Total glucosides of peony ameliorates Sjögren's syndrome by affecting Th1/Th2 cytokine balance

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Abstract. The present study aimed to investigate the molecular mechanisms underlying the effects of total glucosides of peony (TGP) in the treatment of Sjögren's syndrome (SS). A total of 40 mice with SS were evenly assigned into four groups, including: Control group; TGP group, receiving 1 mg TGP daily; hydroxychloroquine (HCQ) group, receiving 0.25 mg HCQ daily; and a combined group, receiving 1 mg TGP and 0.25 mg HCQ daily. After 8 weeks, quantitative polymerase chain reaction and an enzyme-linked immunosorbent assay were used to detect the levels of interferon-γ (IFN-γ), interleukin-4 (IL-4), Fas and FasL in each group of mice. In addition, immunohistochemical analysis was used to determine the expression levels of IFN-γ and IL-4. IFN-γ, IL-4, Fas and FasL levels were significantly increased in the control group compared with the other three groups ($P<0.05$). Furthermore, the expression levels of these factors were reduced in the combined group in comparison with the HCQ group ($P<0.05$). The ratios of IFN-γ to IL-4 were decreased in the TGP and combined groups compared with the control group ($P<0.05$). The present results indicate that TGP ameliorates SS by affecting the Th1/Th2 cytokine balance and decreasing the expression levels of IFN-γ, IL-4, Fas and FasL. Therefore, TGP may represent a potential novel therapeutic agent for the treatment of SS.

Introduction

Total glucosides of peony (TGP) are the primary active ingredients of *Paeoniaceae paeonia* and have been widely used in the treatment of Sjögren's syndrome (SS) (1,2). Previous studies have demonstrated that TGP is able to attenuate the symptoms exhibited by patients with SS, including a dry mouth and eyes, and the SS-associated decrease in erythrocyte sedimentation rate (ESR) (3-5). However, the molecular mechanisms underlying the activity of TGP in SS therapy remain unclear. The levels of Th1-secreted interferon-γ (IFN-γ) and Th2-secreted interleukin-4 (IL-4) are positively correlated with the extent of lymphocytic infiltration in the labial glands of patients with SS (6), and the ratio of IFN-γ to IL-4 indicates the Th1/Th2 cytokine balance (7).

The Fas and Fas ligand (FasL) system is an important signaling pathway for inducing apoptosis in cells (8), and Fas/FasL-mediated apoptosis is reported to be associated with cancer cell death (9). Fas antigen and its ligand FasL have an important role in peripheral T cell clearance and the cytotoxicity of cytotoxic T lymphocytes (CTLs) (10). It is established that activated T cells and CTLs are critical for modulation of the immune response, meaning that the function of Fas and FasL are also crucial to immune reactions (11).

Furthermore, the levels of Fas and FasL in the submandibular glands have been observed to be significantly increased in SS mice compared with control mice (12). In the labial glands of patients with SS, the levels of Fas and FasL are associated with IFN-γ and IL-4 levels and are positively correlated with lymphocytic infiltration (13). Previous results thus indicate that an increase in the levels of IFN-γ, IL-4, Fas and FasL may be crucially involved in the pathogenesis of SS.

To clarify the molecular mechanism underlying the efficacy of TGP for the treatment of SS, the present study evaluated the effects of TGP on the levels of IFN-γ, IL-4, Fas and FasL in serum and in the submandibular glands in an SS mouse model.

Materials and methods

Animals. A total of 40 female, 8-week-old, non-obese (weight, 25-35 g) diabetic (NOD) mice with SS were provided by Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). The diets of all mice comprised 14% casein, 47% cornstarch, 15% gelatinized cornstarch, 10% sugar, 4% soybean oil, and 10% other (including fiber, vitamins and minerals). All animals were maintained at 24˚C in 60% humidity in a pathogen-free environment. All the protocols in present study were approved by ethical committee of The First

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Key words: total glucosides of peony, Sjögren's syndrome, NOD mice, Th1/Th2, Fas, FasL

Table I. Protein levels of IFN-γ and IL-4 in NOD mice, expressed as the mean ± standard deviation.

