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Effects of total glucosides of peony on AQP-5 and its mRNA expression in submandibular glands of NOD mice with Sjogren's syndrome

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Abstract. – OBJECTIVE: The aim of this study was to observe the effects of total glucosides of peony (TGP) on pathological change, Aquaporin-5 (AQP-5) and its mRNA expression in submandibular glands of non-obese diabetic (NOD) mice with Sjogren's Syndrome, to investigate its regulation on secretion of salivary glands.

MATERIALS AND METHODS: 40 NOD mice were randomly divided into model group, TGP group, hydroxychloroquine group, combination group (n = 10). For TGP group, the mice were intragastrically administrated with 0.4 ml TGP dilution per day in accordance with 300 g/kg dose; for hydroxychloroquine group, the mice were intragastrically administrated with 0.4 ml hydroxychloroquine per day in accordance with 60 mg/kg dose; for the combination group, the mice were intragastrically administrated with 0.4 ml TGP dilution and 0.4 ml hydroxychloroquine. 8 weeks later, the mice were sacrificed, and submandibular glands were collected by anatomy. Pathological changes of submandibular gland were observed under a light microscope; AQP-5 protein in submandibular glands was detected by immunohistochemical staining; and AQP-5 mRNA expression in submandibular glands was detected by RT-PCR.

RESULTS: The lymphocytic infiltration score of model mice was significantly higher than that of other groups. The pathological morphology and score of NOD mice were significantly improved after administration, and the combination group was superior to the hydroxychloroquine group and TGP group ($p < 0.05$). AQP-5 mRNA expression level of the model group was lower than other groups ($p < 0.05$); the expression levels in the TGP group and the combination group were higher than the hydroxychloroquine group ($p < 0.05$).

CONCLUSIONS: TGP may improve pathological damage of submandibular glands of NOD mouse with Sjogren's syndrome by upregulating AQP-5 and its mRNA expression in submandibular glands.

Key Words:
Total glucosides of peony, Primary Sjogren's syndrome, NOD mice, Aquaporin-5.

Introduction

Total glucosides of peony (TGP) is a group of glycosides extracted from a herbal, white peony root, accounting for more than 90% of the total, and is the main active ingredients of white peony[1]. Modern pharmacology studies have shown that TGP has bi-directional immune modulation, anti-inflammatory and analgesic effects[2]. Clinical studies suggest that TGP plays a definite role in primary Sjogren's Syndrome (pSS) treatment, reducing blood sedimentation and improving the symptoms of dry mouth, dry eyes, etc., so as to improve the quality of patients' life[3]. AQP-5 is a Aquaporin, mainly distributed in apical membrane and basolateral membrane of mucous acini and basolateral membrane of serous acini of normal salivary gland and intercalated duct, striated duct, and secretor of salivary glands, closely related to saliva secretion. The saliva secretion in AQP-5 gene knockout mice decreases significantly[4]. Studies indicate that the distribution of AQP-5 in salivary glands of mouse model with Sjogren's syndrome is abnormal, that is, the secretion of apical membrane of acini reduces or disappears, and the expression in basolateral membrane of serous acini increases[5]. The results of quantitative analysis of RT-PCR showed that expression of AQP-5 mRNA in submandibular glands of SS mouse decreased significantly[6]. The changes in some procedures of transcription and transportation of AQP-5 in labial gland were closely associated with SS[7]. Mainly based on the preliminary investigations, this study was to explore the effects of TGP on AQP-5 and its mRNA expression in serum and submandibular glands of non-obese diabetic (NOD) mice with Sjogren's syndrome to clarify the possible mechanism for the treatment of Sjogren's syndrome through animal experiments.

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Materials and Methods

Experimental animals

40 specific pathogen-free (SPF)-level female NOD mice aged 8 weeks, provided by Shanghai Laboratory Animal Center were used, with an Animal Certificate: 2007000512526. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Chinese Medical University.

Experimental groups

40 NOD mice were randomly divided into four groups, i.e. model group, TGP group, hydroxychloroquine group (HCQ group), and combination group (n = 10). The mice were raised at a constant temperature, constant humidity environment and were supplied with water by drinking bottles.

