

Total glucosides of paeony alleviates experimental Sjögren's syndrome through inhibiting NLRP3 inflammasome activation of submandibular gland cells

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Abstract

Objective

The mechanisms by which total glucosides of paeony (TGP) mitigates Sjögren's syndrome (SS) remains elusive. In the present study, we aim to explore the relationship between the therapeutic effects of TGP in the treatment of SS and NLRP3 inflammasome activation in submandibular gland (SG) cells.

Methods

Female non-obese diabetic (NOD) mice were selected as the model of SS. The mice were divided into PBS and TGP treatment group. For treatment, TGP (400mg·kg⁻¹) was administered intragastrically every day for 4 weeks. The SS-like symptoms and pathological changes of the SG of mice were compared between the PBS and TGP group. The activation of NLRP3 inflammasome in SG was detected by RT-qPCR, immunohistochemistry and western blot. The SG cells stimulated by lipopolysaccharide (LPS) and adenosine triphosphate (ATP) for activation of NLRP3 inflammasome were treated with or without TGP. Then, NLRP3 inflammasome activation was assessed. The IL-1 β and IL-18 in homogenate of SG, serum and supernatant were detected by ELISA.

Results

Compared with balb/c mice, NOD mice showed SS-like symptoms and lymphocyte infiltration in SG, and the expression of NLRP3 inflammasome in SG was significantly increased. The SS-like symptoms were alleviated, and lymphocyte infiltration in SG was reduced, and the level of NLRP3 inflammasome in SG mice was decreased after TGP treatment. TGP also significantly inhibit the activation of NLRP3 inflammasome of SG cells in vitro.

Conclusion

Collectively, our results indicated that TGP alleviates SS through inhibition of the activation of NLRP3 inflammasome of SG. These findings clarified the mechanism underlying the therapeutic effects of TGP on SS, and provided new evidence for the further application of TGP in the treatment of SS.

Key words

total glucosides of paeony, Sjögren's syndrome, NLRP3 inflammasome, submandibular gland

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Introduction

Sjögren's syndrome (SS) is a chronic systemic autoimmune disease, which mainly invades exocrine glands such as lacrimal glands and salivary glands, and resulting in clinically manifestation as dry mouth and eyes (1, 2). The prevalence rate of SS is approximately 0.5%, mostly in women aged 50–70 years (3). So far, there is no specific treatment for this disease. Some therapeutic drugs have shown certain effects. However, they also increase the risk of infection and malignant tumours in patients (4). Therefore, exploring novel medicine with good therapeutic effects and few side effects is urgently needed.

Total glucosides of paeony (TGP) are the main active ingredient of white peony, which is mainly composed of paeoniflorin, hydroxypaeoniflorin, peony albiflorin, benzoylpaeoniflorin and other components (5). Studies have shown that TGP had various effects, such as anti-inflammatory, liver protection and immune regulation (6–8). Clinical studies have shown that TGP had certain curative effects in the treatment of rheumatoid arthritis, systemic lupus erythematosus and SS (9–11). Therefore, TGP has been one of the medicines for the treatment of autoimmune diseases. However, the mechanisms of the beneficial effects of TGP on SS remain to be elucidated.

NOD-like receptor thermal protein domain associated protein 3 (NLRP3) is an important member of the NOD-like receptor family, and its inflammasome consists of NLRP3, apoptosis-associated speck-like protein containing a carboxyterminal card (ASC) and cysteine protease-1 precursor (pro-caspase-1) (12). The activated NLRP3 inflammasome can prompt ASC to cleave pro-caspase-1 to caspase-1, which in turn triggers the release of IL-1 β and IL-18, thereby causing an inflammatory response (13, 14). In recent years, studies have shown that NLRP3 inflammasome were involved in the development of SS, and activation of NLRP3 inflammasome was closely related to disease activity, and inhibition of its activation could delay the disease process (15, 16). The submandibular gland (SG) is injured organ of SS (17). It has been

reported that inhibiting activation of NLRP3 inflammasome reduced apoptosis and inflammatory response of SG cells, resulting in improving SS-like symptoms (18). However, it is unclear whether the therapeutic effects of TGP on SS was related to inhibiting NLRP3 inflammasome activation in SG cells. Therefore, in the present study, we attempted to explore the regulatory effects of TGP on NLRP3 inflammasome activation in SG cells of NOD mice with SS-like symptoms.

Materials and methods

Mice

Previous studies showed that non-obese diabetic (NOD) mice displayed typical SS-like symptoms, for example infiltration in salivary and lacrimal glands and autoantibodies in serum (19). Therefore, NOD mice are recognised as SS model mice. The female NOD mice and Balb/c mice (control mice) were purchased from Changzhou Cavens Experimental Animal Co., Ltd. (Changzhou, China). Mice were kept under pathogen-free conditions in the animal centre of the Nanjing Drum Tower Hospital Clinical College of Nanjing University of Chinese Medicine. All animal experiments were implemented in compliance with the guidelines of the Nanjing Drum Tower Hospital Clinical College of Nanjing University of Chinese Medicine.

