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Protective Effect of *Dendrobium officinale* Polysaccharides on Experimental Sjogren's Syndrome

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Abstract

Sjogren's syndrome (SS), usually described as a chronic inflammation which results in xerostomia (dry mouth) and xerophthalmia (dry eyes). According to the theory of traditional Chinese medicine, body fluid impairment causes the dryness, inducing water secretion deficiency. Discovery of a family of water-specific membrane channel proteins, the aquaporins, provides an interesting molecular mechanism of water permeability and transportation which were found abnormal in tissues of SS patients. Thus, this dryness may lead to the dysfunction in organs as various systematic manifestations. We established an autoallergic mouse model *in vivo*, and human salivary gland cell line A-253 *in vitro*. Polysaccharides of *Dendrobium officinale* (DP) were administrated as treatment, which was described to nourish yin and promote the body fluid. Results showed that immunization with SG autoantigen induced decrease of body weight and increased water intake, decreased AQP5 expression in a series of organs related to body fluid. Sera from model mice induced apoptosis of A-253 cells with activation of caspase-3. Administration of DP could reverse these pathological changes in both the animal and cell model. Thus, DP may be a promising candidate for the treatment of SS by up-regulating the expression of AQP-5 and protecting cells from apoptosis.

KEYWORDS: *Dendrobium officinale*, polysaccharides, AQP5, Sjogren's syndrome, apoptosis

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INTRODUCTION

In Sjogren's syndrome (Eriksson P), salivary and lacrimal glands are the major targets of chronic inflammation which results in xerostomia (dry mouth), xerophthalmia (Tsifetaki, Kitsos et al.) and other systematic manifestations (Eriksson P 1999; SUGAI 2001) Histological evaluation shows large scale and persistent inflammatory infiltrations indicating further functional injuries. Most frustrating is that current western therapy provides only marginal symptomatic relief.

Discovery of a family of water-specific membrane channel proteins, the aquaporins, provide an interesting molecular mechanism of water permeability and transportation which were found abnormal in tissues of SS patients (Borgnia, Nielsen et al. 1999). The aquaporin 5 (AQP5), usually found on the luminal side of the membrane of humans and murines was demonstrated to have the function of water transportation in transgenic mice lacking AQP5 (Ma, Song et al. 1999). Furthermore, an abnormal distribution of AQP5 had been reported within salivary glands of SS patients (Steinfeld, Cogan et al. 2001), but this topic was controversial because another group could not verify this finding (Beroukas, Hiscock et al. 2002; Gresz V Fau - Kwon, Kwon Th Fau - Gong et al. 2004). On the other hand, according to the theory of Chinese medicine, it is body fluid impairment causing the dryness, inducing water secretion deficiency (Hong Y 2007). Therefore, we made the hypothesis that water deficiency caused the hydropenia organs gradually losing their functions or suffering various disorders, which ultimately led to immunity system damage in SS.

Since the current medication usually had more or less side effects such as headache, increased sweating, nausea, vomiting (Tsifetaki, Kitsos et al. 2003), SS still remained incurable. Rituximab (RTX) (Meijer, Pijpe et al. 2009), a chimeric monoclonal antibody targeting the CD20 antigen on the B cell surface, has been currently under investigation of autoimmune diseases, such as SS, systemic lupus erythematosus and rheumatoid arthritis and fatigue reduction (Dass, Bowman et al. 2008). It was reported by clinical experiment, as the return of symptoms included decrease of salivary flow, increase of rheumatoid factor and return of B cells and subjective symptoms. However, it was also claimed to accompany with many common side effect as diarrhea; flushing; indigestion; lightheadedness; mild fever and chills, especially for the first period of medication. Herbal extracts and traditional Chinese medicine have been used to treat SS with certain degree of success. These natural materials may become an alternative medicine in treatment.

Dendrobium Officinale (DO), according to Chinese Pharmacopoeia (2005 Edition) has distinguishing effect to clear unhealthy heat, nourish Yin and promote body fluid (Z. 2005). Clinically, it was usually prescribed in combination with other herbs for thirst caused by dehydration due to febrile diseases (Krane, Melvin et al. 2001). Combining the clinical use of DO, its water extract mainly consist of polysaccharide was investigated its effect on SS in this study.

We hypothesized that the polysaccharides extracted from DO may have a promising effect on our experimental SS model.

MATERIALS AND METHODS

Extraction procedure of DP

The dry stem of *Dendrobium Officinale* (batch no: DO20080112-3) were collected from Zhejiang, China and their identity was confirmed by experts in the Department of Pharmacognosy, China Pharmaceutical University. Live samples and voucher specimens were kept in the specimen room and the green house of China Pharmaceutical University. All material was rinsed with double distilled water to remove surface contaminants. Dry scions were grinded into fine particles, and sieved through 100-mesh. These powdered stems of DO (35g) were pre-extracted for 24h in a Soxhlet system with acetone (200mL) and subsequently for 24h with MeOH (200mL) to inactivate the enzymes, and remove pigments and low-molecular-weight substances. Exhaustive extraction of the residues with hot distilled water (200mL*3) gave a crude extract. It was taken up in water mixed with 15% trichloroacetic acid to remove the proteins and kept in ice bath for 1h then centrifuged at 12,000g for 1 h. The above supernatant was submitted to graded precipitation with four volumes of ethanol, and the mixture was kept overnight at 4°C to precipitate the polysaccharides. The precipitate was collected by centrifugation (10,000rpm, 30min), washed successively with ethanol, and repeated 3 times. Lyophilization gave 7.5155g crude polysaccharide (DP), which by means gave yield of 21.47% (Hua, Zhang et al. 2004; D 2008).