	IFN-γ, pg/ml		IL-4, pg/ml	
Groups	Serum	SG	Serum	SG
Control	393.62±10.16	478.23±12.35	66.36±3.85	367.67±6.13
TGP	230.50±23.65[a,b]	240.24±11.67[a]	48.79±5.26[a]	180.21±7.96[a]
HCQ	282.87±9.54[a]	205.18±12.63[a]	53.55±6.04[a]	210.44±8.74[a]
Combined	200.68±21.64[a,b]	112.71±15.31[a]	44.16±5.88[a,b]	133.65±6.05[a,b]

n=10 for each group. [a]P<0.05 vs. control group; [b]P<0.05 vs. HCQ group. IFN-γ, interferon-γ; IL-4, interleukin-4; SG, submandibular gland; TGP, total glucosides of peony; HCQ, hydroxychloroquine.

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A total of 40 NOD mice with SS were randomly assigned into 4 groups of 10 mice, as follows: i) Control group, which was fed normally; ii) TGP group, receiving 1 mg TGP per day; iii) HCQ group, receiving 0.25 mg hydroxychloroquine (HCQ) per day; and the iv) combined group, receiving 1 mg TGP plus 0.25 mg HCQ per day. After 8 weeks, blood samples were obtained from the femoral artery, the mice were euthanized by intraperitoneal injection of urethane (2.0 g/kg; Sigma-Aldrich, St. Louis, MO, USA) and their submandibular glands were isolated for evaluation. All animal experiments complied with the experimental animal ethics guidelines of China Zoological Society.

Drugs. TGP tablets (Ningbo Lihua Pharmaceuticals Co. Ltd., Ningbo, China) were diluted in distilled water at 15 mg/ml. HCQ tablets (Shanghai Zhongxi Pharmaceutical Co., Ltd., Shanghai, China) were diluted in distilled water at 3 mg/ml.

Reagents. ELISA kits (Shanghai Langdun Biotechnology, Shanghai, China) were used to detect mouse IFN-γ and IL-4 (cat. no. BP-E60055). The following primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA): goat polyclonal anti-IFN-γ (dilution, 1:1,000; cat. no. sc-9344), goat polyclonal anti-IL-4 (dilution, 1:1,000; cat. no. sc-1260), rabbit polyclonal anti-Fas (dilution, 1:2,000; cat. no. sc-716) and rabbit polyclonal anti-FasL (dilution, 1:2,000; cat. no. sc-956). Biotinylated goat anti-rabbit (cat. no. SP-9001) and biotinylated mouse anti-goat (cat. no. PV-9003) secondary antibodies (dilution, 1:2,000; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) were used to visualize these. A DAB substrate kit (OriGene Technologies, Inc., Beijing, China) was used to detect chemiluminescence. *In situ* hybridization detection kits were used in the detection of Fas and FasL (Wuhan Boshide Biological Engineering Co., Ltd., Wuhan, China).

ELISA analysis of IFN-γ and IL-4 protein levels in serum. Blood samples were placed in serum separator tubes, maintained at room temperature for 30 min then centrifuged for 15 min at 1,400 x g. Serum was transferred into a 1.5-ml centrifuge tube and stored at 4˚C. The levels of IFN-γ and IL-4 in the serum samples were determined using EM1001 and EMIL4 ELISA kits, respectively (Thermo Fisher Scientific, Inc., Waltham, MA, USA), in accordance with the manufacturer's instructions.

Immunohistochemical streptavidin-peroxidase (S-P) assay to evaluate IFN-γ, IL-4, Fas and FasL expression in submandibular glands. IFN-γ, IL-4, Fas and FasL protein levels in the mouse submandibular glands were determined using an immunohistochemical S-P kit (cat. no. 21124; Pierce Biotechnology, Inc., Rockford, IL, USA), in accordance with the manufacturer's instructions. Image analysis was performed using a true color multifunctional cell image analysis management system in Image-Pro Plus 7.0 (Media Cybernetics, Inc., Rockville, MD, USA). The optical densities of five randomly-selected fields were measured and their average values were used for statistical analysis.

