Triptolide reduces salivary gland damage in a non-obese diabetic mice model of Sjögren’s syndrome via JAK/STAT and NF-κB signaling pathways

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Triptolide (TP) has anti-inflammatory and immunosuppressive effects. However, the effect of triptolide on Sjögren’s syndrome (SS) is rarely reported. In this paper, we studied the effects of triptolide on non-obese diabetes mice model of SS. In this study, salivary flow rate was measured every two weeks, and autoantibodies in the serum were detected. Salivary gland index and spleen index were detected, pathological changes of salivary gland were detected by hematoxylin-eosin staining, inflammatory factors were detected by enzyme linked immunosorbent assay, lymphocytes were detected by flow cytometry, proliferation of T cells and B cells were detected, and related proteins were detected by Western blot. Triptolide increased salivary flow rate and salivary gland index, and decreased spleen gland index. Moreover, triptolide reduced the infiltration of lymphocytes to salivary glands, decreased the level of autoantibodies in serum, and reduced the inflammatory factors in salivary glands and IFN-γ induced salivary gland epithelial cells. Further, triptolide inhibited activator of JAK/STAT pathway and NF-κB pathway. In conclusion, triptolide could inhibit the infiltration of lymphocytes and the expression of inflammatory factors through JAK/STAT pathway and NF-κB pathway. Thus, triptolide may be used as a potential drug to treat SS.

Key Words: triptolide, Sjögren’s syndrome, salivary glands, JAK/STAT pathway, NF-κB pathway

Sjögren’s syndrome (SS) is a kind of systemic inflammatory autoimmune disease involving exocrine glands.¹⁻³ It is characterized by destruction and dysfunction of lacrimal and salivary glands, followed by dry mouth and dry eyes.³ The etiology and pathogenesis of SS are not clear, which may be caused by genetic predisposition, environmental trigger, autoimmunity, and other relevant factors.⁴⁻⁵ It was found that the infiltrated cells in the salivary gland of SS patients included B cells, T cells, macrophages, epithelial cells, and dendritic cells, which together led to the occurrence of chronic inflammatory response.⁶ Among environmental factors, bacterial and viral infections are more closely related to the pathogenesis of SS. When the virus infects the glands, the Toll-like receptor (TLR) in the glandular epithelial cells recognizes the viral antigen, and then activates downstream nuclear factor-κB (NF-κB) and other signaling pathways to induce cell surface adhesion factors and immune inflammatory factors.⁷⁻⁸ T and B lymphocytes are activated after interacting with glandular epithelial cells and dendritic cells, further inducing autoantibodies and related inflammatory factors, and then inducing memory B lymphocytes, which cause lymphocytes to continue to act on target organs and the disorder of immune system.⁹⁻¹⁰ Then patients will have a series of clinical manifestations such as dry mouth, dry eyes, and glandular swelling. IFN-γ is mainly produced by activated NK cells and T cells, which stimulate the immune response of salivary gland epithelial cells, and enhancing inflammation and gland damage. IFN-γ-induced inflammation in salivary gland epithelial cells is suitable as a cell model for studying Sjögren’s syndrome.¹¹⁻¹³

Triptolide (TP) is an epoxide diterpenoid lactone, an ethanol extract of Tripterygium wilfordii, which could pass through the blood-brain barrier.¹²⁻¹³ Studies have confirmed that TP has anti-inflammatory, immunosuppression, anti-tumor, and other effects.¹⁴⁻¹⁶ In clinical, TP is used in the treatment of rheumatism and rheumatoid arthritis (RA), systemic lupus erythematosus and other autoimmune diseases.¹⁷⁻¹⁸ TP could selectively prevent epidermal cells stimulated with TNF-α or IL-1β from secreting inflammatory cytokines and chemical factors by inhibiting the transcriptional activity of NF-κB.¹⁹⁻²⁰ TP could affect the distribution of T cell and B cell subsets in patients with RA and maintain the dynamic balance of CD4⁺/CD8⁺, so as to correct the cellular immune dysfunction in patients with RA.¹⁹⁻²⁰

However, there are little studies on the treatment of SS. In this paper, we used non-obese diabetes (NOD) mice, as an animal model of SS, to detect whether TP could improve SS. We examined the salivary secretion rate, salivary gland inflammation, activity and proliferation of immune cells, and related signaling pathways in NOD mice. Furthermore, we investigated the role of TP at the cellular level using salivary epithelial cells.

Materials and Methods

Animal. All 48 female NOD mice (8 weeks, female) were provided by Shanghai Lab. Animal Research Center. Mice were kept in the sterilized cages on a 12 h/12 h dark/light cycle with free access to standard chow and tap water, and constant temperature and humidity. The experiment was approved by the Institutional Animal Care and Use Committee of Affiliated Hospital of Nanjing University of Chinese Medicine.