Drugs and reagents

TGP was purchased from Ningbo Liwa Pharmaceutical Co., Ltd (Ningbo, China). Mouse IL-1 β and mouse IL-18 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Abclonal (Wuhan, China). RNA isolater Total RNA Extraction Reagent, HiScript III RT SuperMix for qPCR and ChamQ Universal SYBR qPCR Master Mix were purchased from Vazyme Biotech Co., Ltd (Nanjing, China).

TGP treatment

The NOD mice develop histopathological changes in the salivary glands and autoantibodies in serum, as those patients with SS do. It is important to consider the stages of SS-like disease in NOD mice and select mice at the ap-

appropriate age for different therapeutic interventions. In our previous study, we dynamically detected SS-like symptoms of NOD in different age. We found that lymphocyte infiltration in salivary glands (SG) were showed in 8-, 10- and 12-week-old NOD mice, while no lymphocyte infiltration was observed in SG of 4-week-old NOD mice. Our results also showed that the salivary flow rate, which is one of the important indexes for SS, decreased significantly by age (20). Peck *et al.* have provided a detailed picture of SS-like pathology and biological markers as it progresses, which was consistent with our study (21). Therefore, in the present study, we initiated total glucosides of paeony treatment at 8-week-old in NOD mice. Female NOD mice (7 weeks old) were allowed to acclimatise for 1 week prior to treatment. The mice were randomly divided into two groups, PBS and TGP treatment. The TGP powder was dissolved in sterilised PBS. The mice in the TGP group were gavaged daily with 400 mg·kg⁻¹TGP solution (400ul) according to previous study (22), and the control mice were gavaged with the same volume of PBS. The mice were sacrificed after treatment for 1 month.

Saliva flow rate measurement

The saliva flow rate of mice was measured according to previous study (23). Briefly, each mouse was intraperitoneally injected with 0.3% pentobarbital sodium. Then, 0.01 mg pilocarpine was injected intraperitoneally to induce saliva secretion. The saliva was collected for 15min at room temperature.

Histological assessment of SG

After mice were sacrificed, SG of mice was collected and immediately fixed in 4% paraformaldehyde. After dehydrating, the paraformaldehyde-fixed tissues were embedded in paraffin. Serial 4µm sections were cut. The SG sections were stained with haematoxylin and eosin (H&E). Images were captured by FSX100 Imaging Device (Olympus, Tokyo, Japan). Histological scores were evaluated by a widely used scoring system (24). The size and degree of lymphocytes in salivary gland tissue are used to assess the severity of

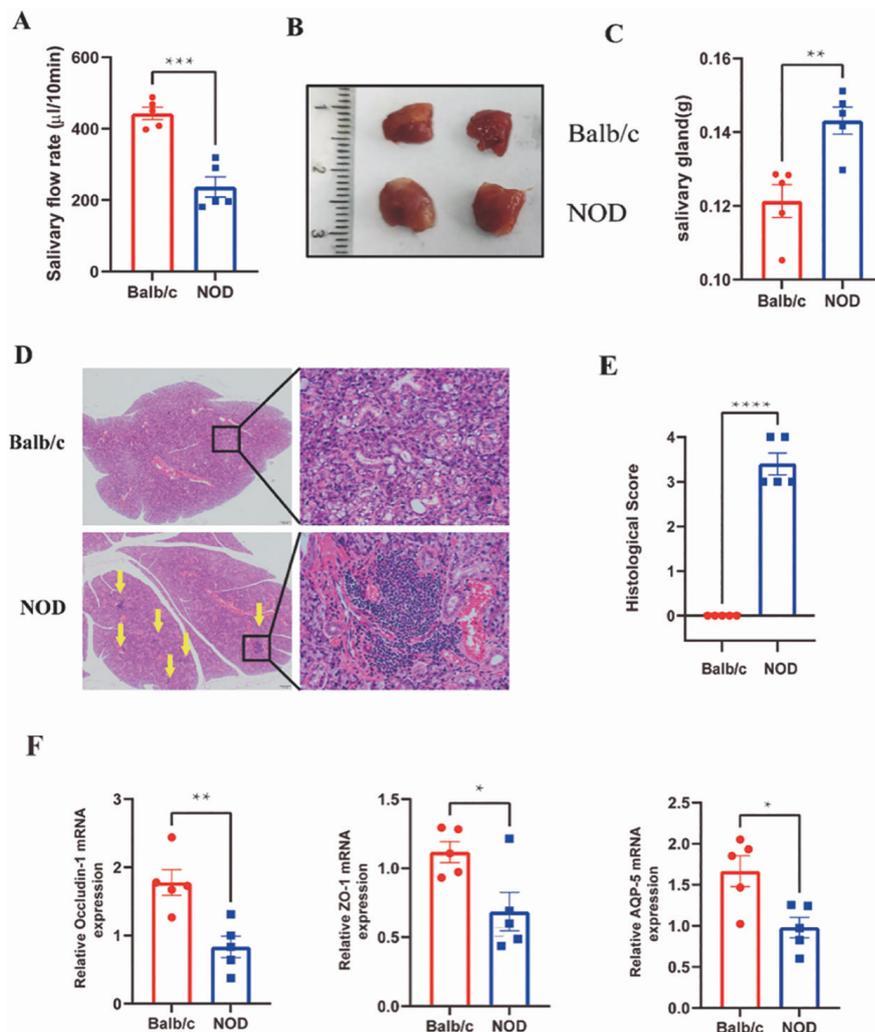


Fig. 1. SS-like symptoms in NOD mice. **A:** Salivary flow rate. **B:** Submandibular gland appearance of mice. **C:** Submandibular gland weight. **D-E:** Histological analysis of the submandibular glands. **F:** The mRNA levels of tight junction molecules and aquaporin 5 in the submandibular gland. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001, n=5.

