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Synergetic effect of *Andrographis paniculata* polysaccharide on diabetic nephropathy with andrographolide

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ABSTRACT

A water-soluble polysaccharide (APP), with a molecular weight of 4.6×10^4 Da, was isolated from *Andrographis paniculata* and gas chromatography (GC) analysis showed APP was composed of galactose, mannose, fucose, arabinose and rhamnose with molar ratios of 15.4:2.5:4.3:1.5:1.6. The synergetic effect of APP in combination with andrographolide on renal complication in streptozotocin (STZ) induced diabetic mice was investigated. Wistar rats were made diabetic by injection of STZ, and APP and/or andrographolide was administered to diabetic mice for continuous two weeks. APP plus andrographolide not only increased the body weight and creatinine clearance rate (Ccr), but also decreased the levels of serum creatinine, serum urea nitrogen, urinary albumin excretion (UAE), serum urea and blood glucose in diabetic rats, as well as the relative kidney weight. In summary, APP plus andrographolide can improve the metabolic abnormalities of diabetic mice and prevent or delay the progression of diabetic renal complications, which may be useful as a therapeutic agent for inhibiting the progression of diabetic nephropathy.

1. Introduction

The prevalence of diabetes mellitus and its clinical complications are rapidly increasing worldwide. Diabetic nephropathy (DN) has already become the leading cause of end-stage renal disease in developed countries and is thus forming an increasing clinical problem [1]. To prevent and treat diabetic nephropathy, current methods using agents such as angiotensin-converting enzyme inhibitors, angiotensin-II receptor blockers and antihypertensive drugs have been tried in clinical practice [2–4]. Despite these treatments, large numbers of patients still develop intractable diabetic nephropathy. This has prompted much interest in the use of traditional medicines for the treatment of this condition today.

Andrographis paniculata (Burm. f.) Nees, commonly known as 'king of bitters', is a herbaceous plant belonging to the Family Acanthaceae [5]. It is a traditional medicine widely used in Asian countries for the treatment of cold, fever, laryngitis and infection. The extract of the plant is a rich source for flavonoids and labdane diterpenoids [6,7]. Of the diterpenoids, andrographolide, a bitter diterpenoid lactone, is the primary active ingredient in *A. paniculata* [8], constituting 70% of the plant extract fraction [9,10]. Andrographolide are reported to have a broad range of biological activities, such as anti-inflammatory [11,12], anti-allergic [13], anti-platelet aggregation [14], hepatoprotective [15,16] and anti-HIV activities [17,18], and others. Besides these, it had been documented that the ethanol or aqueous extract of *A. paniculata* could decrease the blood glucose level in normal rats or STZ-diabetic rats [19].

Preliminary animal experiment identified that andrographolide failed to attenuate the renal damage in diabetic mice. Many documents had reported that polysaccharide could dramatically improve the renal complication in diabetes [20–22]. Thus there is a need to assess whether *A. paniculata* polysaccharide (APP) could synergize its antidiabetic ingredient andrographolide to relieve STZ-induced diabetic nephropathy in mice.

2. Experimental

2.1. Materials and reagents

Whole plant material of *A. paniculata* was purchased from local market in Chengdu. Sepharose CL-6B and DEAE-cellulose was purchased from Amersham Pharmacia Co. (Sweden). Streptocotocin

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(STZ), andrographolide (purity 98%) and T-series dextran were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

2.2. Preparation of neutral polysaccharide from A. paniculata

The whole *A. paniculata* was extracted with 3 volumes of 95% EtOH at 75 °C for 5 h under reflux to remove lipid, and the supernatant was removed. Then the residues were dried and extracted with distilled water at 80 °C for 3 times and 2 h for each time. The whole extract solution was filtered and centrifuged before concentration, and then submitted to precipitation with three volumes of ethanol at 4 °C overnight. The precipitate collected by centrifugation was suspended in distilled water to remove the protein by the Sevag method [23], and exhaustively dialyzed against water for 2 days. Finally the concentrated supernatant was lyophilized to give crude *A. paniculata* polysaccharides, coded as CAPP. The CAPP was dissolved in distilled water and filtered. After the filtering solution was loaded onto DEAE-cellulose anion-exchange chromatography column (3 cm × 30 cm), the column was eluted with continuous gradient concentrations of NaCl aqueous solution (0.15 → 2 M, pH 6–7) stepwise at 4 mL/min, and each tube fraction was collected by the automated fraction collector. Total carbohydrate and protein content of each tube was measured at 490 nm by Dubois's method and Micro-Kjeldahl method, respectively. The water eluted fraction was collected, dialyzed and further purified on a Sepharose CL-6B column (2.6 cm × 100 cm) with 0.15 M NaCl at a flow rate of 1 mL/min to yield one main fraction, which was collected, dialyzed and lyophilized to give white purified polysaccharide fraction (APP).