Drug delivery

The normal group and model group were fed normally every day. For the treatment groups, on the basis of the normal feeding, at the beginning of experiments, the mice were administrated with drugs at a fixed time every day, and the dosage was calculated according to equivalent dose conversion table of animal and human. For TGP group, the mice were intragastrically administrated with 0.4 ml TGP dilution per day in accordance with 300 g/kg dose. For hydroxychloroquine group, the mice were intragastrically administrated with 0.4 ml hydroxychloroquine dilution per day in accordance with 60 mg/kg dose; for the combination group, the mice were intragastrically administrated with 0.4 ml TGP dilution and 0.4 ml hydroxychloroquine. 8 weeks later, the mice were sacrificed, and bilateral submandibular glands were collected for index detection.

HE staining

The collected submandibular glands were fixed in 10% neutral formalin, and paraffin-embedded and sliced. The slices were stained by HE, and observed under a light microscope for histomorphology. Evaluation was conducted according to the method of Cutzler et al[8]: 0 for occasional lymphocytic infiltration; 1, a few scattered lymphocytic infiltrations; 2 for moderate lymphocytic infiltration (without lesions), accompanied by mild parenchymal damage; 3 for occasional 0-1 lymphocytic infiltration lesion/5 low power fields, accompanied by moderate parenchymal damage; and 4 for 2-3 lymphocytic infiltration lesions/5 low power fields, accompanied by severe parenchymal damage.

ELISA

Serum AQP-5 detection, ELISA assays were carried out in strict accordance with instructions.

Immunohistochemical SP assays

Immunohistochemical SP assays were used to detect AQP-5 in mouse submandibular glands, and the specific steps were conducted in strict accordance with instructions.

Multifunctional true-color cell image analysis management system was used for image analysis and result analysis: 400×images were selected, and the cells with brown cytoplasm or membrane were recorded as positive cells. Meaningful tissue photos were selected, recorded, numbered, analyzed, and read for data. Five fields were randomly selected for mean optical density determination, and the mean values were used as the representative values of the slices for statistical analysis.

Real-time PCR

Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control, and the experiment was conducted according to the instructions of kit, and the specific steps were as following: Total RNA of submandibular glands was extracted using Trizol kit, and 1 μL RNA was used in RT-PCR analysis. For AQP-5, the upstream primer was 5,-AAATGGTGAAGGTCGGTGTG-3, and the downstream primer was 5,-TGAAGGGGTCGTTGATGG-3; and for GAPDH, the upstream primer was 5,-GGCCCTCTGCATCTTCTCCTC-3, and the downstream primer was 5,-GCCCCCACGATCGGTCCTAC-3; the product was 108 bp in length, with a Tm value of 60. According to the primer design, the product of PCR amplification was 216 bp, with a Tm of 60. PCR reaction parameters were: 94°C 5 min, and 40 cycles of 94°C 15 s and 60°C 45 s. The fluorescence was detected at 60°C.

The reactions of AQP-5 and the internal control GAPDH were conducted in two tubes separately; excluding probes and primers,