Quantitative polymerase chain reaction (qPCR) analysis of Fas and FasL mRNA levels in submandibular glands. Briefly, total RNA was extracted using TRIzol (Beijing TransGen Biotech Co., Ltd., Beijing, China) and DNase (final concentration, 10 mM; Takara Biotechnology Co., Ltd.), and cDNA was synthesized from 1.5 μg of total RNA using a Takara RNA PCR kit (AMV) v. 3.0 (Takara Biotechnology Co., Ltd.). This reverse transcription was performed in one cycle, as follows: 30˚C for 10 min; 42˚C for 30 min; 95˚C for 5 min; and 4˚C for 5 min. qPCR was performed using 10 μl Premix Ex Taq PCR probes (2X concentration; cat. no. RR390A; Takara Biotechnology Co., Ltd.), 1 μl cDNA and 9 μl ddH_2O in a 200-μl PCR tube, in accordance with the manufacturer's instructions, with GAPDH as an internal control. Primers were synthesized as follows: Fas, forward 5'-AGGCCGCCCGCTGTTTTC-3' and reverse 5'-ACGAACCCGCCTCCTCAGC-3' (product length, 145 bp); FasL, forward 5'-GCCGCCACTGACCCC TCTAA-3' and reverse 5'-CCACACTCCTCGGCTCTTTT-3' (product length, 242 bp); and GAPDH, forward 5'-AAATGG TGAAGGTCGGTGTG-3' and reverse 5'-TGAAGGGGTCGT TGATGG-3' (product length, 108 bp). PCR was conducted as follows: Incubation at 94˚C for 5 min, followed by 40 cycles at 94˚C for 15 sec and at 60˚C for 45 sec. The fluorescence value was determined at 60˚C. These reactions were all conducted using an AriaMx Realtime PCR System (cat. no. G8830A; Agilent Technologies, Inc., Santa Clara, CA, USA).

An identical quantity of control RNA, templates and Premix Ex Taq PCR probes was used across all reactions. The average mean threshold cycle (C_q value) was obtained

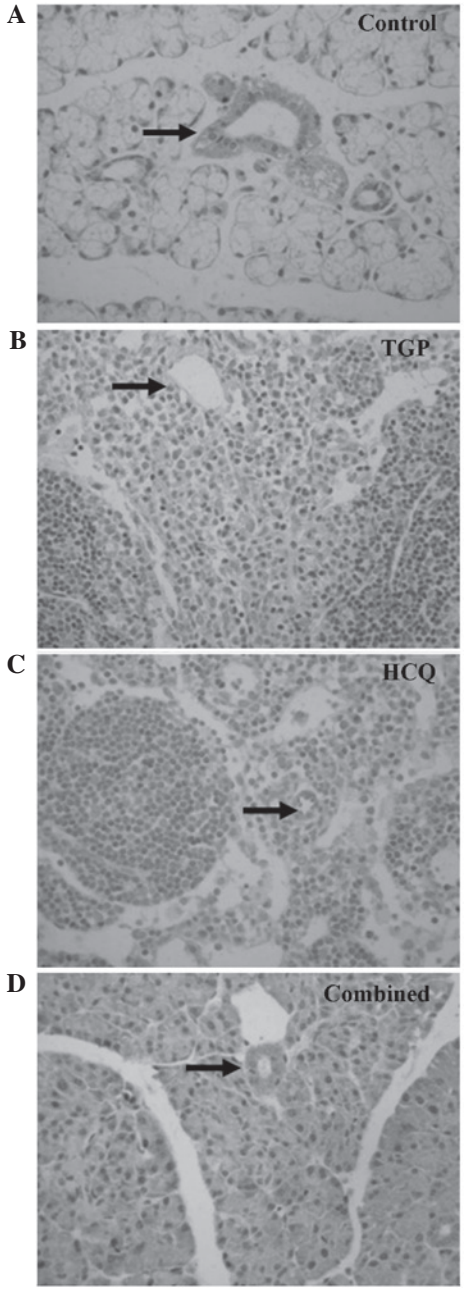

Figure 1. Immunostaining of interferon-γ (indicated by arrows) in submandibular glands of (A) control (magnification, x400), (B) TGP (magnification, x200), (C) HCQ (magnification, x100) and (D) combined treatment group mice (magnification, x200). TGP, total glucosides of peony; HCQ, hydroxychloroquine.

