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Original Article

Protective Effect of Zengye Decoction (增液汤) on Submandibular Glands in Nonobese Diabetic Mice*

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ABSTRACT **Objective**: To investigate the protective effect of Zengye Decoction (增液汤, ZYD) on the submandibular glands (SMGs) in nonobese diabetic (NOD) mice. **Methods**: Twenty-seven female NOD mice were randomly equally divided into 3 groups: the model group, the hydroxychloroquine (HCQ) group, and the ZYD group. Nine C57/B6 mice served as the normal group. After 1-week acclimation, the HCQ and ZYD groups were intragastrically administered with HCQ and ZYD, respectively, and the normal and model groups were administered with normal saline. Changes in the salivary flow rate were observed. Mice from all 4 groups were sacrificed at the age of 20 weeks. The serum and SMGs were collected. Serum cytokines gamma-interferon (IFN-γ), interleukin-10 (IL-10) were detected by enzyme-linked immunosorbent assay. Histological changes in the submandibular glands were examined by hematoxylin and eosin staining. The mRNA expression of IFN-γ, IL-10 and vasoactive intestinal peptide (VIP) in the submandibular glands were measured by real-time polymerase chain reaction. **Results**: Compared with the model group, the salivary flow of the ZYD group significantly increased ($P<0.05$), the extent of the histological changes was ameliorated ($P<0.05$), and the Th1/Th2 cytokine imbalance was remedied ($P<0.05$). In the ZYD-treated mice, the VIP mRNA was up-regulated ($P<0.05$). **Conclusions**: ZYD is beneficial in protecting structure and function of SMGs in NOD mice. The mechanism may be associated with the correction of the Th1/Th2 cytokine imbalance, and with the prevention of a progressive decline of the VIP level.

KEYWORDS Sjögren's syndrome, Th1/Th2, vasoactive intestinal peptide, Zengye Decoction, Chinese medicine

Zengye Decoction (增液汤, ZYD), which was first recorded in *Treatise on Differentiation and Treatment of Seasonal Diseases* (Wen Bing Tiao Bian) written by WU Ju-tong, is composed of *Radix scrophulariae*, *Ophiopogon japonicus*, and *Radix rehmannia*. It is a classic formula used for moisturizing dryness, promoting the production of body fluids, and primarily treating functional constipation associated with yin deficiency. There is a lower expression of vasoactive intestinal peptide (VIP) in the intestinal tissue from patients with slow transit constipation (STC).[1] It was found that ZYD could upregulate the expression of VIP in the intestinal tissue of STC model mice.[2] The spontaneous nonobese diabetic (NOD) mouse model of Sjögren's syndrome (SS) provides a valuable tool to study the onset and progression of the autoimmune response and secretory dysfunction. Studies have shown a progressive decline of VIP expression in the submandibular glands of NOD mice.[3] VIP, a neuropeptide present in the lymphoid microenvironment, was initially discovered as a gastrointestinal hormone; it exhibits abundant functions that range from neurotransmitter, vasodilator, and bronchodilator effects to acting as a trophic agent, secretagogue, and immunodulator.[4] It can inhabit proinflammatory cells (e.g., macrophages and microglia of central nervous system cells), produce cytokines and chemokines, promote Th2 differentiation, and inhibit Th1 cells at inflammatory

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sites of aggregation, and prevent the occurrence of chronic inflammation. Local delivery of a recombinant serotype 2 adeno-associated virus that encodes the human VIP transgene can have disease-modifying effects in the submandibular glands (SMGs) of NOD mice.[4] The current study was designed to explore whether ZYD can up-regulate the expression of VIP in SMGs, and protect the SMGs in NOD mice.

METHODS

Drugs

ZYD was prepared by decocting the crude drugs *Radix Scrophulariae*, *Ophiopogon japonicus*, and *Radix rehmannia* in the proportion of 5:4:4. The decoction was concentrated to 0.5 g/mL of the crude drugs.[5] The crude drugs were all purchased from the Nanjing Tongrentang Co., Ltd. (Nanjing, China). The botanical identities of the herbs were defined by the Medicinal Plant Department, Nanjing University of Chinese Medicine (Nanjing, China). Hydroxychloroquine (HCQ, batch No. 120103) was purchased from Shanghai Zhongxi Pharmaceutical Co., Ltd. (Shanghai, China). The drugs were ground and diluted with normal saline to the required concentration (5 mg/mL).