For quality control, Phenol-sulfuric acid method was applied to determine the concentration of polysaccharides. For HPLC analysis, the column condition was performed as: a reversed-phase column (Xbridge C18, 5µm, 250mm * 4.6 mm i.d., Thermo, USA) was used and the mobile phase conditions were acetonitrile (A) and 0.05% Sodium dodecyl sulfate (SDS) in 0.1% acetic

acid (B) using gradient program of 80-60% (B) in the first 5 min, 60% (B) in 5-30 min and 60-40% (B) in the last 20 min (Powell 1985).

A-253 cell culture and treatment

A-253 cells from human epidermoid carcinoma of the submaxillary gland (ATCC, HTB-41) were cultured in McCoy's 5A modified medium (ATCC) supplemented with 10% fetal bovine serum (FBS, Hyclone), 1% (v/v) antibiotic solution (100 unit/ml of penicillin and 100ug/ml of streptomycin, Sigma) and incubated in a humidified 5% CO₂-95% air incubator at 37°C.

Cell viability: To determine the proliferative and anti-apoptosis in vitro, MTT assay method was carried out after the treatment of DP and H₂O₂ on A-253 cells. Fresh FBS-free medium was replaced before each treatment. Cells without any treatment were set as the normal group as the control in the following experiments.

Up-regulation effect of DP: Based on our preliminary experiments, DP of 1ug/ml was added in cell culture for 48h. Cells were then washed and fixed with paraformaldehyde onto glass slides then incubated with rabbit anti-AQP5 (1:500) for overnight at 4°C and goat anti-rabbit IgG-FITC (1:500, Millipore) for 2h at room temperature. Finally, the slides were mounted and examined under a fluorescence microscope.

Anti-apoptosis effect of DP induced by H₂O₂: After the pre-treatment of DP 1ug/ml for 48h, A-253 cells were incubated with H₂O₂ of 500um for 30min. Hoechst 33258 (CalBioChem) was employed to label the nucleolus with 2 mg/ml for 5 min at room temperature. Cells were immediately observed under fluorescence microscopy using a blue reference filter at 450nm.

Animal model and treatment

8-week-old female C57BL/6 mice of inbred strains, weighing 18-20g, were obtained from Laboratory Animal Center of University of Hong Kong. Animals were housed 4 per cage and had free access to water and food. The surrounding temperature and humidity maintained at 22°C and 50% respectively, with consistent light of 12h and 12h dark cycle. Mice were allowed to acclimatize for a

minimum of one week. Mice were randomly divided into four groups used for experiments: normal, model, high dose (HD) and low dose (LD) group (n=8). Based on our preliminary experiments, since the LD group was calculated based on the dosage reported polysaccharides from *Ophiopogon Root* (Wang, Yan et al. 2007), we have found the recovery started to be promising till our high dose, quadrupling the LD. Mice of HD and LD group were administrated with DP at a dose of 20mg/ml and 5mg/ml, respectively. Normal and model group were treated with an equivalent volume of saline (Wang, Yan et al. 2007). Body weight and water intake were measured every 2 days.

Model establishment

As described previously (White and Casarett 1974), with modification (Wang, Yan et al. 2007), a SS model was induced by immunization with submandibular gland (SG) autoantigen. Preparation of the SG autoantigen was conducted on ice. Three C57BL/6 mice were sacrificed by overdose of pentobarbital. The bilateral SG from the mice were immediately removed under sterile conditions, dissected free from fat and connective tissues, weighed and homogenized at 10,000 rpm for 20s in 2ml of sterile saline solution per 100mg SG at 4°C. Sample was further centrifuged at 3000*g for 15 min at 4°C. Supernatant was collected and protein concentration was determined by BCA assay (Sigma) (Abelson JN, Simon MI et al. 1990), then dissolved in PBS, adjusted to 800µg/ml, and emulsified in an equal volume of complete Freund's adjuvant (Sigma) to a concentration of 400µg/ml. On day 0, mice were injected subcutaneously with 0.1ml per mouse of the emulsion. On day 14 the boosting injection was carried out with the same dose of autoantigen emulsified in incomplete Freund's adjuvant (Sigma). Normal mice were immunized with 0.1ml per mouse of PBS.

Histochemistry

All procedures were performed according to the standardized laboratory methods. Specimens of submandibular gland and lung were placed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer sections of each paraffin-embedded tissue were cut and then examined by hematoxylin and eosin (HE) staining for determination of lymphocyte infiltration.