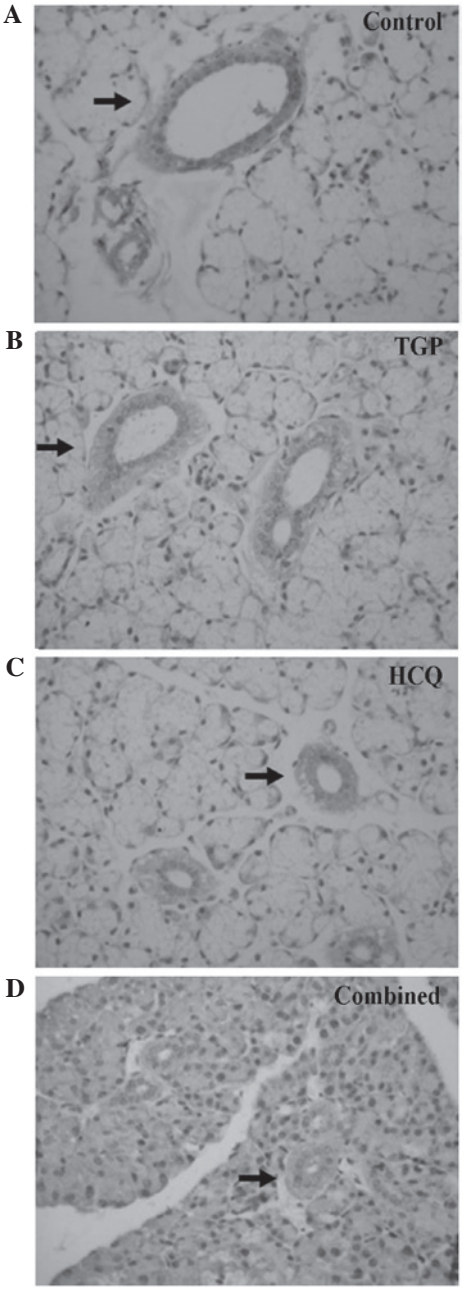

Figure 2. Immunostaining of interleukin-4 (indicated by arrows) in submandibular glands of (A) control (magnification, x400), (B) TGP, (C) HCQ and (D) combined treatment group mice (magnification, x200). TGP, total glucosides of peony; HCQ, hydroxychloroquine.

and data were analyzed using the relative quantitative analysis method (14). The $2^{-\Delta\Delta Cq}$ method was used to calculate relative mRNA level, and ΔC_q values were used for statistical analysis. The amplification and melting curves were analyzed using the AriaMx Realtime PCR System. The control untreated group and was used as a basis of comparison for the other groups.

In the present study, $\Delta C_q = C_{q\ target\ gene} - C_{q\ internal\ reference\ gene}$, and $\Delta\Delta C_q = [(C_{q\ target\ gene} - C_{q\ internal\ reference\ gene})$ in the experimental group] - $[(C_{q\ target\ gene} - C_{q\ internal\ reference\ gene})$ in the control group]. A smaller ΔC_q indicates a higher expression level of Fas and FasL mRNA in the submandibular gland tissue. A higher $2^{-\Delta\Delta Cq}$ indicates a greater difference in Fas and FasL mRNA expression between the experimental and control groups.