tissue injury, in which lymphocytic lesions are defined as groups with more than 50 lymphocytes per 4 mm². The grades were as follows: 0=no lymphocytic infiltration, 1=mild lymphocytic infiltration, 2=moderate lymphocytic infiltration, but less than one lymphocytic focus per 4 mm², 3=one lymphocytic focus per 4 mm², 4=more than one lymphocytic focus per 4 mm².

Immunohistochemical staining

Mouse SG sections were deparaffinized in xylene and hydrated in water. After antigen retrieval, SG sections were treated with 3% H₂O₂ for 20 min at room temperature to inactivate the endogenous peroxidase. The sections were blocked with goat serum at room temperature for 30min. Then, SG sec-

tions were incubated with primary antibodies, including NLRP3(1:200, AiFang biological AF300649), Caspase-1 (1:200, ABclonal A0964), and ASC (1:200, ABclonal A1170) at 4°C overnight respectively. The second antibody (horseradish peroxidase labelled goat anti-rabbit immunoglobulin) (1:500, Servicebio GB15001) was added and incubated at room temperature for 1 h. The DAB (AiFang biological AFIHC004, Hunan, China) chromogenic solution was added to slides for staining. Next, the sections were counterstained with haematoxylin and examined by light microscopy.

Quantitative real-time polymerase chain reaction

RNA was extracted from submandibu-

lar gland tissue by Trizol. RNA was reverse transcribed into complementary DNA. The mRNA expression of NLRP3, caspase-1 and ASC was detected by RT-qPCR. The primers (Supplementary Table S1) were designed and synthesised by Genscript Biotech Corporation.

Western blot analysis

Western blot was performed as described previously. Briefly, proteins were extracted from mouse SG in lysis buffer. Then, samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Next, the PVDF membrane was incubated with primary antibodies, and then incubated with secondary antibody. Then, detection was performed with ECL luminescent substrate system and analysed by ImageJ software. Primary antibodies were anti-NLRP3 Rabbit pAb (1:1000, ABclonal A5652, Wuhan, China), anti-Caspase1 Rabbit pAb (1:1000, ABclonal A0964, Wuhan, China), anti-ASC Rabbit mAb (1:1000, Cell Signalling Technology D2W8U Boston, America) and anti-Cleaved-caspase-1 Rabbit mAb (1:1000, Cell Signalling Technology E2G2I Boston, America). Secondary antibody was horseradish peroxidase (HRP)-labelled goat anti-rabbit antibody (ABclonal, Wuhan, China).

Enzyme-linked immunosorbent assay

The IL-1 β and IL-18 in serum and SG homogenate of mice in each group were detected by ELISA kits from ABclonal according to the manufacturer's instruction.

Cell culture

Mouse SG cells were isolated under aseptic condition. The SG cells were inoculated in 6-well plates with 2×10^6 cells per well. The cells were stimulated by lipopolysaccharide (LPS) and adenosine triphosphate (ATP) to activate NLRP3 inflammasome (25). The SG cells were divided into three groups according to treatment: control, LPS+ATP, and LPS+ATP+TGP treatment group. The LPS+ATP group was stimulated with 1 μ g/ml LPS for 3 h, and