Determination of AQP5 and apoptosis

We adopted the approach of western blot to visualize the expression of AQP5 in organs. Proteins were collected from organs of mice for AQP5 determination. Protein samples were loaded on 12% SDS-polyacrylamid precast gels. All the experiments were carried out in duplicate. After the transfer to nitrocellulose, membranes were then incubated overnight with rabbit anti-AQP5 pAb (AB15858, 1:1000, Millipore). As the identical manner of incubation with secondary antibody, bands were visualized by ECL Advanced Solution (GE Healthcare Life Sciences). Beta-actin protein was determined as the loading control (SC-47778, 1:1000, Santa Cruz).

The intracellular activation of caspase-3 during the apoptotic process was also determined, since caspase-3 was known as an executioner of apoptosis (Williams, Hornig et al. 2008). Based on the method of IgG penetration verified by Sisto (Sisto, Lisi et al. 2006), we examined the apoptosis on A-253 cells by the treatment with diseased sera purified from model mice. For western blot, membranes were incubated overnight with rabbit anti-caspase-3 pAb (9661S, 1:1000, Cell Signaling Technology) and anti-rabbit IgG-HRP (1:1000, Millipore), then visualized by ECL Advanced Solution.

Statistical Analysis

Data were analyzed for normality using the Wilks Shapiro Test. Comparisons was made using the unpaired Student's t-test or the analysis of variance (ANOVA), followed by the Newman-Keuls test. In all instances values of $p < 0.05$ were considered as statistically significant.

RESULT

Quantitative and qualitative analysis of DP

The purity of crude polysaccharides in the water extract is 95.3%. For HPLC analysis, it showed 2 single and symmetrical peaks from DP at the retention time of 60% mobile phase (B), indicating that 2 kinds of proteins may be present in DP (Guoxiang M 1994) (data not shown).

Effect of DP on A-253 Cell Line

Proliferative Effect

Compared with Normal group, DP could stimulate the proliferation of A-253 cells in the dose-dependent manner.

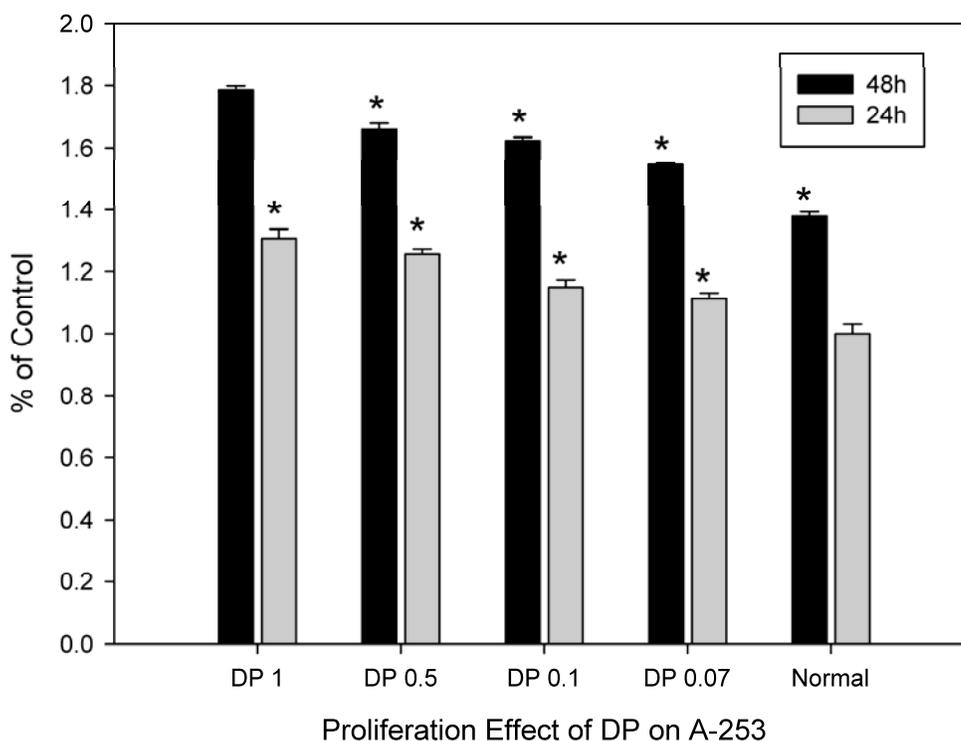


Fig. 1 MTT assay result from proliferative effect of DP. A-253 cells were incubated with the concentration of 1, 0.5, 0.1, 0.07 $\mu\text{g/ml}$ DP for 24h and 48h. The experiment was performed four times independently. All results were reproducible. * $p < 0.05$ compared with model group.

The MTT assay was performed in order to examine the proliferative effect of DP. The MTT reagent is a yellow tetrazolium salt that produces a dark-blue formazan crystal when incubated with viable cells. Therefore, the level of the reduction of MTT into formazan demonstrated the level of A-253 cell metabolism. Fig 1 showed the results from MTT assays of A-253 cultured with DP at various concentrations.

Up-regulation effect

Immunoperoxidase labeling with a rabbit anti-AQP5 antibodies (1:500) was performed on paraffin sections of A-253 cells. Cells were pre-treated with DP of 1ug/ml for 24h. In the normal group, AQP5 antibody labeling was clearly observed and distributed mostly on the membrane (Fig.2).