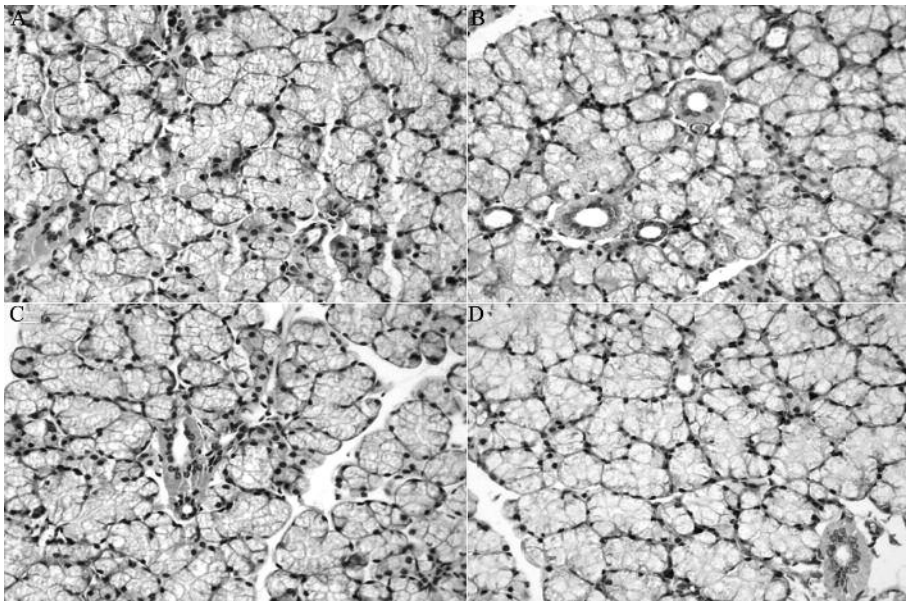

Figure 1. Comparison of pathomorphology of mouse submandibular glands (HE staining × 40 times). **A,** Model group; **B,** TGP group; **C,** HCQ group; **D,** The combination group.

RNA templates and other reagents were consistent in two tubes. The mean value of threshold cycle (CT value) was calculated. The relative quantitative analysis method $2^{-\Delta\Delta CT}$ was used to analyze results[9], relative mRNA expression levels, and ΔCT values were statistically analyzed; meanwhile, Applied Biosystems 7500 quantitative PCR instrument (Foster City, CA, USA) was used to describe the amplification curve and melting curve of each group. In this experiment, the model group was used as the untreated group for comparison with other groups.

ΔCT = CT value of target gene in the experimental group – CT value of the internal control gene of the same group.

ΔΔCT = (CT of target gene-CT of internal control gene) of the experimental group – (CT of target gene-CT of internal control gene) of model group.

The smaller ΔCT indicates the higher expression of AQP-5mRNA in submandibular glands, and the higher $2^{-\Delta\Delta CT}$ indicates the higher expression of AQP-5mRNA in sample relative to the model group.

Statistical Analysis

Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). The measurement data were presented as mean ± standard deviation (± SD). Comparison among groups used one way ANOVA method. $p < 0.05$ was considered as statistical significance.

Results

Pathomorphology comparison

Observation of histomorphology of submandibular glands (Figure 1)

Pathological observation of submandibular glands indicates the model group demonstrated moderate lymphocytic infiltration in mesenchyme, varied acini, partial damaged acini, local glandular atrophy and fibrous hyperplasia. In other groups, the pathomorphology of mice submandibular glands was characterized by a few scattered single lymphocytic infiltration, varied acini, and occasional damages of a few acini and duct structures.

Comparison of pathological scores of mouse submandibular glands (Table I)

The pathological score shows the pathological score of submandibular glands of the model mice was significantly higher than that of other groups,

Table I. Comparison of pathological scores of mouse submandibular glands (points, $\bar{x} \pm s$)

Group	Animal number	Score result
Model group	10	2.900 ± 0.738
TGP group	10	$1.600 \pm 0.516^{\#}$
HCQ group	10	$1.800 \pm 0.422^{\#}$
The combination group	10	$1.100 \pm 0.316^{\#\Delta}$

Comment: Compared with model group $^{\#}p < 0.05$; Compared with HCQ group $^{\Delta}p < 0.05$