Treatment and groups. TP (purity >99%) was obtained from Sigma (St. Louis, MO). TP was dissolved in 0.05% DMSO (Sigma). The NOD mice were administrated with TP by means

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of gastric gavage. The treatment was kept daily for 90 days. The dosage selection for TP (10 \( \mu \)g/(kg·day), 20 \( \mu \)g/(kg·day), and 40 \( \mu \)g/(kg·day)) was referred to the previous study. Forty-eight NOD mice were divided into 4 groups: Control group (Vehicle) \((n = 12)\), NOD mice treated with 10 \( \mu \)g/(kg·day) TP \((n = 12)\), NOD mice treated with 20 \( \mu \)g/(kg·day) TP \((n = 12)\), NOD mice treated with 40 \( \mu \)g/(kg·day) TP \((n = 12)\).

Before the above experiments, we carried out pre-experiments. Thirty NOD mice were divided into 3 groups: Control group (Vehicle) \((n = 10)\), NOD mice treated with 20 \( \mu \)g/(kg·day) TP \((n = 10)\), NOD mice treated with 12.5 mg/(kg·day) hydroxychloroquine (HCQ) \((n = 10)\).

Measurement of salivary secretion. The volume of saliva secreted by mice was detected every 2 weeks. The protocol of saliva collection is as follows: After anesthesia with 3% chloral hydrate, 0.01 mg pilocarpine was injected intraperitoneally. 5 min later, the head of the mouse was lowered. The capillary tube was placed in the mouth of the mouse to collect saliva for 10 min. After collecting saliva, the saliva flow rate (\( \mu \)l/min) was calculated.

Calculation of salivary gland index and spleen index. After the animals were killed, the salivary gland and spleen were removed and weighed, and the salivary gland index and spleen index were calculated. The salivary gland index \((%) = \text{spleen weight (g)/mouse weight (g)}\) and the spleen index \((%) = \text{spleen weight (g)/mouse weight (g)}\).

ELISA assay. After the mice were anesthetized with 3% chloral hydrate, the blood was extracted from the eyeball, collected with EDTA anticoagulant tube, centrifuged at 4°C, 3,000 rpm for 5 min, and the supernatant was frozen and stored at −80°C. TNF-\( \alpha \), IL-2, and IL-6 are detected by ELISA kit (Roche Diagnostic, Mannheim, Germany), and the operation steps are in accordance with the instructions. The OD value is read at a 450 nm absorbance using the Microplate reader (Bio-Rad, Hercules, CA).

Hematoxylin & eosin staining. At the end of the intervention, salivary glands of mice were taken, fixed overnight in 4% paraformaldehyde solution, paraffin embedded, sectioned 4 \( \mu \)m, stained with hematoxylin & eosin (HE), observed and photographed under the microscope. Histopathological evaluation was according to Cutler’s method: grade 0: scattered lymphocyte; grade 1: a small amount of lymphocyte infiltration; grade 2: moderate lymphocyte infiltration (no lymphadenopathy), mild submucosal gland vascular and ductal edema; grade 3: 1 lymphadenic focus (more than 50 infiltrating lymphocytes are equivalent to 1 lymphadenic focus) per 5 low power microscopes, moderate vessels and glands; grade 4: there are 2–3 lymphocytic focus per 5 low power microscopes, atrophic acini, and severe vascular and glandular edema. Grade 1 is equivalent to 1 point, and more than 2 points are considered as disease.

Analysis of lymphocyte distribution in mouse salivary gland by flow cytometry. Salivary glands of mice were taken out for grinding, and then filtered through a 200 mesh screen to make the single cell suspension. The suspension was centrifuged at 1,600 rpm for 5 min, and the supernatant was discarded. 5 ml red blood cells (RBC) lysis solution was added to the suspension for 8 min, and washed twice using the PBS containing 0.5% BSA, and then the suspension was counted again. The concentration of the suspension was 4 \( \times \) 10^7 cells/ml. The cells were resuspended in 1 ml RPMI1640 medium, 10 \( \mu \)l cell stimulant was added, and incubated in 5% CO\(_2\), 37°C for 4 h. Cells were washed twice using the PBS containing 0.5% BSA and then resuspended. Then CD3\( \epsilon \) (145-2c11), CD4 (gk1.5), CD8\( \alpha \) (53-6.7), CD19 (1D3), and CD45.1 (A20) were added and incubated in dark at room temperature for 15 min. After washed twice, 10,000 positive cells were collected from each sample. The cells were detected using flow cytometer (BD Biosciences, San Jose, CA). The proportion of positive cells was analyzed by FLOWJO software.