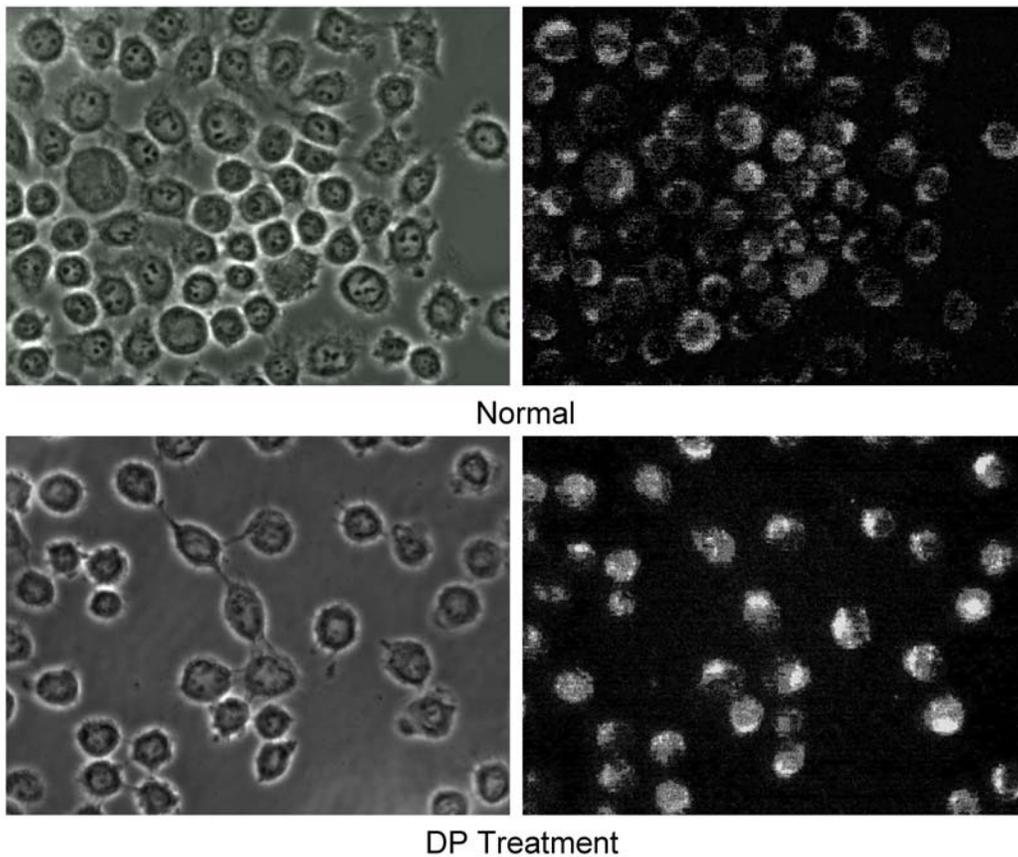


Fig.2 AQP5 up-regulation effect of DP on A-253 cells. There was no morphological change after the DP treatment, but stronger expression of AQP5 on the membrane was observed compared to normal cells.

By contrast, DP treatment showed much more extensive labeling surround the membrane. These results suggested that DP administration could increase the expression of AQP5.

Anti-apoptosis effect induced by H₂O₂ treatment

To evaluate the cytoprotective effect of DP on apoptosis induced by H₂O₂, the loss of cell viability of A-253 were measured as in Fig3. As shown in the Figure, A-253 cells treated with DP revealed a recovery after exposure to H₂O₂. Corresponding to the proliferation, DP prevented H₂O₂-induced cell death also in the manner of dose-dependent.

