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Dendrobium officinale polysaccharides ameliorate the abnormality of aquaporin 5, pro-inflammatory cytokines and inhibit apoptosis in the experimental Sjögren's syndrome mice

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ABSTRACT

Sjögren's syndrome (SS) is a chronic autoimmune disease with exocrine glands disorder. Our previous work demonstrated the protective effect of *Dendrobium officinale* polysaccharides (DP) both on the phenotypes of patients and animal model with SS. In this study, we expand these observations to explore the possible mechanisms. The experimental SS mice model was established with or without the administration of DP (20 mg/ml). The time frame of lymphocytes infiltration, apoptotic indicators such as Bax, Bcl-2 and caspase-3 were determined in submandibular gland (SG), as well as the subsequent mRNA expression of cytokines such as TNF-α, IL-1beta and IL-6. The expression and localization of aquaporin-5 (AQP-5) was examined by Western blot and immunofluorescent staining. As the result, DP could suppress the progressive lymphocytes infiltration and apoptosis, and balance the chaos of pro-inflammatory cytokines in the SG. Further, DP ameliorated the abnormalities of AQP-5 and maintained its functional importance of saliva secretion. In addition, the protection of AQP-5 by DP from human TNF-α was supported by an in vitro study on A-253 cell line. Our study further supported the efficacy of DP as the promising candidate for the therapy of SS.

1. Introduction

The genus *Dendrobium*, consisting of 1100 species or more, is the one of the largest genera in Orchidaceae [1]. With the rising interest [2,3], *Dendrobium officinale* Kimura et Migo (DO), the most precious species, is currently specified in the China Pharmacopoeia (2010) as an individual entry among all the 76 species in China, including 74 species and 2 varieties. DO is an epiphytic perennial herb endemic to China. It is mainly distributed in the tropical and subtropical areas such as Anhui, Zhejiang, Fujian, Guangxi, Sichuan and Yunnan provinces of China at an elevation of about 1600 m on the mountain [4]. For its clinical use, DO has been applied for maintaining tonicity of stomach and promoting the body fluid production [5]. Polysaccharide, as the major active constituent and rich in *Dendrobium* species [6], is recently reported to be positive in the immunomodulation [7]. However, no investigation regarding the pharmacological effect of *D. officinale* polysaccharide (DP) is available to the best of our knowledge.

Sjögren's syndrome (SS), a chronic autoimmune disorder of the exocrine glands with associated lymphocytes infiltration, is usually clinically manifested as dry eyes and dry mouth [8]. The dysfunction of the exocrine indicated the severe inflammation, and possible apoptosis. The pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and 6 [9,10] in the exocrine glands in response to immune-mediated inflammation, are found over-expressed in the SS patients. In addition, the abnormality of aquaporin 5 (AQP-5) in saliva secretion is also usually seen in the SS patients. The expression of AQP-5 is functionally important, supported by the fact that salivary fluid secretion was decreased by 50% in AQP-5 knockout mice [11]. The specific expression of AQP-5 in the apical plasma membranes (APM) of acinar cells in the submandibular gland (SG) has been implicated to mediate the water transport into saliva [12].

Our previous study showed the protective effect of DP on patients with SS [5], and further, we proved its efficacy on an experimental SS mice model [13]. In both of the studies we found DP could restore the saliva secretion. However, the underlying mechanism that whether DP can suppress the inflammatory cytokines and apoptotic indicators is unknown. The present studies were designed to examine the possible mechanism that DP may protect the SG from the progressive destruction in the experimental SS model mice.

2. Materials and methods

2.1. Plant material and polysaccharide extraction

The 20 g dry stems of *D. officinale* (batch no. DO20080112-3) were collected from Zhejiang Province, China, and identified by experts in

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the Department of Pharmacognosy, China Pharmaceutical University. DP extraction was carried out as previously described [13]. Briefly, dry stems were grinded into fine particles (100-mesh). The powdered stems were pre-extracted by acetone and subsequently methanol in a Soxhlet system for 8 h, or till the elution was colorless to remove pigments and impurities. The residues were isolated by 400 ml hot distilled water for 3 times and gave a crude extract. Proteins were further removed by trichloroacetic acid and the supernatant was neutralized and concentrated to 200 ml. The crude extracts were then submitted to graded precipitation with 80% ethanol for three times. The crude polysaccharides were then applied to Sephadex G-100 and G-200 columns separately. Columns were equilibrated in PBS (flow rate of 30 ml/h). Each fractionated elution was monitored at wavelengths of 206 and 254 nm. The void volume fractions were combined and analyzed by phenol–sulfuric acid method. The purified elute was lyophilized into fine powders. The yield of DP extraction is 22.6% with the purity of 99.47% determined by the phenol–sulfuric acid method [14]. All the dissolved DP was filtered through a 0.22 μm membrane (Jet Biofil) before use. An endotoxin detection kit (Pyrosate 0.25 EU/ml, Association of Cape Cod, Inc.) was used to ensure that DP was free from endotoxin.