Proliferation of T cells and B cells assay in spleen. The spleen was separated from NOD mice, and washed with Hank’s Balanced Salt Solution (HBSS) for 3 times. The cleaned spleens were ground and filtered with 200 mesh screen, and the cells were collected by being centrifuged at 4°C, 1,500 rpm for 3 min. After RBC lysis solution added, the cells were incubated at room temperature for 3 min, and then centrifuged at 4°C, 1,500 rpm for 3 min so that spleen cells were obtained. After being washed twice with PBS, the spleen cells were re-suspended with 1640 medium, and cultured at 37°C 5% CO\(_2\) in a cell culture incubator. Spleen cells were inoculated into 96 well plates at a density of 1 \( \times \) 10^5/well. 100 \( \mu \)l cell suspension was added to each well, and then 10 \( \mu \)l TP was added to the wells. The final concentration of TP in 96 well plate was 10 ng/ml, 20 ng/ml, or 40 ng/ml. Then add 10 \( \mu \)l of Concanavalin A (ConA) or 10 \( \mu \)l of lipopolysaccharide (LPS) (from E. coli). The final concentration of ConA in 96 well plate was 10 \( \mu \)g/ml, and that of LPS in 96 well plate was 2 \( \mu \)g/ml. After 48 h, 10 \( \mu \)l Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China) used for cell proliferation was added to the wells, and then the absorbance was detected under 490 nm after 4 h.

Salivary gland epithelial cells (SGECs) isolation and culture. SGECs were isolated from NOD mice according to the previous method. Simply, the salivary gland tissue was obtained, and rinsed using precooled and sterile PBS containing 100 units/ml penicillin and 100 \( \mu \)g/ml streptomycin. Then, it was placed in serum-Free Keratinocyte Medium (keratinocyte SFM), ground with 50 mesh steel mesh to make single cell suspension, centrifuged for 5 min at 1,500 rpm to remove the supernatant, and resuspended with keratinocyte SFM containing 0.4 \( \mu \)g/ml hydrocortisone, 100 unit/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 25 \( \mu \)g/ml bovine pituitary extract. Following that, cells were inoculated into 24 well plate coated with type I collagen (Iwaki, Tokyo, Japan). After reaching confluence, cells were digested with trypsin and passage, and then the fresh cell culture medium was replaced. The cultured epithelial cells were used for the third generation in the experiment.

Western blot. Radioimmunoprecipitation (RIPA) buffer (Solarbio, Beijing, China) was used to extract salivary gland tissue protein, Bicinchoninic Acid (BCA) Protein Assay Kit (Vazyme, Piscataway, NJ) was used to detect protein concentration, and Western blot was used to detect protein expression as previous studies. Band scan 5.0 software was used to measure the gray value of each band, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference. The primary antibodies were used as below: JAK1/2 (1:1,000, 664666-1-Ig; Proteintech, Chicago, IL), p-JAK1/2 (1:1,000, sc-101716; Santa Cruz Biotechnology, Delaware Ave, CA), STAT3 (1:1,000, sc-8059; Santa Cruz Biotechnology), IkB\( \alpha \) (1:1,000, 10253-2-AP; Proteintech), p-STAT3 (1:1,000, sc-8059; Santa Cruz Biotechnology), IkB\( \beta \) (1:1,000, 10268-1-AP; Proteintech), p-IkB\( \beta \) (1:1,000, sc-52943; Santa Cruz Biotechnology), NF-kB (1:1,000, 10745-1-AP; Proteintech), p-NF-kB (1:1,000, sc-136548; Santa Cruz Biotechnology), GAPDH (1:2,000, ab8245; Abcam, Cambridge, UK). The second antibodies used were as below: Goat Anti-Rabbit IgG H&L (HRP) (1:5,000, ab6721; Abcam), Goat Anti-Mouse IgG H&L (HRP) (1:5,000, ab205719; Abcam).

Statistical analysis. The data were analyzed by SPSS13.0 software. The data were expressed by mean ± SD. Difference between groups was compared by one-way ANOVA, and Tukey test is used for inter group comparison. \( P<0.05 \) was defined as the difference with statistical significance.

Results

Protective effects of resveratrol against salivary gland index, spleen index, and salivary flow rate in NOD mice. The results showed that the salivary gland index of NOD mice in TP group were significantly higher than those in the control group (Fig. 1A), and the spleen index of NOD mice in TP group were
significantly lower than those in the control group (Fig. 1B). The salivary flow rate of NOD mice in the control group decreased with the prolongation of time. The salivary flow rate in the 6 to 12th week was significantly lower than that in the TP group. With the increase of TP concentration, the difference between salivary flow rate and control group was larger (Fig. 1C).