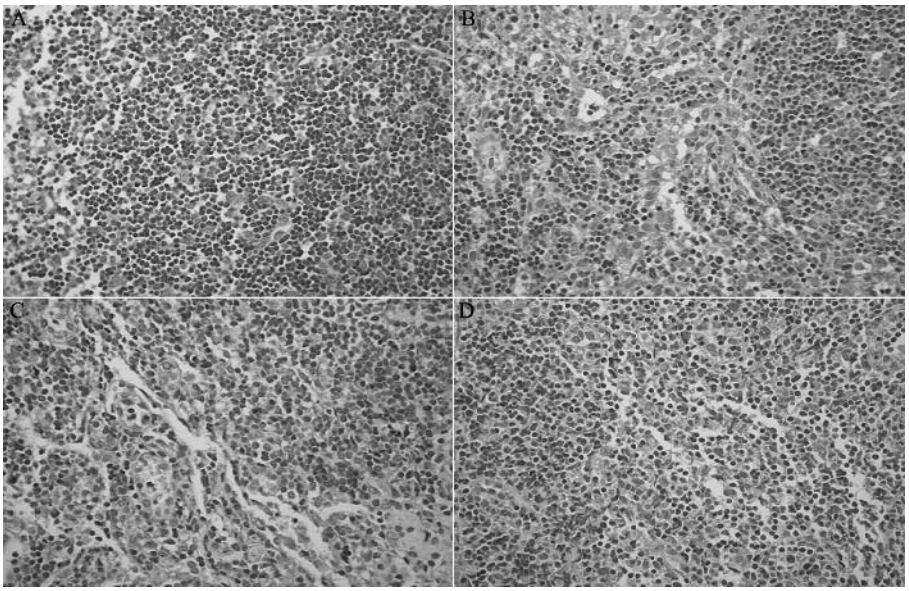

Figure 2. Results of immunohistochemical SP detection for AQP-5 contents in mouse submandibular glands (DAB staining × 400 times). **A,** Model group; **B,** TGP group; **C,** HCQ group; **D,** The combination group.

with a significant statistical difference ($p < 0.05$). The combination group was lower than that of the HCQ group, with a significant statistical difference ($p < 0.05$).

AQP-5 contents comparison

Results of immunohistochemical SP detection for AQP-5 contents in mouse submandibular glands was shown in Figure 2. In the model group, the staining of acini, intercalated ducts, secretors, conduits of submandibular glands diminished or disappeared. In the combination group, the intercalated ducts, conduits and acini of submandibular glands showed many low-to-moderate stained brown particles. In TGP group and HCQ group, the intercalated ducts, conduits and acini of submandibular glands showed scattered brown particles.

As shown in Table II, AQP-5 expression in submandibular glands of the model mice was lower than other groups, and with a statistically significant difference ($p < 0.05$) compared with the combination group; the combination group was higher than the other groups, but with HCQ group compared statistically significant difference ($p < 0.05$); the TGP group was higher than the HCQ group, but without a significant difference ($p > 0.05$).

AQP-5 mRNA expression

As shown in Table III, relative AQP-5mRNA expression in submandibular glands of model mice was lower than other groups, with a statistically significant difference ($p < 0.05$). It is also found that expression of TGP group and the combination group was higher than the hydroxychloroquine group, with a statistically significant difference ($p < 0.05$). Compared with the model group, the expression level of AQP-5mRNA, TGP group and the combination group was significantly higher than that of hydroxychloroquine group.

Comparison of dissolution curves and amplification curves of AQP-5mRNA and internal control GAPDH results suggest that the melting curves of AQP-5mRNAPCR products in fluorescence quantitative analysis showed a single peak, and AQP-5 mRNA amplification curves of all samples showed an exponential growth and reached a plateau, with high amplification efficiencies, which suggests a single amplification product, to avoid false positive results in the detection process.

Table II. Comparison of AQP-5 mean optical densities of mouse submandibular glands ($\bar{x} \pm s$)

Group	Animal number	AQP-5
Model group	10	0.138±0.067
TGP group	10	$0.250\pm0.085^{\#}$
HCQ group	10	0.200±0.105
The combination group	10	$0.390\pm0.110^{\#\Delta}$

Comment: Compared with model group $^{\#}p < 0.05$; Compared with HCQ group $^{\Delta}p < 0.05$

Table III. Comparison of relative AQP-5 mRNA expression in mouse submandibular glands ($\bar{x} \pm s$).

Group	Animal number	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$
Model group	10	9.409±1.914	0.000±1.914	1.000
TGP group	10	5.121±1.920$^{\#\Delta}$	-4.288±1.920	19.535
HCQ group	10	7.701±2.261$^{\#}$	-1.708±2.261	3.267
The combination group	10	4.408±0.954$^{\#\Delta}$	-5.001±0.954	32.022

Comment: Compared with model group $^{\#}p < 0.05$; Compared with HCQ group $^{\Delta}p < 0.05$

Discussion

Sjogren's syndrome (SS) is an autoimmune disease mainly invading salivary gland, lacrimal gland and other exocrine glands, and the pathological changes of glands are mainly lymphocytic infiltration, resulting in acinar destruction, duct atrophy, and loss of glandular secretion function, and causing dry mouth, dry eyes and other symptoms. The disease is mainly found in 40 to 50-year-old female, and the ratio of female to male is 9:1[10]. The main clinical manifestations are dry mouth, difficult swallowing, rampant caries, dry eyes, less tears, joint pain, mycteroxerosis, epistaxis, and dryness and itching of vaginal mucous. Most of SS symptoms appear later, with complex clinical manifestations, and there is no unified SS classification criterion. Studies of the pathogenesis and interventions of SS by reproducing SS animal models have attracted attention.