Statistical analysis. Statistical analysis was performed using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. One-way analysis of variance was used to compare the groups, and the Student-Newman-Keuls method was used for post-hoc analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TGP reduces IFN-γ and IL-4 protein levels in NOD mice. As indicated in Table I, the levels of serum IFN-γ and IL-4 in NOD mice were significantly higher in the control group

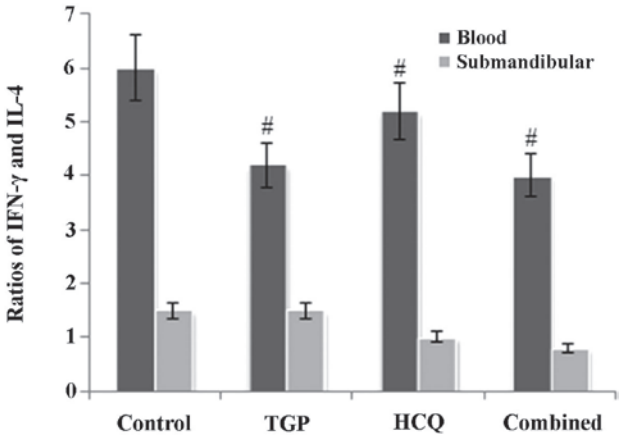

Figure 3. Ratio of IFN-γ/IL-4 in serum and submandibular glands in mice. #P<0.05 vs. control group. n=10 in each group. IFN-γ, interferon-γ; IL-4, interleukin-4; TGP, total glucosides of peony; HCQ, hydroxychloroquine.

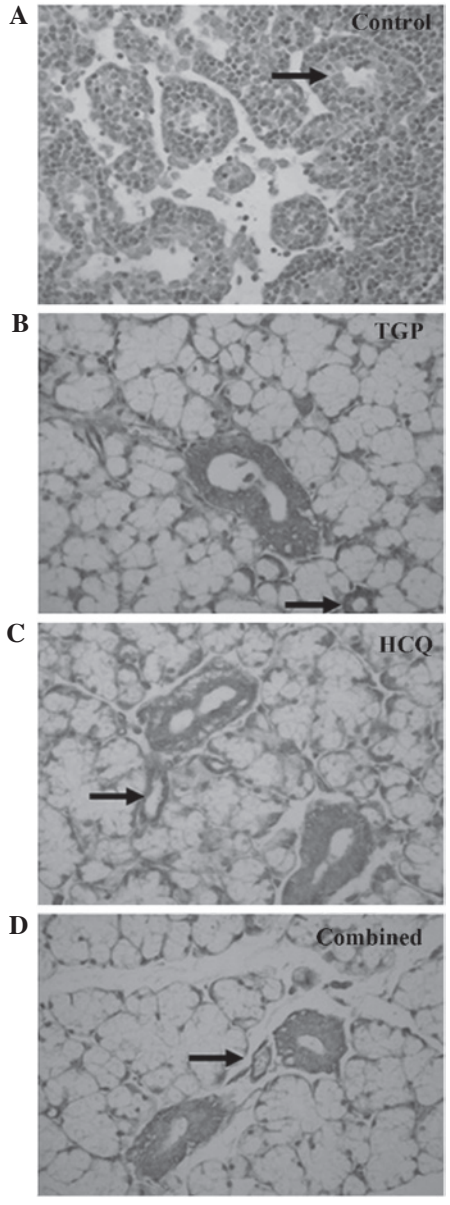

Figure 4. Immunostaining of Fas (indicated by arrows) in submandibular glands of (A) control (magnification, x100), (B) TGP, (C) HCQ and (D) combined treatment group mice (magnification, x400). TGP, total glucosides of peony; HCQ, hydroxychloroquine.