2.2. Preparation of PMP derivatives of monosaccharide

Hydrolysis of polysaccharides was carried out as previously described by Yang et al. [15]. Briefly, DP sample (2.0 mg) was dissolved in 2 ml of 4 M trifluoroacetic acid in an ampoule, and kept in an oil bath at 110 °C for 8 h under the nitrogen atmosphere. The reaction mixture was cooled to room temperature and centrifuged at 1000 rpm for 5 min. The supernatant was collected and neutralized to pH 7.0 with 0.4 M NaOH. Subsequently, hydrolyzed DP samples were labeled with 1-phenyl-3-methyl-5-pyrazolone (PMP). The PMP derivatization of monosaccharides was further carried out as described by Zhang et al. [16]. Briefly, 7 monosaccharide standards or the hydrolyzed samples of DP were dissolved in the 0.4 M aqueous NaOH (50 μl) and a 0.5 M methanol solution (50 μl) of PMP. Each mixture was allowed to react at 70 °C for 30 min, and neutralized with 0.4 M HCl (50 μl) after cooling to room temperature. The solution was dissolved in chloroform (1 ml). After vigorous shaking and centrifuging, the organic phase was carefully discarded to remove the excess reagents. The extraction process was repeated three times and the aqueous layer was filtered through a 0.22 μm membrane and diluted with water (150 μl) before HPLC analysis. The concentration of samples prior to injection into the HPLC system was about 0.5 mg/ml.

2.3. HPLC analysis

The analysis of PMP-labeled monosaccharides was carried out on the Waterson system equipped with a Waters 600S LCD pump, a Waters 717 plus auto-sampler and Waters 996 PDA detector. The analytical column was a reversed-phase X-bridge C18 column (5 μm, 250 mm × 4.6 mm i.d., Thermo, USA). The wavelength for UV detection was 250 nm. Elution was carried out at a flow rate of 0.8 ml/min at room temperature. The mobile phase consisted of 50 mM sodium phosphate (PH 6.9) with 15% (A) and 40% (B) acetonitrile, using a gradient elution of 0–8–20% buffer B by a linear increase from 0–10–30 min.

2.4. Mice

Female, 8-week-old C57BL/6 mice of inbred strains, weighing 18–20 g, were obtained from Laboratory Animal Center of University of Hong Kong. Mice were bred and maintained at the Laboratory Animal Unit under specific pathogen-free conditions. The ambient temperature and humidity were maintained at 22 °C and 50%, respectively. The lighting conditions were 12 h of light and 12 h of darkness. Mice were allowed to acclimatize for a minimum of one week.

2.5. Induction of SS model

The SS model was induced by immunization with submandibular gland autoantigen as previously described [13]. Briefly, five C57BL/6 mice were sacrificed by using an overdose of pentobarbital. The bilateral SG from the mice was immediately removed under sterile conditions, dissected free from fat and connective tissues, weighed and homogenated at 10,000 rpm at 4 °C for 20 s in 2 ml of sterile saline solution per 100 mg SG. The sample was further centrifuged at 3000 × *g* for 15 min at 4 °C. The supernatant was collected and protein concentration was determined by the BCA assay (Sigma-Aldrich) [17], then adjusted to 800 μg/ml in PBS, and emulsified in an equal volume of Freund's complete adjuvant (Sigma-Aldrich) to a concentration of 400 μg/ml. On day 0, each of the mice was injected subcutaneously with 0.1 ml of the emulsion. On day 14, the booster injection was carried out with the same dose of autoantigen emulsified in Freund's incomplete adjuvant (Sigma-Aldrich). Control mice were immunized with 0.1 ml of PBS per mouse. Mice were randomly divided into three groups as control, model and DP. Mice of DP group were administrated with DP at the dose of 20 mg/ml in physiological saline 200 μl per day, with control and model group receiving saline alone. Water intake was measured every 2 days. The protocol for animal study had been approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong meeting the standards required by the UKCCCR guidelines (Project number CULATR-1727-08).

2.6. Serum collection

On day 25, mice were sacrificed; blood samples collected in endotoxin-free non-additive silicone coated tubes were allowed to clot at room temperature for 30 min before centrifugation (1500 × *g*, 4 °C, 10 min) and the serum was removed and stored at −80 °C until analyzed. After blood sampling, SG from each mouse was excised and weighed to calculate the SG index (calculated as organ weight/body weight) × 1000. The SG was then used for histological examination.

2.7. H&E and immunofluorescent staining

After blood sampling, SG from each mouse was fixed in a 4% paraformaldehyde (PFA) solution. Paraffin-embedded tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) for determination of lymphocyte infiltration. Lymphocytes in H&E-stained SG sections were measured by the use of IPP software (ver4.5, Image-Pro Plus, Media Cybernetics, USA) and compared with control group sections.

For immunofluorescent staining, thin sections were blocked by the 1% goat serum and incubated with AQP-5 pAb (1:250) followed by staining with goat anti-rabbit IgG-FITC (1:400, Millipore). Fluorescence microscopy employing a Zeiss fluorescent microscope was used for image capture. AxioVision digital imaging system was used to optimize signal-to-noise ratio.

2.8. Gelatin zymography and MMP-9 activity assay

Gelatinase level in the serum samples of mice was measured by gelatin zymography, as previously described with slight modification [18]. Diluted serum (all at a dilution of 1:10) or SG samples were incubated in loading buffer without β-mercaptoethanol for 30 min at room temperature and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Sigma-Aldrich). MMP standard and protein marker were preloaded to indicate the molecular weight. After electrophoresis, the proteins were renatured