**TP inhibits autoimmune damage in Sjogren’s syndrome mice.** For pre-experiment, we found that the effect of TP is more obvious than that of HCQ in Supplemental Fig. 1*. Moreover, there were obvious lymphocytic infiltration and lymphoid foci formation (Fig. 2A). The histopathological score was 3.1 ± 0.21. In the TP treatment group, there were a small amount of lymphocytic infiltration and no lymphoid foci formation. The histopathological scores were respectively 2.4 ± 0.18, 1.8 ± 0.17, and 1.5 ± 0.19 (Fig. 2B). The antibody levels of IgG, IgM, IL-2, IL-6, and TNF-α in the serum of NOD mice in TP treatment group were significantly reduced compared with control group (Fig. 2C and D). CD4⁺, CD8⁺, and B220⁺/CD45⁺ cells were significantly reduced (Fig. 2E). Furthermore, we found that the proliferation of T cells and B cells in the spleen of NOD mice treated with TP was significantly reduced compared with treated with 0.05% DMSO (Fig. 2F).

**TP inhibits JAK/STAT and NF-κB signaling pathways in salivary glands of NOD mice.** We studied the effects of TP on JAK/STAT and NF-κB signaling pathways in salivary glands of NOD mice. The results of Western blot showed that TP significantly inhibited JAK/STAT signal pathway through inhibiting the phosphorylation of JAK1/2 and STAT (Fig. 3A), and significantly inhibited NF-κB signal pathway through inhibiting the phosphorylation of p65 and IκBα (Fig. 3B).

**TP reduces IFN-γ-induced inflammatory damage in SGECs by inhibiting JAK/STAT and NF-κB signaling pathways.** We detected the cytotoxic effect of TP on SGECs, and the results showed that the cell activity did not change significantly when SGECs was treated with 10 ng/ml, 20 ng/ml, and 40 ng/ml TP for 72 h (Fig. 4A). Thus 10 ng/ml, 20 ng/ml, and 40 ng/ml TP was used in the next study. IFN-γ significantly induced the increase of inflammatory factor (IL-2, IL-6, and TNF-α) secreted by SGECs, but TP significantly inhibited the expression of inflammatory factor referred to the concentration of TP (Fig. 4B). Furthermore, we tested the effect of TP on IFN-γ-induced activation of JAK/STAT and NF-κB pathways involved in SGECs activation. TP did not show any significant effect on the expression level of JAK1/2, STAT3, IκBα, NF-κB, but p-JAK1/2, p-STAT3, p-IκBα, and p-NF-κB were significantly inhibited (**p<0.001**) as shown in Fig. 4C and D.

**Discussion**

NOD mice, being similar symptoms with SS patients, show such as early lymphocytic infiltration of salivary glands and lacrimal glands, serum immunological characteristics (auto-antibodies) and salivary flow reduction. Therefore, NOD mice are often used as SS animal model at present. The treatment of autoimmune diseases with traditional Chinese medicine is a hot topic recently, such as TP. TP has strong immunosuppressive and immunoregulatory effects, and is widely used in the treatment of a variety of clinical autoimmune diseases. Some studies have shown that TP has an inhibitory effect on the development of type I diabetes in NOD mice. While the effects on spontaneous