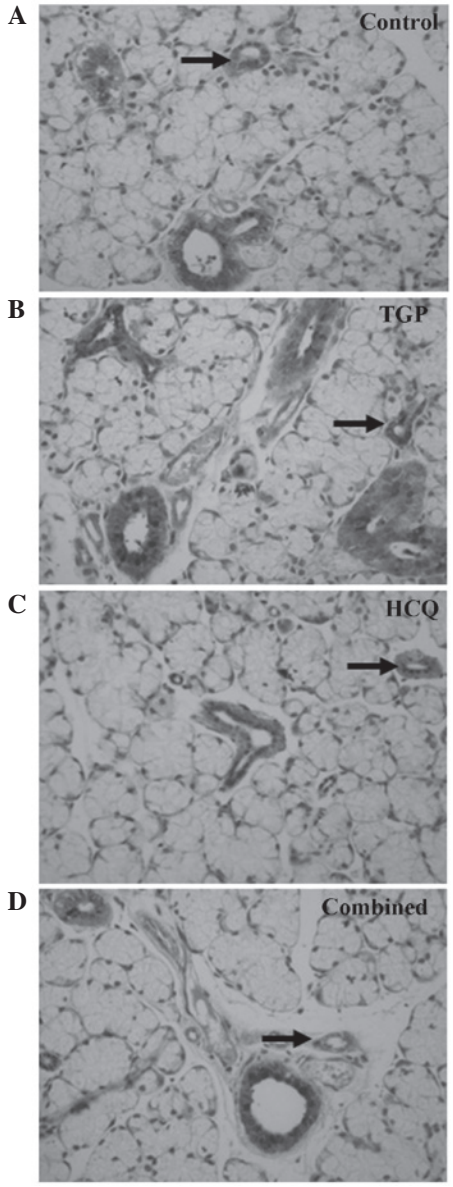

Figure 5. Immunostaining of FasL (indicated by arrows) in submandibular glands of (A) control, (B) TGP, (C) HCQ and (D) combined treatment group mice (magnification, x400). TGP, total glucosides of peony; HCQ, hydroxychloroquine.

compared with the other groups (P<0.05). The levels of IFN-γ in the TGP and combined groups were reduced compared with the HCQ group (P<0.05). The levels of IFN-γ and IL-4 in the submandibular glands were increased in the control group compared with the other groups (P<0.05). Furthermore, the levels of IL-4 were significantly reduced in the serum and the submandibular glands in the combined group compared with the HCQ group (P<0.05).

As indicated by the immunohistochemical S-P assay (Figs. 1 and 2), particles of intense IFN-γ staining were present in the apical and basolateral membranes of the submandibular gland acini, the intercalated ducts and the duct epithelia in the control NOD mice. An increased number of these IFN-γ particles were present in the submandibular glands of the control group compared with the other groups, and fewer particles were present in a number of the intercalated ducts, duct epithelia and gland cells of the HCQ group.

Table II. Relative mRNA levels of Fas and FasL in mouse submandibular glands

	ΔC_q, mean ± SD		$\Delta\Delta C_q$, mean ± SD		$2^{-\Delta\Delta Cq}$	
Group	Fas	FasL	Fas	FasL	Fas	FasL
Combined	6.91±1.23[a,b]	10.13±1.03[a,b]	0.00±1.74	0.00±1.46	1.000	1.000
HCQ	12.53±1.87	13.93±2.36	5.62±2.24	3.80±2.57	0.020	0.072
TGP	9.23±1.35[a,b]	11.38±1.69[a]	2.32±1.83	1.25±1098	0.200	0.420
Control	13.26±1.68	15.25±3.18	6.35±2.08	5.12±3.34	0.012	0.029

n=10 in each group. [a]P<0.05, vs. control group; [b]P<0.05 vs. HCQ group. SD, standard deviation; TGP, total glucosides of peony; HCQ, hydroxychloroquine.

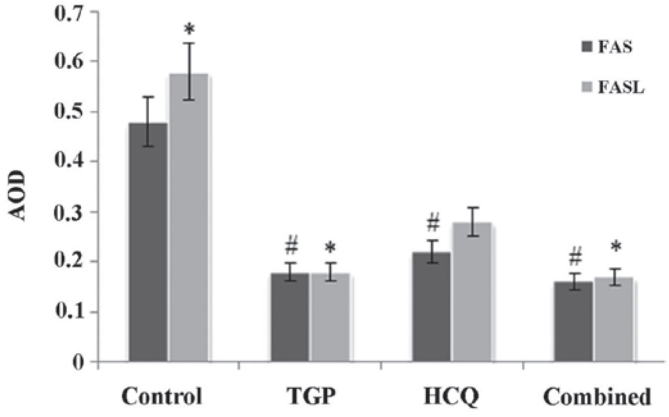

Figure 6. Fas and FasL levels in mouse submandibular glands. #P<0.05 vs. control group; *P<0.05 vs. HCQ group. n=10 in each group. AOD, average optical density; TGP, total glucosides of peony; HCQ, hydroxychloroquine.