Major Reagents and Instruments

Enzyme-linked immunosorbent assay (ELISA) kits for interferon-γ (IFN-γ, batch No. CK-E11382M) and interleukin-10 (IL-10, batch No. CK-E20011M) were both purchased from R&D system (USA); TRIZOL, from the Invitrogen Biotechnology Company; RNA reverse transcription kit, from the Promega Company (USA); and real-time quantitative polymerase chain reaction (RT-PCR) kit, from the TaKaRa Company (Japan) were used.

Instruments used in the study were as follows: BX50WI microscope (Olympus, Japan); Multiskan spectrum microplate (Themo Co., GER); MyCyler polymerase chain reaction (PCR) (Bio-Rad Co., USA); and Dolphin-Doc gel imaging system (Wealtec Co., USA).

Animals

Eight-week-old female inbred strains of NOD/Ltj (NOD) and C57/BL6 mice [Licence No. SCXK(Hu)2012-0002] that weighed 16–18 g were purchased from the Shanghai Slac Laboratory Animal Co, Ltd. (Shanghai, China). The animals were housed with 9 animals per cage and had free access to water and food. The surrounding temperature and humidity were maintained at 19–22 ℃ and 50%–60%, respectively. The mice were allowed to acclimatize without handling for 1 week. All experiments were started between 9:00 a.m. and 10:00 a.m. in a mouse room.

Treatment Regimens

Twenty-seven NOD mice were divided into 3 groups using random digits table method: the model group, the HCQ group, and the ZYD group (n=9 per group). The normal control group consisted of 9 C57/BL6 mice. The mice in the ZYD and HCQ groups were treated daily with ZYD (10 g/kg of the crude drugs, 20 mL/kg) and HCQ (0.1 g/kg, 20 mL/kg), respectively. The animals in the normal and model groups received an equivalent volume of saline (20 mL/kg). These doses were based on the results from preliminary experiments and related reports in the medical literature.[1] All treatments were administered by gastric infusion once daily for 11 weeks. One mouse in the normal, model and ZYD groups, respectively, died of intragastric administration.

Measurement of Salivary Flow

The salivary flow of the mice was examined at the age of 15, 17, and 20 weeks. The animals were fasted for 1 h before the procedure. A preweighed cotton swab (10 mg) was placed immediately below the lower cheek pouch of each mouse. At 3-min intervals, the cotton swab was weighed for the quantification of the salivary production. Salivary flow was measured by subtracting the original weight of the cotton swab from the weight obtained after the procedure and normalized to milligrams of saliva per 100 g of body weight. Therefore, the salivary flow was expressed every 3 min as milligrams of saliva per 100 g of body weight.

Determination of Serum IFN-γ and IL-10 Levels

At 20 weeks of age, the blood of all mice was collected through glass capillary tubes from the retro-orbital sinus. The serum levels of IFN-γ and IL-10 were measured by ELISA in accordance with the manufacturer's instructions. The absorbance was read a microplate reader at 450 nm. The IFN-γ and IL-10 concentrations were expressed as pg/mL and ng/L, respectively. The ELISA kits' minimum detectable level was 50 pg/mL for IFN-γ and 25 ng/L for IL-10.

Histological Assessment of SMGs

Immediately after blood sampling, the mice

were killed by cervical dislocation, and then dissected. The SMGs of each mouse were excised. All procedures were performed in accordance with standardized laboratory methods. A portion of each gland was fixed in 10% formalin overnight. The tissues were thereafter dehydrated in a series of graded ethanol solutions, and then embedded in paraffin. Baseline histological slides containing 4-mm-thick sections stained with hematoxylin and eosin (HE) were reviewed in a blinded manner by a well-trained pathologist. The SMG sections were reviewed and graded by using the Chisholm-Mason scale, as previously described.[6] With this scoring system, the SMG sections were graded from 0 to 4, based on the presence of lymphocytic foci that consisted of 50 or more lymphocytes per 4 mm^2. Grade 0 indicated no lymphocytes; grade 1, infiltration of lymphocytes without any foci; grade 2, the presence of at least 1 focus; grade 3, multiple foci; and grade 4, multiple foci and evidence of gland destruction. Histological observations and photomicrography were performed by using an Olympus BX50WI microscope.

