levels were significantly increased when treated with IFN- γ , whereas they were markedly decreased after treatment with luteolin. *P<0.05. IFN, interferon; p, phosphorylated; STAT, signal transducer and activator of transcription; COX, cyclooxygenase; TNF, tumor necrosis factor.

levels were slightly elevated in TG mice, which may be due to the thyroid damage caused by thyroiditis. Luteolin appears to reduce the release of T4 into the blood; however, no statistical significance was detected.

Western blot analysis of RAW264.7 cells demonstrated that luteolin exerted anti-inflammatory effects by significantly inhibiting the IFN- γ -induced increase of COX2 and p-STAT1 (Y701) and p-STAT3 (Y705) expression, whereas total STAT1 and STAT3 remained unchanged. COX-2 is an inducible enzyme (45), which is highly expressed in cells involved in the inflammatory response including monocytes/macrophages and mast cells. Cytokine TNF- α detection in supernatants also demonstrated that luteolin exhibited anti-inflammatory effects by significantly reducing TNF- α secretion *in vitro*.

Additional experiments are required to elucidate the anti-inflammatory mechanisms of luteolin, which may include the IL-6/ STAT1 and STAT3 pathways discussed in the present study or other pathways. Even if the immunosuppressive effects are mediated solely through the IL-6 pathway, addi-

tional proteins or molecules involved in the process remain to be discovered.

In conclusion, treatment with luteolin exhibited a significant immunosuppressive effect by attenuating lymphocytic infiltration and the destruction of the thyroid epithelia in thyroid glands, which is likely to have occurred via the inhibition of IL-6/STAT1 and STAT3 signaling pathway in the glands. The present study provides evidence for a promising novel therapeutic strategy for the early intervention of autoimmune thyroiditis. Further investigation is required to fully elucidate the mechanisms involved.

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