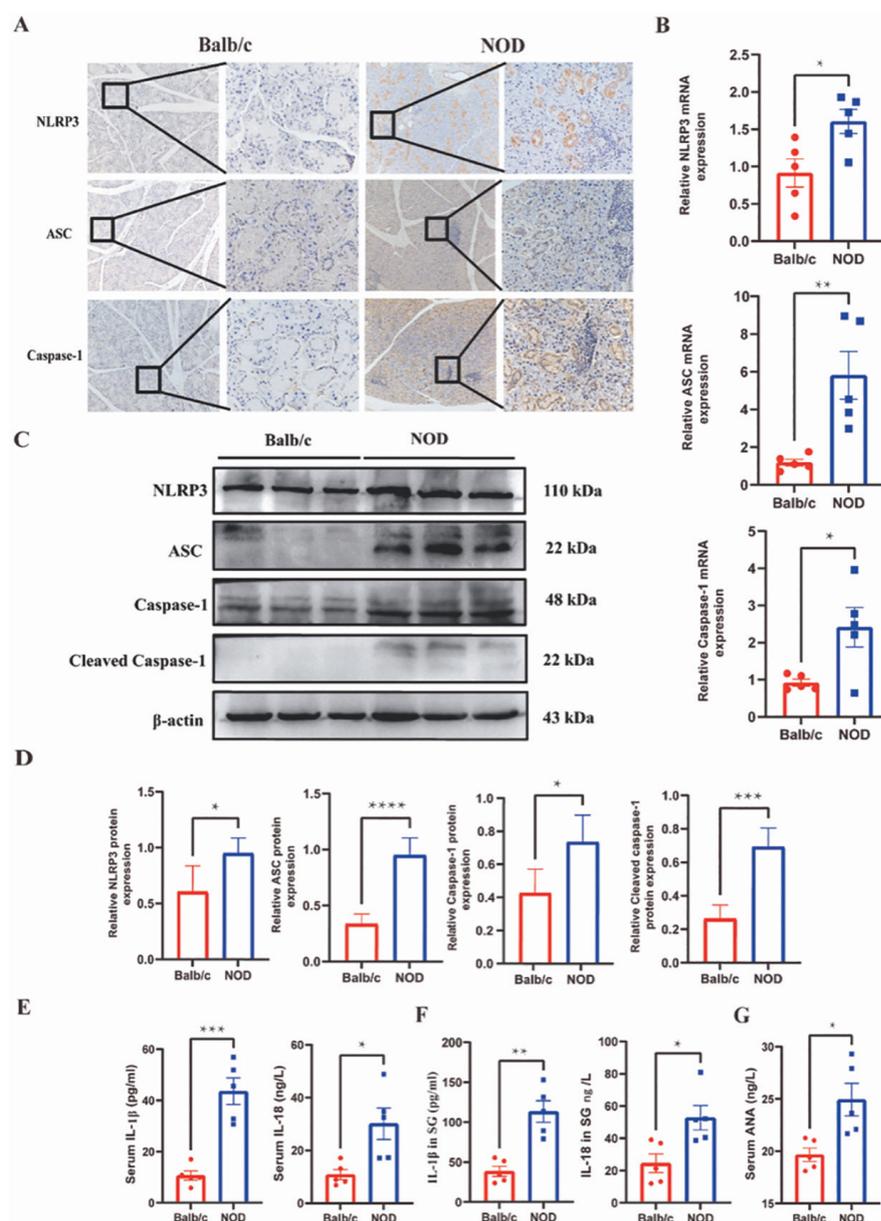


Fig. 2. Activation of NLRP3 inflammasome in the submandibular gland of NOD mice. **A:** Immunohistochemical staining of NLRP3 inflammasome in submandibular gland. **B:** The mRNA levels of NLRP3 inflammasome in submandibular gland. **C-D:** The protein of NLRP3 inflammasome in submandibular gland. **E:** Serum IL-1 β and IL-18, **F:** IL-1 β and IL-18 in homogenate of submandibular gland. **G:** Serum ANA.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 5$.

then stimulated with 5mmol/L ATP for 30 m. The cells in the LPS+ATP+TGP group were treated with 1 μ g/ml LPS and 100 μ g/ml TGP (Suppl. Fig. 1 A-B), and then incubated 5mmol/L ATP for 30 min.

Statistical analysis

All data are presented as mean \pm SEM. All statistical analyses were carried out with GraphPad Prism9 software (GraphPad Software, La Jolla, CA, USA). For two values sets comparison,

Student's t-tests were used. For multiple comparisons, one-way ANOVA followed by Bonferroni's test were performed. $p < 0.05$ was considered a significant difference.

Results

SS-like symptoms in NOD mice

We first determined the SS-like symptoms in 8-week-old NOD mice. We found that spleen and body weight did not show significantly difference between 12-week-old female NOD and

Balb/c mice (Suppl. Fig. 2 A-B). However, saliva flow decreased, and the weight of SG increased in NOD mice compared with Balb/c mice (Fig. 1 A-C). There was severe lymphocyte infiltration in SG of NOD mice, while no lymphocyte infiltration was observed in SG of Balb/c mice (Fig. 1 D-E). Furthermore, Occludin-1, zo-1 and aquaporin-5 were decreased in SG in NOD mice (Fig. 1 F). Collectively, these observations indicated that 8-week-old female NOD mice displayed typical SS symptoms.

Activation of NLRP3 inflammasome in SG of NOD mice

Several lines of evidence have demonstrated that aberrant activation of NLRP3 inflammasomes contribute to pathogenesis of SS (26-28). However, whether NLRP3 activation was involved in SG damage in SS remains to be elucidated. Thus, we examined the NLRP3 inflammasome in the SG of NOD mice. Results of immunohistochemistry showed NLRP3 inflammasome in SG of NOD mice, while no NLRP3 inflammasome in SG of Balb/c mice (Fig. 2A). Furthermore, mRNA expressions of NLRP3, ASC and caspase-1 in SG of NOD mice were significantly higher than those of Balb/c mice (Fig. 2 B-D). In addition, IL-1 β and IL-18 in serum and homogenate of SG from NOD mice were significantly higher than those of Balb/c mice (Fig. 2E-F). Anti-nuclear antibody (ANA) in serum of NOD mice was also significantly increased (Fig. 2G). Taken together, these results indicated that NLRP3 inflammasome activation is augmented in SG of NOD mice.

TGP alleviates SS-like symptoms in NOD mice

Given that TGP can effectively treat autoimmune diseases (29,30), we next examine the effects of TGP on SS mice. We found that saliva flow was significantly increased, and the weight of SG was significantly decreased in NOD mice with TGP treatment (Fig. 3A-C).