Determination of IFN-γ, IL-10, and VIP in SMGs

The mRNA expression of cytokines in the SMG was determined by RT-PCR. The Th1/Th2 cytokine balance was expressed by the ratio of IFN-γ and IL-10. Total ribonucleic (RNA) samples were extracted from the SMG of NOD mice by using TRIZOL reagent in accordance with the manufacturer's instructions. The concentration of RNA was measured by the Multiskan spectrum microplate at 260 nm. Reverse-transcribed complementary deoxyribonucleic acid (cDNA) was amplified by using specific primers for IFN-γ, IL-10, VIP, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), and by using the previously described conditions. The following sequences were used for the forward and reverse primers.

IFN-γ: 5'-GTGATTGCGGGGTTGTATCT-3' and 5'-TGTCATTCGGGTGTAGTCACA-3'; IL-10: 5'-TCCTTGGAAAACCTCGTTTG-3' and 5'-CTTCAATTGCTTCCCAAGGA-3'; VIP: 5'-TTCACCAGCGATTACACAGCAG-3' and 5'-TCACAGCCATTTGCTTTCTG-3'; GAPDH: 5'-TCAACGGCACAGTCAAGG-3' and 5'-ACCAGTGGATGCAGGGAT-3'.

The PCR products were size-fractionated on 2% agarose gels. They were visualized by staining with ethidium bromide and using a size molecular marker. For the real-time experiments, the expressions of IFN-γ, IL-10, and VIP were determined as previously described.

Statistical Analysis

Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Statistical significance of differences was determined by *t*-test or variance analysis. Differences were considered significantly at $P<0.05$.

RESULTS

Salivary Flow

The whole saliva was collected. Whole saliva primarily represents a combination of parotid and SMG secretions, and a very minor component from the sublingual, minor salivary, nasal, and tracheal glands. There were no differences between the four groups at the age of 15 weeks. The mean salivary flow of the normal group remained at the baseline during the study. However, the other groups showed a progressive decline in salivary flow starting at 17 weeks of age. The salivary flow was thereafter significantly lower in the model group than in the normal group ($P<0.05$). The mice treated with HCQ and ZYD exhibited less reduction in salivary flow, compared with the model group ($P<0.05$). Significant differences between the HCQ and ZYD groups occurred at 17 weeks and 20 weeks of age (Table 1).

Table 1. Comparison of Salivary Flow of Mice among Groups ($\bar{x} \pm s$)

Group	*n*	Salivary Flow (mg/100 g body weight)		
		15 weeks	17 weeks	20 weeks
Normal	8	7.4±2.4	7.9±2.2	7.3±1.8
Model	8	6.5±1.6	4.9±1.2*	3.8±1.0*
HCQ	9	6.7±1.4	5.7±1.1△	4.5±1.2△
ZYD	8	7.0±1.8	5.8±1.0△	4.8±1.6△

Notes: *$P<0.05$, compared with the normal group; △$P<0.05$, compared with the model group

Histological Findings

The presence and degree of pathology in the form of lymphocytic infiltrates varied among individual mice. As shown in Figure 1, the model group mice clearly showed an infiltration of lymphocytes in the SMG, whereas the normal group mice showed little or no infiltration of lymphocytes in the SMG. After HCQ and ZYD treatments, the lymphocytic infiltration in the

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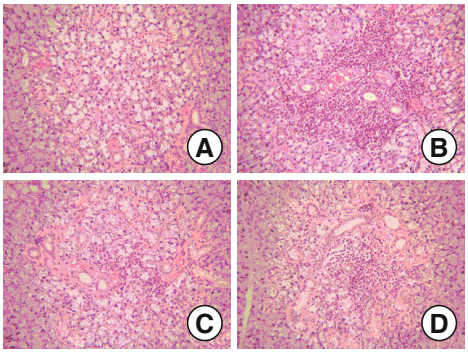

Figure 1. Lymphocytic Infiltration of SMGs in Mice (HE staining, × 100)

Notes: A: normal group; B: model group; C: HCQ group; D: ZYD group

HCQ and ZYD groups was slightly lower than that in the model group.

The mean grade of lymphocytic infiltration in the model mice was higher than that in the normal group (3.2 ± 0.4 *vs*. 0.2 ± 0.4, *P*<0.05). After 11 weeks of treatment with HCQ or ZYD, lymphocytic infiltration was significantly decreased (0.6 ± 0.9 and 1.0 ± 1.4, respectively) as compared with the model group (*P*<0.05).