However, the IFN-γ staining in the HCQ-treated group was more intense compared with the combined group. Marked expression of IL-4 particles was detected in the basement membrane of the submandibular gland acini and ducts in the control group. Diffuse staining was observed in the basement membrane of submandibular gland acini and ducts in all groups, with less intense expression observed in the combined and TGP groups.

TGP reduces the ratio of IFN-γ to IL-4 in NOD mice. As reported in Fig. 3, the ratio of IFN-γ to IL-4 in mouse serum was higher in the control group compared with the other groups (P<0.05). In addition, the ratio of IFN-γ to IL-4 in the submandibular glands of the control group mice was increased compared with the other groups; however, this difference was not statistically significant (P>0.05).

TGP reduces Fas and FasL protein levels in mouse submandibular glands. Fas and FasL expression was detected using immunohistochemical staining (Figs. 4 and 5). High levels of Fas and FasL were observed in the cytoplasm and membrane of submandibular gland cells and in the duct epithelia of the control mice. Diffuse expression of Fas and FasL in the cytoplasm and on the membrane of submandibular gland cells and in the duct epithelia of the mice was also observed in the TGP, HCQ and combined groups; however, FasL expression in these groups was lower compared with the control group.

As shown in Fig. 6, the expression of Fas and FasL proteins in the submandibular glands was significantly increased in the control group compared with the other groups (P<0.05). There was no statistical difference in the expression of Fas protein amongst the 3 treatment groups, whilst FasL protein expression was significantly lower in the TGP group compared with the HCQ group (P<0.05). FasL protein expression was significantly lower in the combined group compared with the HCQ group (P<0.05).

TGP reduces the levels of Fas and FasL mRNA in mouse submandibular glands. The amplification curves of Fas and FasL mRNA in all samples indicated exponential growth, eventually reaching a plateau, indicative of its high-efficiency amplification. The melting curves for Fas mRNA and FasL PCR products presented with a single peak, indicating a single amplification product (data not shown).

As demonstrated in Table II, the levels of Fas mRNA in mouse submandibular glands were significantly increased in the control group compared with the combined and TGP groups (P<0.05). Furthermore, Fas mRNA levels were lower in the TGP and combined groups compared with the HCQ group (P<0.05).

The levels of FasL mRNA in mouse submandibular glands were significantly higher in the control group compared with the combined and TGP groups (P<0.05). The relative mRNA levels of FasL mRNA were significantly lower in the combined group compared with the HCQ group (P<0.05).

Discussion

Previous pharmacological studies have demonstrated that TGP is able to exert bidirectional immunomodulatory, anti-inflammatory, anti-oxidative and analgesic effects (15,16). TGP has been repeatedly used in the treatment of SS, with effective clinical results (3). The combined use of TGP and HCQ, however, may have more significant therapeutic effects in the treatment of SS. A combined therapy may reduce ESR, improve symptoms including a dry mouth and eyes and can increase saliva flow with reduced side effects (17). Therefore, the present study aimed to elucidate the molecular mechanism underlying this combination treatment. SS has complex

clinical manifestations, and there is currently no uniform classification criterion for SS (18). Previous studies have primarily relied on animal models to investigate the pathogenesis and treatment of SS (19). The NOD mouse frequently develops insulin-dependent diabetes and lymphocytic infiltration in the submandibular and lacrimal glands and other organs (20). These types of pathological damage are similar to those observed in SS. The NOD mouse is therefore an appropriate animal model for the study of SS (21).