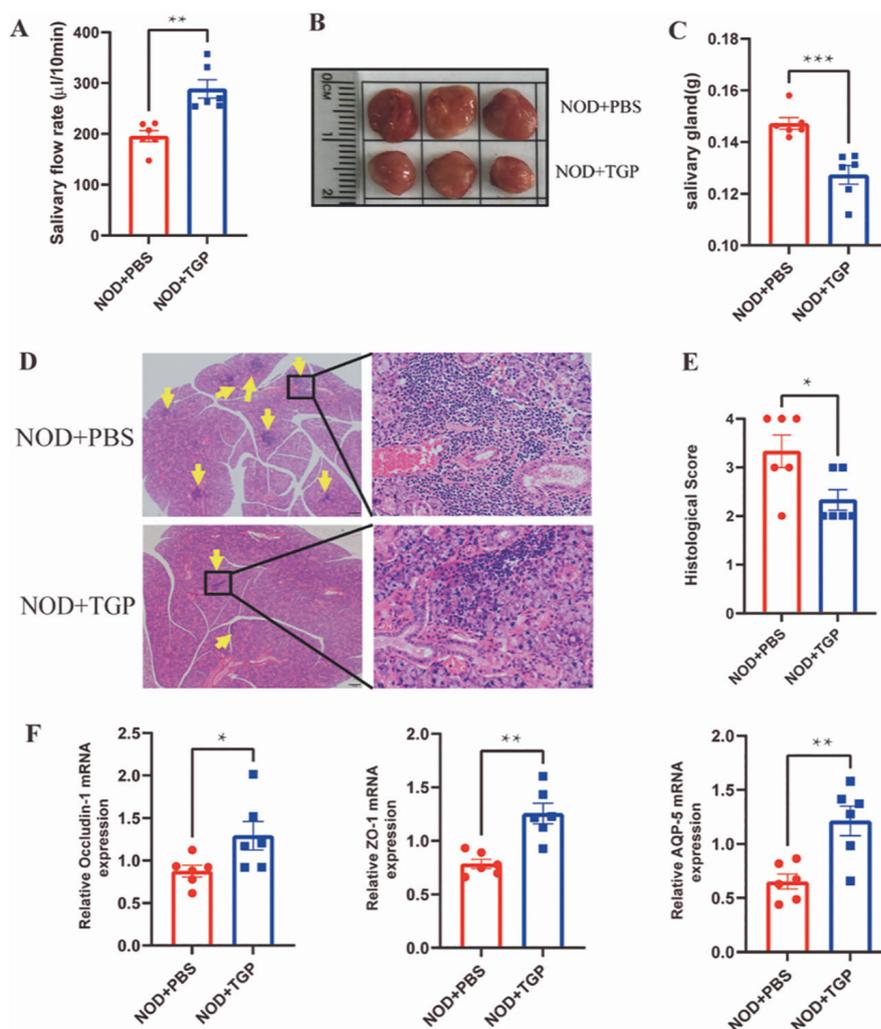


Fig. 3. TGP improves SS-like symptoms in mice. **A:** salivary flow rate. **B:** submandibular gland. **C:** Submandibular gland weight. **D-E:** Histological analysis of the submandibular glands. **F:** The mRNA levels of tight junction molecules and aquaporin 5 in the submandibular gland. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 6$.

However, there was no significant difference in spleen and body weight of mice between PBS and TGP treatment group (Suppl. Fig. 2A-B). In addition, we observed that the lymphocyte infiltration in the SG tissue of the mice in TGP group was significantly reduced (Fig. 3D-E). Furthermore, we found that occludin-1, zo-1 and aquaporin-5 in SG tissues of mice in TGP group were significantly increased compared with PBS group (Fig. 3F). These findings indicated that TGP effectively alleviated SS-like symptoms in NOD mice.

TGP inhibited NLRP3 inflammasome activation in SG of NOD mice

Next, we aimed to elucidate the mechanism by which TGP mitigated SS. Previous study demonstrated that SS-like

symptoms in NOD mice is related to activation of NLRP3 inflammasome (31). Herein, we detected NLRP3 inflammasome in SG of NOD mice after TGP treatment. Immunostaining of NLRP3 hinted that NLRP3 inflammasome in SG of TGP-treated mice is lower than that of PBS-treated mice (Fig. 4A). The results of RT-qPCR showed that NLRP3, ASC, and caspase-1 mRNA was significantly decreased in SG of NOD mice after TGP treatment (Fig. 4 B). Similarly, western blot analysis confirmed that NLRP3, ASC, Caspase-1 and cleaved-caspase-1 in SG of mice in TGP group were significantly down-regulated (Fig. 4C-D). In addition, serum ANA was significantly decreased after TGP treatment (Fig. 4E). As expected, IL-1 β and IL-18 in serum and homogenate of SG were