Serum Level of Cytokines and mRNA of Cytokines in SMGs

As shown in Table 2 and Figure 2, the serum and the SMG had consistent changes. The IFN-γ and IL-10 levels and the IFN-γ/IL-10 ratio in the serum and in the SMG of the model group were significantly higher than those in the normal group (*P*<0.05). The intragastric administration of HCQ and ZYD resulted in a significant decrease in the IFN-γ/IL-10 ratio (*P*<0.05). The IFN-γ level decreased significantly in the HCQ group (*P*<0.05). No significant difference in the IFN-γ level was observed between the ZYD and the model groups. However, there was a significant increase of the IL-10 level in the ZYD group in comparison to the model group (*P*<0.05).

Table 2. IFN-γ and IL-10 Levels and Ratio of IFN-γ/IL-10 in Mice Serum ($\bar{x} \pm s$)

Group	*n*	IFN-γ (pg/L)	IL-10 (ng/L)	IFN-γ/IL-10 (pg/ng)
Normal	8	404.3 ± 36.3	285.1 ± 9.0	1.42 ± 0.16
Model	8	527.7 ± 93.9*	320.6 ± 33.2*	1.63 ± 0.16*
HCQ	9	431.5 ± 43.9△	313.3 ± 37.1	1.38 ± 0.16△
ZYD	8	532.9 ± 45.2	358.0 ± 21.0△	1.49 ± 0.14△

Notes: *P<0.05, compared with the normal group; △P<0.05, compared with the model group

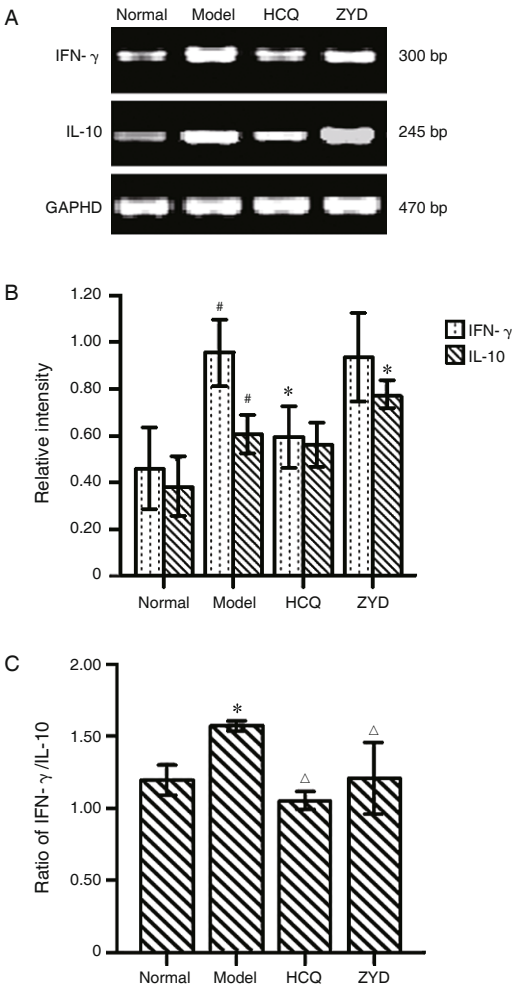

Figure 2. Relative Expression Levels of IFN-γ and IL-10 mRNA in SMGs of Mice ($\bar{x} \pm s$, *n*=3)

Notes: *P<0.05, compared with the normal group; △P<0.05, compared with the model group

VIP mRNA in SMGs

As shown in Figure 3, the mRNA expression of VIP in the SMG is lower in the model mice than in the normal mice (*P*<0.05). The mice of the ZYD group conversely showed a significant increase in VIP mRNA expression compared with the model group (*P*<0.05). However, the HCQ-treated mice failed to show an apparent difference in comparison to the model group.

DISCUSSION

SS is an autoimmune condition in which dry eyes (i.e., keratoconjunctivitis sicca) and dry mouth (i.e., xerostomia) result from lymphocytic infiltration of the lacrimal and salivary glands.[7] The NOD mouse model provides a valuable tool to study the onset and progression of SS-like disorders.[8] The results of the current experiment show that the salivary flow gradually decreased with increasing age of the NOD

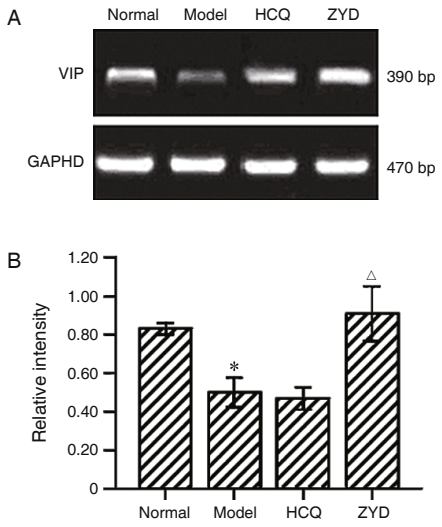

Figure 3. Relative Expression of VIP mRNA in SMGs of Mice ($\bar{x} \pm s$, n=3)

Note: *P<0.05, compared with the normal group; △P<0.05, compared with the model group

mice. At the age of 20 weeks, the infiltration of a large number of lymphocytes could be observed in the SMG. This is very similar to the pathogenesis of SS.