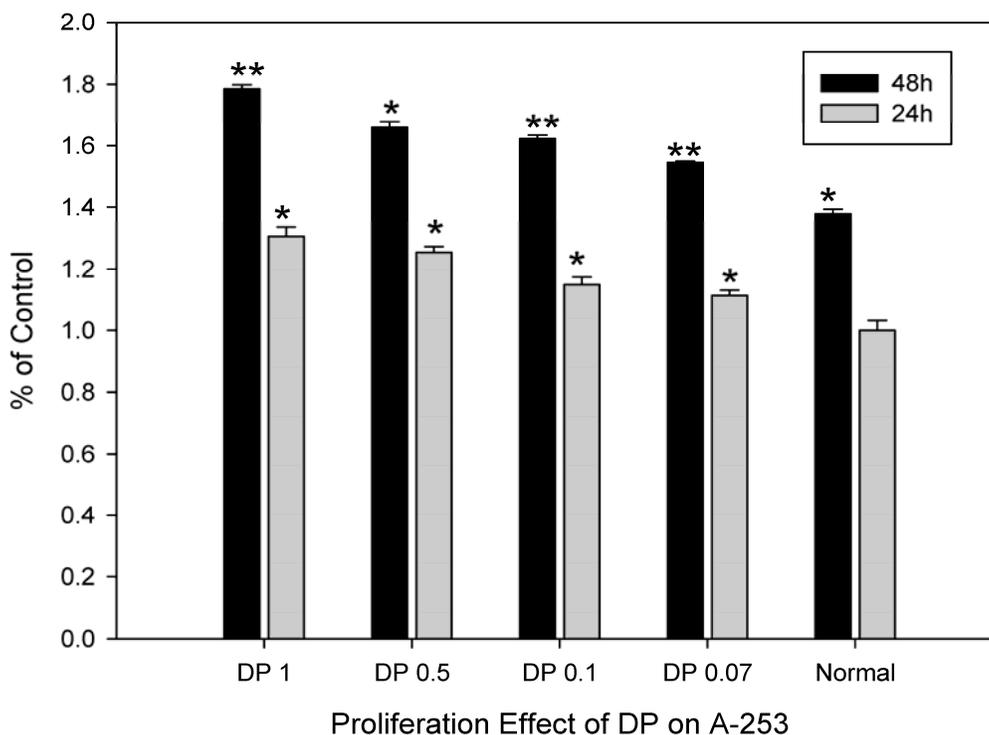


Fig. 3 MTT assay result from anti-apoptosis effect of DP induced by H₂O₂ treatment. A-253 was pretreated with 1ug/ml DP before the addition of H₂O₂ of 1mm and 2mm respectively. This experiment was performed three times independently. Results were reproducible. *p<0.05, and **p<0.01 compared with model group.

Moreover, the nuclei of the A-253 cells were stained with Hoechst 33258 and assessed by microscopy to visualize the apoptosis. The microscopic pictures in Fig. 3A revealed that the normal cells had intact nuclei, while the H₂O₂-treated cells (Fig.4.C, E) showed nuclear fragmentation and collapse, which was indicative of apoptosis. However, cells added with DP for 24h prior to H₂O₂ treatment, appeared less fragmentation and collapse of nuclear (Fig.4 D, F).

Animal Model

Body weight/ water in take

We monitored the body weight of SS mice during the experiment. As in Fig.5A, the mice of normal group showed steady gain of body weight during the experim-

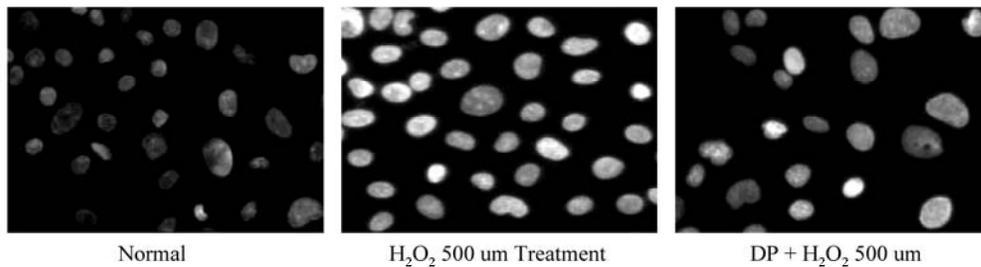


Fig.4 Anti-apoptosis effect of DP on A-253 cells against H₂O₂ induction. Cells were treated with DP at 1ug/ml for 24h before the addition of H₂O₂. Incubated with H₂O₂ treatment of 500um for 30min, cells were observed under the fluorescence microscope

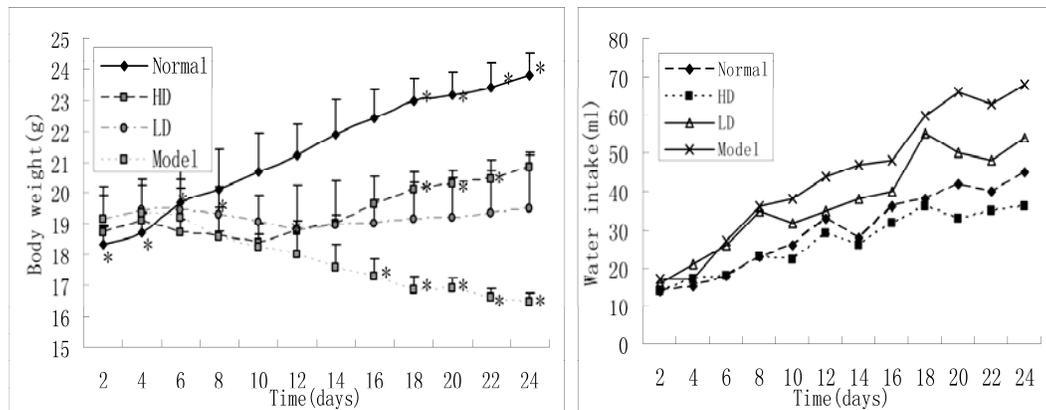


Fig. 5 Effect of DP on model mice of body weight (A) and water intake (B). HD, LD and model group were immunized with SG autoantigen to induce autoallergic SS model. Each point represents mean±S.E.M. All results were reproducible. *p<0.05 compared with model group

-ental period. However, the model mice resulted in a gradual loss and this reduction became significant from day 8 compared to normal mice, and this

phenomenon was gradually abolished after the treatment of DP. Additionally, the HD group was found more effective than LD group respect to the model group.