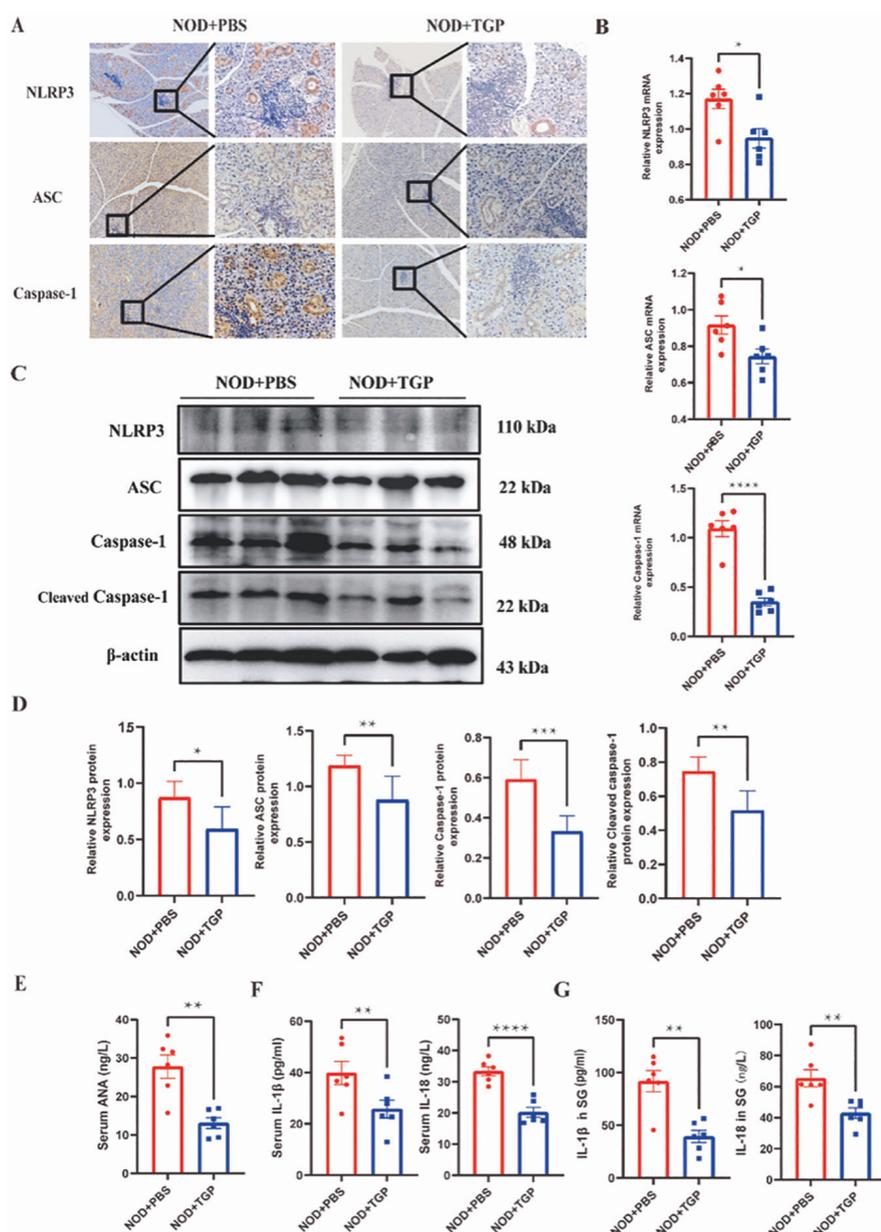


Fig. 4. TGP reduced NLRP3 inflammasome activation in the submandibular gland of NOD mice. **A:** Immunohistochemical staining of NLRP3 inflammasome in submandibular gland. **B:** The mRNA levels of NLRP3 inflammasome in submandibular gland. **C-D:** The protein of NLRP3 inflammasome in submandibular gland. **E:** Serum ANA. **F:** Serum IL-1 β and IL-18. **G:** IL-1 β and IL-18 in homogenate of submandibular gland. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 6$.

significantly reduced after TGP treatment (Fig. 4F-G). Collectively, these findings suggested that TGP inhibited activation of NLRP3 inflammasome of SG inf SS mice.

TGP inhibited NLRP3 inflammasome activation in SG cells *in vitro*

The inhibition of TGP on NLRP3 inflammasome activation *in vivo* promoted us to determine the effects of TGP on SG cells *in vitro*. SG cells from

NOD mice were isolated and cultured. The concentration of TGP we selected was based on the effect of TGP in the viability of SG cells (Suppl. Fig. 1A-B). We found that the NLRP3, ASC and Caspase-1 mRNA in the SG cells were significantly increased after stimulation by LPS+ATP. Remarkably, the overactivation of NLRP3 inflammasome (hallmarked by NLRP3, ASC and Caspase-1) in the SG cells inducing by LPS+ATP were significantly reversed

by TGP treatment (Fig. 5A). Consistently, western blot analysis showed that protein of NLRP3, ASC, caspase-1 and cleaved-caspase-1 in SG cells in the LPS+ATP group were significantly increased, while the effects were abrogated by TGP treatment (Fig. 5B-C). In addition, we observed that IL-1 β and IL-18 in the supernatant of SG cells in the LPS+ATP group were significantly increased, while the increase of IL-1 β and IL-18 was partially restored by TGP (Fig. 5D). These data suggested that TGP inhibited the activation of NLRP3 inflammasome in SG cells *in vitro*.