SS is a chronic systemic autoimmune disease that affects the exocrine glands, including salivary and lacrimal glands; and the majority of patients with SS are women aged 40-50 years (22). Currently, the etiology and pathogenesis of SS is unclear, and there is no specific treatment for SS. There has been increased attention directed towards the immune effects of the helper T cells (Th) and cytokines secreted by Th cells in patients with SS and in animal models. During the onset of SS, Th1- and Th2-type cytokines are in a state of dynamic equilibrium. Th2-type cytokines are dominant during the early stages of lymphocytic infiltration into the exocrine glands in patients with SS (23). However, Th1-type cytokines gradually become more involved during the late stage of SS, when infiltration becomes more severe (24). A previous study revealed that the levels of Th1-type cytokine IFN-γ and tumor necrosis factor-α (TNF-α) in the salivary glands are significantly higher in patients with SS compared with normal control patients (25). IFN-γ is able to reduce the growth and development of salivary gland cells, thus serving an crucial function in early SS onset (26). Cytokine IL-4, secreted by Th2 cells, has a key role in the adaptive immune response during clinical onset of SS, and the knocking out of IL-4 genes has been demonstrated to restore gland secretory functions in SS animal models (6). Previous experiments by the authors of the current study have demonstrated that the levels of Th1- and Th2-specific cytokines in serum and submandibular glands of NOD mice were significantly increased compared with normal BALB/C mice, indicating a Th1/Th2 immune imbalance (8).

Fas and its ligand FasL are crucially involved in the regulation of apoptosis, immune privilege and the maintenance of homeostasis in the body (27). Previous studies have demonstrated that Fas expression in SS exocrine epithelial cells and the classical Fas/FasL system, induce apoptosis, causing human placental and gestational trophoblastic disease (28). Saegusa *et al* (29) reported $CD4^+$ T cell infiltration in salivary gland tissue and large ratio of Fas to FasL ligands in an SS animal model, but that salivary duct epithelial cells continue to secrete Fas. These results suggested that Fas/FasL pathway-mediated apoptosis may be partially responsible for labial gland tissue destruction and dysfunction in SS.

Thl cells are able to induce apoptosis of target cells by expressing FasL (30). Fas mRNA is predominantly expressed in Th2 cells, and FasL mRNA in Thl cells. Thl cells are able to downregulate Th2 and Th0 cells by Fas/FasL-mediated apoptosis; however, imbalance of Thl to Th2 cells causes changes to cytokine secretion and inhibits the normal apoptosis of immune cells, leading to the accumulation of nucleosomes in cells (31). These nucleosomes subsequently stimulate the production of antibodies by autoreactive T-lymphocytes, thereby damaging the body. The combined effects of Th1 and Th2 cells regulate the onset of SS. Previous studies have suggested that the Fas to FasL ratio is correlated with the IFN-γ to IL-4 ratio in SS labial gland lymphocytes, and is positively correlated with lymphocyte infiltration in labial glands (32). These results suggest that the increase in IFN-γ to IL-4 and Fas to FasL ratio is crucially involved in the destruction of SS labial glands and pathogenesis of SS.

The present study demonstrated that the levels of IFN-γ and IL-4 in serum and in submandibular glands were significantly higher in the control NOD mice compared with the other groups. However, IFN-γ levels in serum were lower in the TGP and combined groups compared with the HCQ group. The ratio of IFN-γ to IL-4 in serum was higher in the control group compared with the TGP, HCQ and combined groups. Following TGP intervention, the expression of Fas and FasL in the submandibular glands reduced, and the expression of Fas and FasL mRNA in the submandibular glands was significantly lower in the TGP group compared with the control group. These results suggest that TGP is able to reduce cytokine levels in serum and submandibular glands in SS, and decrease the ratio of IFN-γ to IL-4 in submandibular glands. Furthermore, TGP appears to be able to downregulate the levels of Fas, FasL and their mRNA expression, thereby mediating the Th1/Th2 immune balance and reducing cell apoptosis, thus achieving its therapeutic effect on SS. Future studies are required to aid understanding of the mechanism by which TGP affects cell apoptosis.

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