There are different theories about the immunologic mechanism of this disease, and the contribution of Th1 and Th2 responses in SS is not completely understood. However, there is a growing consensus that Th1 cytokines (e.g., IFN-γ) contribute to the development of autoimmunity in the salivary glands. With regard to Th2 cytokines (e.g., IL-10), which were often detected in salivary glands of SS patients, it is unclear whether it is the cause or the result of the disease. IL-10 is nevertheless a cytokine with a wide spectrum of immunosuppressive activity. Some researchers have evaluated the effect of adeno-associated virus (AAV) vector-mediated viral IL-10 gene expression on local salivary glands in NOD mice and on the lacrimal gland in a rabbit model of induced autoimmune dacryoadenitis; these studies show that IL-10 could have disease-modifying effects in the NOD mice and rabbit models of induced autoimmune dacryoadenitis.[9,10] For the past 20 years, accumulated evidence suggests that a Th1/Th2 imbalance has a role in the pathogenesis of SS and the degree of imbalance is associated with the severity of disease.[11-15]

The classic formula of ZYD has primarily been used to treat functional constipation associated with yin deficiency, and it has recently been used in the treatment of SS. In the current experiment, the salivary flow rate of the mice receiving ZYD significantly increased at 17 weeks of age, compared with that in the model mice. At 20 weeks of age, ZYD administration led to marked improvements in inflammatory changes in the SMGs, as indicated through histological assessment. The administration of HCQ, which has been proven to be effective in treating SS, presented the same effects. Furthermore, the administration of the two drugs revealed an apparent decrease in the IFN-γ/IL-10 ratio in the serum and in the SMGs. The difference nevertheless should not be ignored. In the HCQ group, the lower ratio was achieved mainly by reducing the IFN-γ level, whereas in the ZYD group the lower ratio was mainly achieved by raising the IL-10 level.

VIP, a neuropeptide present in the lymphoid microenvironment, was initially discovered as a gastrointestinal hormone.[4] It also can inhabit proinflammatory cells (e.g., macrophages and microglia of central nervous system cells), produce cytokines and chemokines, promote Th2 differentiation, and inhibit Th1 cells at inflammatory sites of aggregation, and prevent the occurrence of chronic inflammation. It was found that there was a progressive decline of VIP expression in the SMGs of NOD mice, as compared with its expression in normal mice.[3] Local delivery of a recombinant serotype 2 adeno-associated virus that encodes the human VIP transgene can have disease-modifying effects in the SMGs of NOD mice.[4] Our experiment found that the VIP mRNA expression in the SMG of NOD mice receiving ZYD was significantly increased as compared with that in model mice. Mounting evidence supports that VIP can upregulate the level of the anti-inflammatory IL-10.[16-18] This may be one reason that IL-10 level increased in the ZYD group. The polysaccharide component of ZYD may also cause an elevation in the cytokine level. However, no significant difference existed between the HCQ and model groups in the expression of VIP mRNA. This indicated that HCQ and ZYD may have different pharmacological properties. There is also a lower expression of VIP in the intestinal tissue from patients with slow transit constipation.[1] One researcher found that ZYD could upregulate the expression of VIP in the intestinal tissue of model mice with STC.[2] An abnormality in VIP level commonly exists in patients with STC and SS. The effect of ZYD on VIP level may be a scientific basis for the same treatment for different diseases.

In summary, the administration of ZYD could protect the structure and function of SMGs in NOD mice. The mechanism may be associated with the correction of the Th1/Th2 cytokine imbalance, and with the prevention of a progressive decline of the VIP level.

Conflict of Interest

No conflict of interest exits in this manuscript.

Author Contributions

Li CY wrote this article. Wang Y designed the whole experiments and guided the writing of this paper. Wu SL, Sun LX, and Yan TT involved in data collecting and made many valuable suggestions.

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