Discussion

The exact aetiology and pathogenesis of SS are not clear (32). So far, glucocorticoids and immunosuppressants are the most frequently treatments for SS. The major limitation of these approaches is increased risks of severe side effects (33). In recent years, the effects of traditional Chinese medicine in treating SS have gradually been confirmed in clinical practice. TGP exhibits potent immunomodulatory properties and less side effects (34, 35), suggesting certain advantages and broad prospects in the treatment of SS (36, 37). However, the molecular pathways responsible for the therapeutic effects of TGP in SS remain incompletely understood. Herein, we demonstrated that NLRP3 inflammasome activation in SG of SS mice. Notably, we found that TGP ameliorated SS-like symptoms in NOD mice through inhibiting NLRP3 inflammasome activation in SG.

Inflammasome is an intracellular protein complex stimulated by pathogen-associated molecular patterns or danger-associated molecular patterns (38). The complex is present in the cytoplasm of immune cells such as monocytes, macrophages, and dendritic cells. Up to now, several inflammasome subtypes have been identified, the most extensively studied of which is the NLRP3 inflammasome (39, 40). NLRP3 inflammasome activation has been demonstrated to be involved in the progress of various autoimmune diseases. The activation of NLRP3 inflammasome is closely related to disease activity. Inhibition of NLRP3 inflammasome

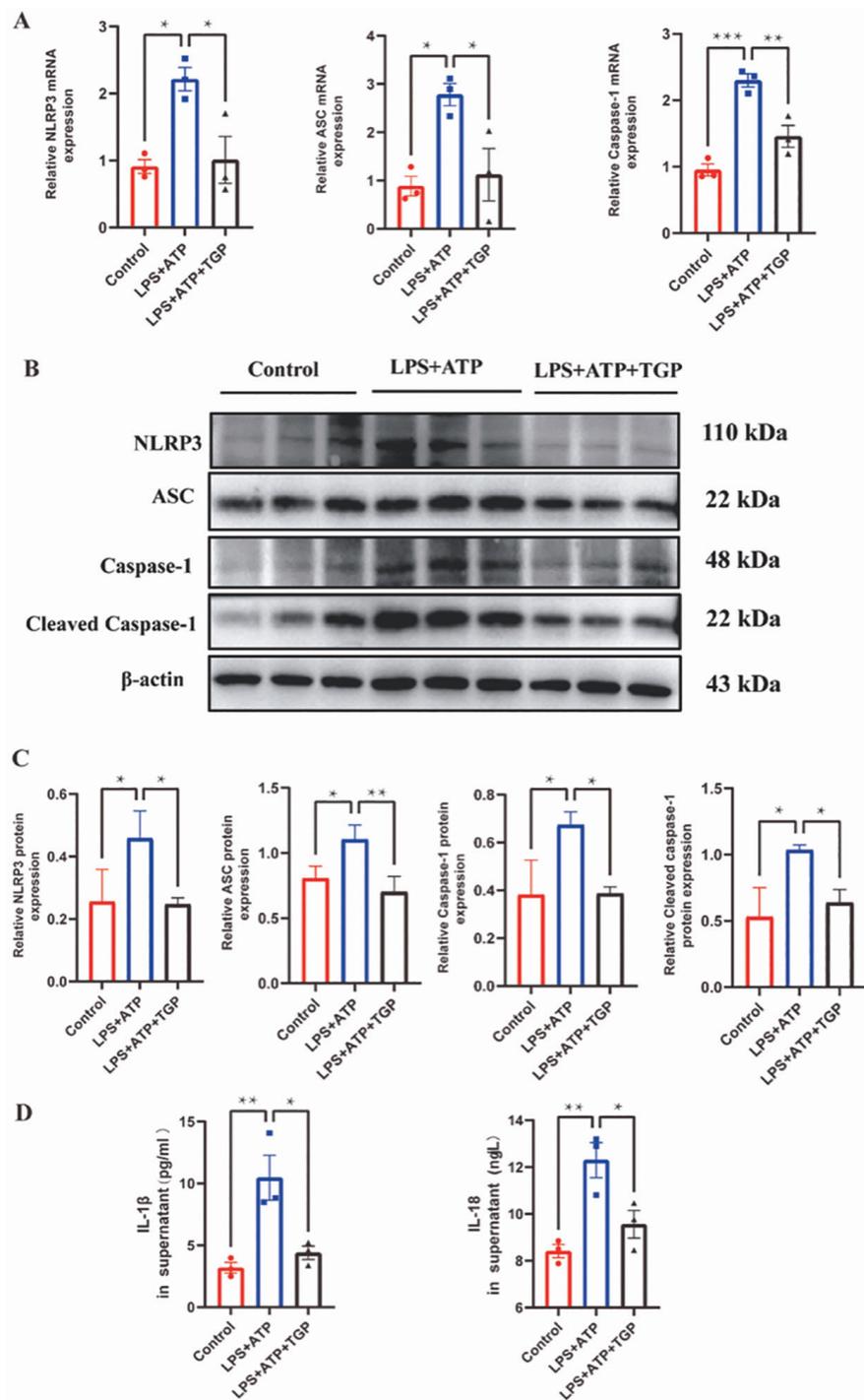


Fig. 5. TGP inhibited NLRP3 inflammasome activation in submandibular gland cells *in vitro*. **A:** The mRNA NLRP3 inflammasome in submandibular gland cells. **B-C:** The protein of NLRP3 inflammasome in submandibular gland cells. **D:** IL-1β and IL-18 in supernatant of submandibular gland cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$.