As shown in Fig5B, water intake in normal group gradually increased with the body weight, and so did the HD and LD group. However, Model group showed more needs of water intake compared to normal group. While the treatment, especially HD group had apparent effect on the water intake and even had less need than normal mice.

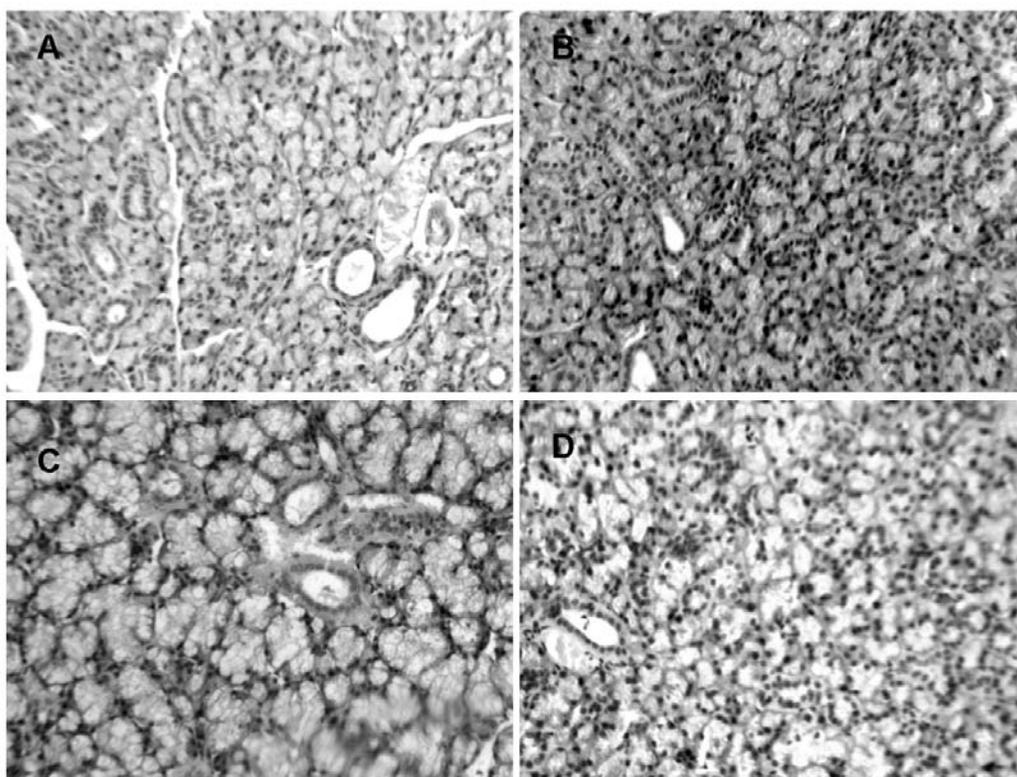


Fig.6 Effect of DP on lymphocytic infiltration of SG in experimental SS mice. Sections were stained with haematoxylin and eosin. Photomicrographs showing significant lymphocytic infiltration based on the presence of lymphocytic foci (B) compare to normal mice (A) and much less infiltration of treatment mice with HD (C), and LD (D). Original magnification: $\times 100$

Histopathologic Findings

The presence and degree of pathology in the form of lymphocytic infiltrates varied among the individual mice, but immunized mice clearly had infiltrates, whereas normal mice had little or none in the salivary glands. As Fig.6 indicated, it apparently showed inflammatory cells observed mainly around the ducts in the SG of auto-immunized animal. On the other hand, compared to normal mice, DP treatment (Fig.6C: high dosage, 6D: low dosage) appeared much less infiltration.