2.3. General method

The total carbohydrate content was determined by the phenol–sulfuric acid method using D-glucose as the standard [24]. Protein was measured by the Micro-Kjeldahl method [25]. Uronic acid content was determined according to a m-hydroxydiphenyl colorimetric method by using D-galacturonic acid as the standard [26].

2.4. Monosaccharide composition

Gas chromatography (GC) was used for identification and quantification of the composition of polysaccharide. Samples were hydrolyzed and acetylated according to the method by Lehrfeld [27]. Firstly, the samples (10 mg) were hydrolyzed with 2 M TFA (2 mL) at 120 °C for 2 h, and the excess acid was completely removed by co-distillation with ethanol. Then the hydrolyzed product was reduced with KBH_4 (30 mg), followed by neutralization with dilute acetic acid and evaporated at 45 °C after adding 1 mg myo-inositol and 0.1 M Na_2CO_3 (1 mL) at 30 °C with stirring for 45 min. After desalted by cation exchange resin, filtered by quantitative filter paper and neutralized by evaporating with methanol, the reaction solution was mixed with 1 mL n-propylamine and anhydrous pyridine for 30 min at 55 °C. Finally dried sample was mixed into 0.5 mL anhydrous pyridine and acetic anhydride for 1 h at 90 °C [28,29] and were analyzed by GC as previously mentioned, with myo-inositol as the internal standard [30].

2.5. Homogeneity and molecular weight determination

The homogeneity and molecular weight of the polysaccharide were identified by high-performance gel permeation chromatography (HPGPC) on a Waters-2414 HPLC apparatus, equipped with a Waters-1515 RI detector and an Ultrahydrogel™-500 analytical column. 20 μL of sample solution (5 mg/mL) was injected in each run, with ultra-pure water as mobile phase at a flow rate of 0.5 mL/min. Different average molecular weights of standard dextrans, T-2000, T-500, T-70, T-40, and T-10, were prepared as 0.1% (w/v) solutions and 20 μL of solutions were injected in each run, and then the retention time was plotted against the logarithms of their respective molecular weights. A calibration curve was prepared from the known MW Dextran T system standards. According to the retention time of sample, its molecular weight was calculated by the calibration curve equation. The purity and homogeneity were judged by the peak shape.

2.6. Animals

Five-weeks-old male Wistar rats (120–130 g) were purchased from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). We followed standard animal experimental procedures approved by the Animal Ethics Committee of the Fourth Military Medical University. All animals were housed in an air-conditioned room at 23 ± 1 °C with a 12-h light/dark cycle and allowed ad libitum access to a standard pelleted diet and water. Before inducing the diabetic animal mode, all the mice were acclimatized for one week.

2.7. Experimental design

After 1-week acclimatization, the rats were intraperitoneal injected with STZ (50 mg/kg body weight) in freshly prepared citrate buffer (0.4 M, pH 4.5) to induce diabetes after an overnight fast. Diabetic rats were confirmed by measuring the 4-h fasting blood glucose level from the tail vein at 72 h after injection with STZ. Animals with a blood glucose level above 16.7 mM were considered to be diabetic and were used in the study [31].

A total of 50 rats were used and divided into 5 subgroups: (I) non-diabetic rats received with citrate buffer only (NC, $n = 10$); (II) diabetic rats received with citrate buffer only (DC, $n = 10$); (III) diabetic rats received with APP (D+APP, 100 mg/kg BW/day, $n = 10$); (IV) diabetic rats received with andrographolide (D+Andro, 10 mg/kg BW/day, $n = 10$); (V) diabetic rats received with APP plus andrographolide (D+APP+Andro, 100 mg/kg plus 10 mg/kg BW/day, $n = 10$). Both normal rats and STZ-diabetic rats receive oral treatment once daily for continuous 2 weeks.

After 14 days of the administration period, the mice were individually housed in metabolic cages for 24 h for urine collection. Then blood samples were collected from the abdominal aorta under sodium pentobarbital anesthesia (50 mg/kg body weight, intraperitoneally), and then the serum was immediately separated from the blood samples by centrifugation for measurement of the biochemical parameters. Prior to sacrifice, the mice were weighted on the balance. The weight of the left kidney at sacrifice was measured in grams and was regarded as the absolute weight. In addition, the ratio of the kidney weight compared with the body weight at sacrifice was calculated as the relative kidney weight as follows: relative kidney weight (%) = [the weigh of kidney/body weight at sacrifice] × 100. Each one of the removed kidneys was stored at −80 °C and another each of the ones was fixed in 10% formalin solution. All animal procedures were in accordance with guidelines set by the Animal Experiment Committee of the Fourth Military Medical University.

2.8. Determination the level of blood glucose in mice

Levels of serum glucose was measured by using the commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China)