activation can delay the disease process (41). Previous study has shown that P2X7 could activate NLRP3 inflammasome. NLRP3 was not activated in P2X7-deficient mice in a mouse model of autoimmune exocrine disease. And P2X7 receptor antagonists reduced salivary gland inflammation and increase

salivary secretion in SS mice (42). In another study, inhibiting the activation of NLRP3 inflammasome reduced apoptosis and inflammatory response of SG cells in NOD/ShiLtJ mice, thereby improving SS-like symptoms in mice (18). Our results showed that NLRP3 inflammasome activation was augmented

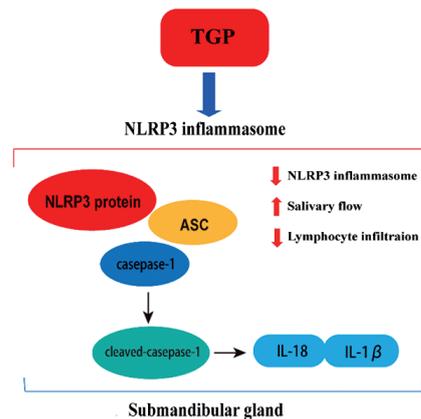


Fig. 6. Scheme of TGP inhibits the activation of NLRP3 inflammasome in submandibular gland of SS model mice. In SS, NLRP3 inflammasome is overactivated in submandibular gland. TGP reduces the production of IL-18 and IL1β by down-regulating NLRP3, ASC and caspase-1 in submandibular gland, thereby alleviating SS-like symptoms.

ed in SG of SS mice. Given SG cells contribute to salivary flow rate and was injured in SS, our results provided direct evidence of NLRP3 inflammasome activation on pathogenesis of SS. To our knowledge, our data demonstrated for the first time excessive inflammasome activation in SG of mice with SS-like symptoms.

Generally speaking, majority of patients with SS only manifest dry eye and dry mouth-related symptoms. Current treatment of SS patients includes local tear and saliva substitutes, and immunosuppressants. However, these therapeutic strategies are frequently ineffective. Therefore, novel therapeutic approaches are needed for treatment of SS. Previous studies demonstrated that TGP has been used for treatment of autoimmune diseases, including SS. In a clinical trial on primary SS, TGP can down-regulate the expression of programmed death-1 on the surface of Treg cells, then enhance the immunosuppressive function of Treg cells, inhibit the activation of T and B lymphocytes and the secretion of autoantibodies, thereby relieve the symptoms of the disease (43). In the present study, the saliva flow of mice in the TGP treatment group was significantly increased, and the lymphocyte infiltration in the SG tissue and serum ANA antibodies was significantly reduced, indicating that TGP can alleviate the symptoms

of SS. In consistent with our results, previous study has shown that TGP can reduce the inflammatory response in NOD mice, and reduce the concentration of cytokines and autoantigens in serum and SG, which in turn alleviated the SS-like symptoms (44). In addition, in SS model mice, TGP can improve mouse constipation by regulating neurotransmitters, and at the same time inhibit intestinal inflammation in mice by regulating the concentration of immunomodulatory peptides (22).

Although TGP has been used clinically to treat SS patients, the mechanism by which TGP alleviates SS is still unclear. In this study, compared with NOD mice in PBS group, The NLRP3 inflammasome in the SG of NOD mice in the TGP group was significantly reduced, indicating that TGP can inhibit the NLRP3 inflammasome in the submandibular gland. The *in vitro* experiments also indicated that TGP reduced NLRP3 inflammasome activation. As reported in other *in vitro* experimental systems, macrophage inflammation in gouty arthritis was simulated *in vitro*, and TGP was found to inhibit NLRP3 inflammasome activation in macrophages (45). These results are consistent with our findings, demonstrating that TGP had inhibitive effects on NLRP3 inflammasome activation.

There are some limitations to the interpretation of our results. First, in our study, mouse submandibular gland cells were prepared according to previous studies (46-48). The epithelium, fibroblasts were not able to be separated by this isolation method. Therefore, the effects of TGP were not seen in epithelium or fibroblasts only. Secondly, in the present study, TGP (400 mg·kg⁻¹) was administered intragastrically in NOD mice every day for 4 weeks. After the last administration, the mice were sacrificed. We did not determine the therapeutic effects of TGP in NOD mice for long time after the last administration. The study by Li *et al.* has demonstrated that pathological damage of submandibular gland in NOD mice was alleviated after TGP treatment for 2 weeks (44). In their study, TGP was intragastrically administrated for 6 and 16 weeks on NOD mice. The therapeutic

effects were evaluated shortly after last administration. Their results indicated that the therapeutic effects of TGP on SS in NOD mice were observed after 2-week treatment. Therefore, it is conceived that the beneficial effects of TGP on SS mice gradually faded without maintaining TGP treatment.

Conclusion

In summary, our findings indicated that SG cells showed excessive NLRP3 inflammasome activation, other than an injured target in SS. We demonstrated that TGP alleviated the symptoms of experimental SS. Our data highlight that the beneficial effects of TGP on SS is related to limit overactivation of NLRP3 inflammasome of SG. These findings not only reveal the new mechanism of TGP in the treatment of SS, but also provide new evidence for the further application of TGP in the treatment of SS.

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