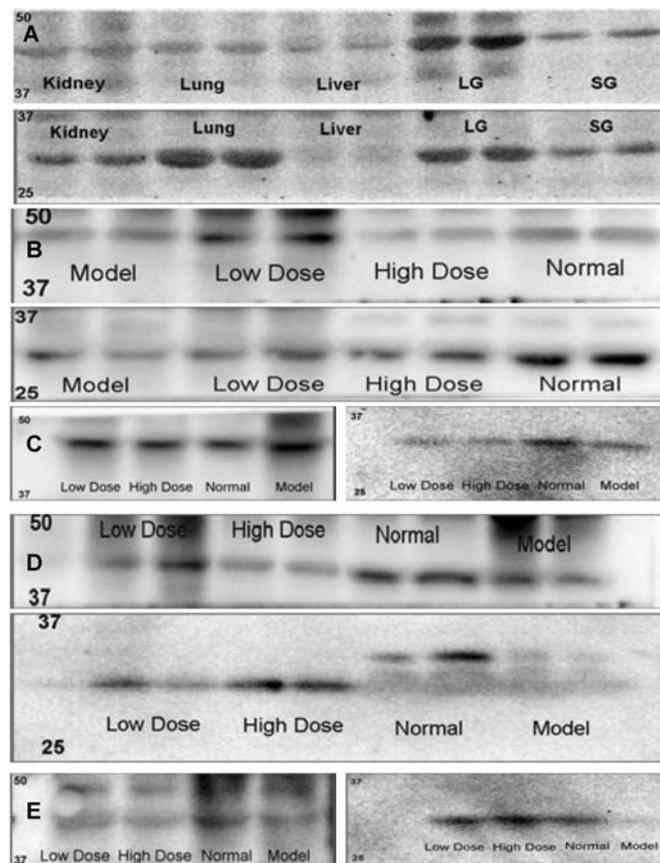


Fig.7 Expression of AQP5 inSS model mice. Mice were sacrificed for tissue collection. Total proteins were isolated with RIPA. Expression of AQP5 (28kDa) was investigated by western blot. (A) Existence of AQP5 in collected organs; (B) Expression of AQP5 in SG; (C) Existence of AQP5 in LG; (D) Existence of AQP5 in Lung; (E) Existence of AQP5 in kidney.

Expression of Aquaporin-5

AQP5 is a protein with a molecular weight of 28 kDa. Expression of AQP-5 was observed in sub- -mandibular gland, l- -acrimal gland (LG), lung, liver and kidney that related to body fluid secretion. Fig.6A showed the AQP5 existence in these organs, indicating there was merely AQP5 in the liver of normal mice. Therefore, except liver, we examined the difference in the organs of AQP5 expression between each group. All results in Fig.6 showed that, AQP5 expressed less in SS model mice compare to normal group, and after DP administration, HD group showed significant recovery, which could be concluded in Fig. 7 as the measurement of Fig. 6.

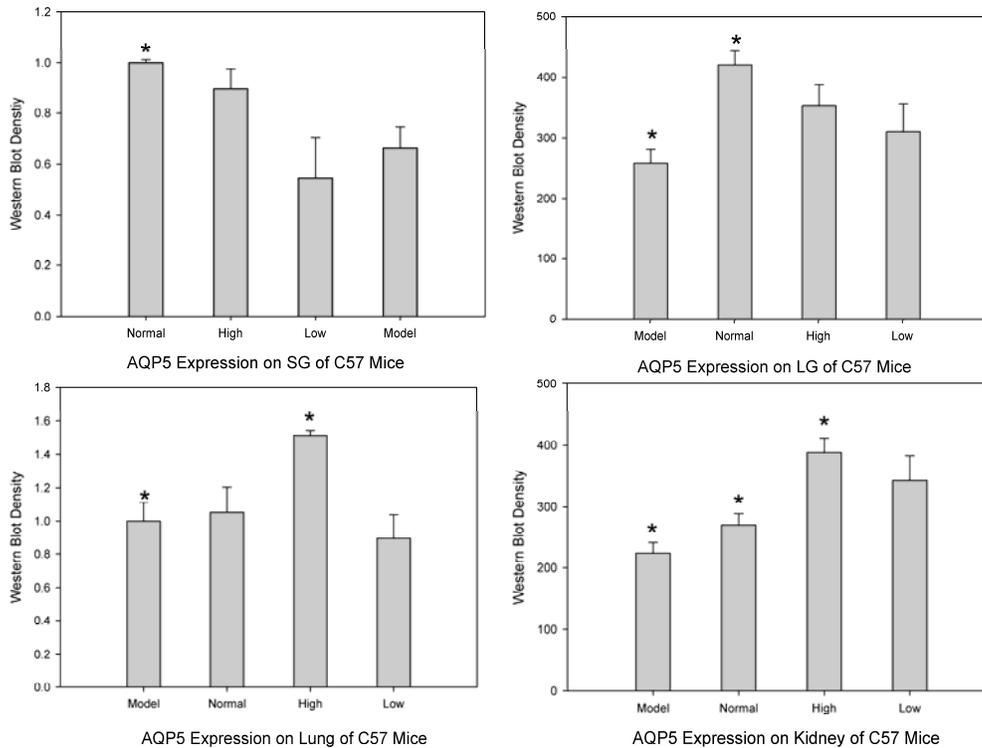


Fig. 8 Expression of AQP5 in organs of SS model. As indicated, group had up-regulation effect on AQP5 compared to experimental SS model. All results were reproducible.

Activity of Caspase-3 during the apoptosis

We examined the cleaved caspase-3 by western blot as in Fig. 8. The active caspase-3 subunits were recognized by anybodies and the presence of the 19 kDa band was evaluated in A-253 cells treated with sera and DP. Measurements of the intensity of protein expression indicated that DP could inhibit the diseased sera induced active form of caspase-3. Beta-actin protein was determined as the loading control. Results showed significant activity of caspase-3 cleavage in model group compared to normal group. In the dose-dependent manner, DP had the effect to inhibit the diseased sera induced apoptosis.