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Fig. 2. Effects of TP on lymphocytic infiltration and inflammatory factors of salivary gland in NOD mice. (A) Salivary gland tissues sections were stained by HE assay. (B) Histopathological evaluation was according to Cutler's method, the pathological scores were calculated. (C) Autoantibodies (IgG and IgM) levels were detected in the serum of NOD mice. (D) Inflammatory factors (IL-2, IL-6, and TNF-α) were detected in salivary gland by ELISA. (E) Lymphocytes (CD4, CD8, and B220/CD19) were detected in salivary gland by flow cytometry. (F) The cell viability of T cells and B cells was detected in spleen by CCK8. *p<0.05, **p<0.01 compared with control group.
SS in the same kind of mice are rarely reported, this study was to observe the effects of TP on NOD mice model of spontaneous SS. In SS, the salivary gland is infiltrated and destroyed by inflammatory cells, resulting in the reduction of exocrine function. On the one hand, the accumulation of cytokines produced by immune cells in salivary glands mediates the damage of glandular epithelium, resulting in the increasing level of autoantibodies. On the other hand, epithelial cells increase the release of inflammatory factors and aggravate the immune response. The destruction of SS exocrine gland is closely related to the continuous lymphocyte infiltration. Immunopathological study showed T cells and B cells are considered to be important links in pathological damage of SS exocrine glands. It has been confirmed that paeoniflorin, a kind of terpenoids, can improve pathological damage of salivary gland by up-regulating the expression of aquaporin-5 (AQP-5). The efficacy of paeoniflorin show a similar effectiveness as HCQ in delaying the onset of SS-like disease in NOD mice by ameliorating inflammation and normalizing autoantibody profiles. In this study, our results showed that with the increase of age, the salivary flow rate of NOD mice in the control group decreased gradually, and there were more serious lymphocytic infiltrating lesions in the salivary gland, which was consistent with the previous experimental results. Moreover, we found that the concentrations of IgG and IgM in mice serum increased gradually, and the expression of inflammatory factors increased. However, compared with the control group, TP treatment group significantly improved the salivary gland flow rate, decreased lymphocyte infiltration, IgG and IgM concentration and the expression of inflammatory factors. It is well known that SS can cause abnormal proliferation of T and B lymphocytes. ConA and LPS are used to act on the corresponding receptors on the membrane surface of T and B lymphocytes, respectively, and activate T and B lymphocytes to promote their mitosis and proliferation. The present results indicated that TP inhibited ConA- and LPS-induced T and B lymphocytes proliferation. Those results indicated that TP could improve the symptoms of SS in NOD mice by inhibiting the infiltration of lymphocytes and inflammatory of glands. TP is well known to high toxicity, but there was no significant tissue damage in NOD mice at the TP dose we used.

NF-κB pathway is over activated in inflammatory diseases, which leads to the formation of inflammatory factors and aggravates the occurrence and development of diseases. Altavilla et al. found that NF-κB is a fast reactive transcription factor, which plays a role in inflammatory response by expressing inflammatory mediators (IL-1, IL-2, IL-6, and TNF-α), adhesion factors and enzymes. Studies have shown that mice that knock out TNFAIP3 (A20), an inhibitor of the NF-κB signaling pathway, exhibit the characteristics of SS. All of these indicate that NF-κB signaling pathway is involved in regulating the development of SS. JAK/STAT signaling pathway is an important pathway of cell and growth factor, which is widely involved in cell immune regulation, proliferation, differentiation, migration, apoptosis, and other

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**Fig. 3.** TP blocks activation of NF-κB and JAK/STAT signaling pathways in salivary gland. (A) The expression of JAK1/2, p-JAK1/2, STAT3, and p-STAT3 were detected in salivary gland by western blot. (B) The expression of IkBα, p-IkBα, p65, and p-p65 were detected in salivary gland by Western blot. *p<0.05, **p<0.01 compared with control group.
Fig. 4. TP inhibits inflammatory factors expression by blocking activation of NF-κB and JAK/STAT signaling pathways in IFN-γ induced SGECs. (A) After 0, 1, 5, 10, 20, 40, 160 ng/ml TP was administrated for 72 h, SGECs viability was detected by CCK8. (B) Inflammatory factors (IL-2, IL-6, and TNF-α) were detected in SGECs by ELISA. (C) The expression of JAK1/2, p-JAK1/2, STAT3, and p-STAT3 were detected in SGECs by Western blot. (D) The expression of IκBα, p-IκBα, p65, and p-p65 were detected in SGECs by Western blot. *p<0.05 compared with control group, †p<0.05 compared with IFN-γ group, ‡p<0.01 compared with IFN-γ group.
processes. The abnormal activation of JAK/STAT can cause inflammatory diseases, polycythemia, and leukemia, etc. JAK is a kind of intracellular non receptor soluble tyrosine protein kinase. STAT, as a transcription gene, plays an important role in various pathophysiological mechanisms and is the downstream substrate of JAK. JAK inhibitors reduced B cell-activating factor in inflammatory diseases, polycythemia, and leukemia, etc. The mechanism of activation of macrophage plasmin and cytokine IFN-γ by JAK/STAT pathway has been found to be the key to persistent inflammation and tissue damage in the exocrine glands of SS.

Our data showed that TP could decrease activator of JAK/STAT pathway and NF-κB pathway in salivary glands of NOD mice and in IFN-γ induced SGECs. The results revealed that TP decreased inflammation and tissue damage in NOD mice SS model by inhibiting JAK/STAT pathway and NF-κB pathway.

Taken together, this study proved that TP could inhibit the development of SS in a NOD mice model, which suggests that TP has a good application prospect in the treatment of autoimmune diseases, especially in SS.

Acknowledgments

Not applicable.

Conflict of Interest

No potential conflicts of interest were disclosed.

References


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