NOD mice are mice with a tendency to spontaneous insulin-dependent diabetes, in which lymphocytic infiltration is found in pancreas, submandibular gland, lacrimal gland and other organs. The frequencies of diabetes and sialadenitis in female mice are higher than those in male mice. Studies show that in NOD mice at 10-12 weeks of age, lymphocytic infiltration appears in exocrine gland and exocrine function reduces at 16 weeks of age. Anti-Ro/SSA, anti-La/SSB and other autoantibodies have been found in serological studies[11]. Our previous studies showed that dietary amount and salivary secretory volume in NOD mice were lowed than in normal mice, infiltrative degrees of lymphocytes in submandibular gland was stronger than normal mice. These were similar to clinical manifestations[12]. Therefore, NOD mice are regarded as the most appropriate animal model to study SS[13].

AQPs are specific channel proteins transporting water on cell membrane, distributing in a variety of glandular cells, among which AQP-5 is expressed in submandibular glands, lacrimal glands and lung. Researchers have shown that[14] TNF-α can up-regulate the expression of AQP-5, so AQP-5 expression may be also related to the action of various proinflammatory cytokines, and viral and other infections may lead to the inhibition of AQP expression through inflammation factors. Studies have showed that[15] in SS patients, especially patients with primary Sjogren's syndrome, AQP-5 protein expression in cavity surface of acinar cells of labial glands is significantly lower than that of non-connective tissue disease group, and is negatively correlated with the score of lymphocytic infiltration lesions in labial gland, indicating that AQP-5 is expressed abnormally in labial glands of patients with pSS, and that in SS pathogenesis, some links of AQP-5 transcription and transit change. Therefore, the study on the impact of drugs on AQP-5 expression in SS submandibular gland will provide a theoretical basis for clinical treatment.

Nowadays, for SS treatment, measures are mainly used to improve the symptoms, control and delay disease progress, and local treatment or systemic treatment can be used according to disease conditions. TGP has a strong immunosuppressive and immunomodulatory effect, and is widely used in clinical treatment of various autoimmune diseases as an important immunosuppressant in China. Previous studies showed that TGP has good therapeutic effects on rheumatic autoimmune diseases[13]. Studies demonstrate that TGP plays a definite role in pSS treatment, reducing blood sedimentation and improving the symptom of dry mouth, and sleep, appetite, physical strength and general conditions, so as to improve the quality of patients' life significantly[3]. Controlled studies on treating SS between TGP and hydroxychloroquine (HCQ) showed that they have equivalent effects, and the safety of TGP was better[16]. TGP combined leflunomide had notable curative effect on treating SS and good security[17]. In NOD mice of early stage, TGP can actively improve pathophysiological features of submandibular glands, significantly improve glandular destruction and atrophy, retain normal glandular secretion function, alleviate the clinical symptoms of dry mouth; regulate the immune system, reduce the autoimmune attack, and significantly reduce the expression of autoantibodies[18,19].

Conclusions

This study shows that model NOD mice demonstrated moderate lymphocytic infiltration in mesenchyme, with varied acini and local gland atrophy. In the administration groups, there were a few scattered single lymphocytic infiltrations in submandibular glands, and the pathological score decreased, which suggests that after treatment, lymphoid infiltration in mouse submandibular glands was improved, and the combination group was superior to the hydroxychloroquine group. Both APQ-5 and mRNA expression in submandibular glands of model mice were lower than other groups, and the combination group and TGP group were higher than that in the hydroxychloroquine group. It is suggested that TGP can significantly reduce the pathological damage of the submandibular glands of NOD mice, increase the expression of AQP-5 and its mRNA, and improve the saliva secretion of salivary glands, and hydroxychloroquine synergistic therapeutic effect, thus alleviating the clinical symptoms of SS patients such as dry mouth and thirst, to achieve the purpose of clinical treatment of SS.

Conflict of interest

The Authors declare that they have no conflict of interests.

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