DISCUSSION

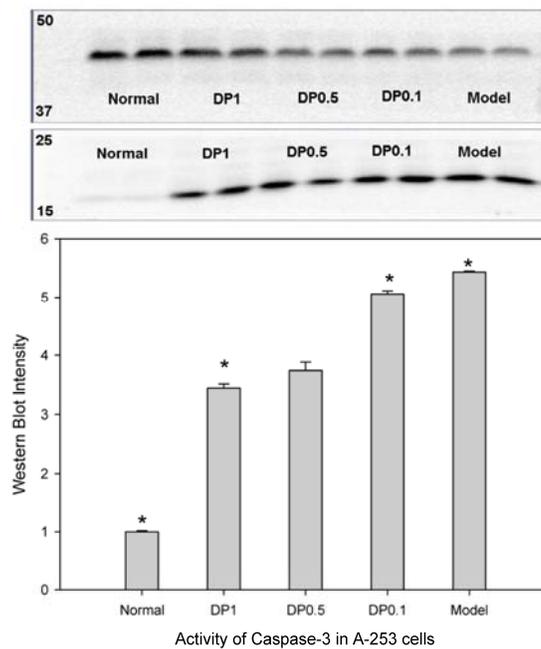


Fig. 9 Sera from model mice induced apoptosis in A-253 cells via caspase-3 activation. Caspase-3 cleavage was analyzed and measured. This experiment was performed three times independently. Results were reproducible. * $p < 0.05$ compared with model group.

In our present study, we determined the preventive effect of DP in vivo and in vitro. DP may behave in the ways of up-regulation on the expression of AQP5, inhibition of cell apoptosis and proliferation of healthy cells. Associated with the regulation of body fluids, the changing expression of AQP5 may result in the water deficiency in the related organ, hence the induction of cell apoptosis in SS, which may penetrate the membrane via Fcγ receptors (Lisi, D'Amore et al. 2008). According to the theory of Chinese medicine, kidney plays the major role affecting the quality of body fluids (SW 2005), which had been verified in western medicine (RF 1963), that kidneys are commonly described as excretory organs regulating volume, concentration and composition of internal fluid environment. Cases were reported that the involvement of kidney was an extraglandular manifestation of SS (Bossini, Savoldi et al. 2001), derived from acute renal failure (ARF) (Sutton and Molitoris 1998). Furthermore, previous study showed that rates of transepithelial fluid transport per unit membrane surface area in these epithelia were substantially lower than proximal tubule and salivary gland (Tradtrantip, Tajima et al. 2009). Since it could be the body fluids impairment causing the dryness in organs, we examined the expression of AQP5 in the kidney of SS model mice (Fig7E). Compared with normal mice, SS model showed lower expression water channel protein AQP5, which may lead to the primary body fluid or its transportation deficiency in the internal fluid environment. Thus, the involvement of kidney seemed to be correlated with other organs in water transportation. However, this issue was not fully investigated in SS which was defined as the systemic disease, that we would further study the relationship of the secretion between kidney and salivary gland. Moreover, as the previous findings by Hamid Rabb (Rabb, Chamoun et al. 2001), down-regulation of AQP5 for body fluids transportation was discovered in the kidney-lung dysfunction in ARF associated with SS, highly related to death of patients. Based on this support, we also found this similar phenomenon in our SS model mice (Fig7D). Associated with saliva flow in the common diagnosis of SS, and increased water intake of experimental SS model, AQP5 in lacrimal gland and submandibular gland were also determined (Fig7B, C) (Tsubota, Hirai et al. 2001). In contrast, after the treatment of DP, not only AQP5 expression in all the above organs had certain increase (Fig7, 8), body weight and water intake also showed the recovery of mice (Fig 5). Clear evidence by fluorescence microscopy method also showed the up-regulation effect of DP on AQP5 in vitro test (Fig.2). Probably, such neural modulation as the role DP might play as the agonist of muscarinic acetylcholine receptor to increase the expression of AQP5 (Kim, Park et al. 2009).

Nevertheless, the mechanism of DP on body fluid promotion should be further studied. Although the purification can not perfectly give the clean DP, for the above results, we may conclude that DP could be the key ingredient which ameliorates the symptom by increase the expression of AQP5 and protection from apoptosis in related organs. Meanwhile, proliferation effect of DP had been proved by MTT test on A-253 (Fig.1). This result indicated the replacement of impaired cells simply by cell proliferation could also contribute to rehabilitation from SS. Additionally, since apoptosis could be another possible mechanism described in the auto-immune disease (Sisto, Lisi et al. 2006), penetration method was established for cell model by triggering the apoptosis and DP had distinctive effect to protect cells from this induction (Fig. 9). Also, compared with another classic apoptotic pathway in vitro, DP could protect cells from H₂O₂-induced apoptosis by maintaining the cell viability and morphology (Fig3, 4). Therefore, besides the stimulation of AQP5, DP may inhibit the damage from apoptosis in SS. The purity of DP was only determined on the basis of glucose, so the practical polysaccharide purity is considered to be higher. The main impurity was found as protein (4.7%) due to the incomplete removal by trichloroacetic acid. But the differences between partial presence and complete removal of protein component should be further studied as there was no report associated with the bioactivity of the protein in DO. As the conclusion, DP, with the effect of promotion of body fluid could be a potential treatment for SS by up-regulation on AQP5 and protection from apoptosis.

Therefore, a better understanding of DP responsible for promotion of body fluids and nourishing yin may allow further investigation on the protection from consequent impairment of the secretory function.

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