Biomedicine & Pharmacotherapy 98 (2018) 643–650

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Protective effects of the Chinese herbal medicine prescription Zhujing pill on retina of streptozotocin-induced diabetic rats

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ARTICLE INFO

Keywords:
Zhujing pill
Diabetic retinopathy
Inflammation
Oxidative stress

ABSTRACT

Objective: In this study, we investigate the protective effects of the Chinese herbal medicine prescription Zhujing pill (ZJP) on the development of diabetic retinopathy (DR) and explore the potential mechanisms.
Materials and methods: Zhujing pill extract (ZJPE) was prepared, and the main components were identified by high-performance liquid chromatography (HPLC). Several serum and tissue (retina) parameters were measured, such as levels of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), intercellular adhesion molecule 1 (ICAM-1), vascular endothelial growth factor (VEGF), interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), advanced glycation end products (AGEs), and high sensitivity C-reactive protein (hsCRP). Aldose reductase (AR) activity and blood-retinal barrier (BRB) breakdown were determined. Finally, retinal electrophysiology and morphological changes were assayed.
Results: In STZ-induced DM rats, ZJPE treatment restored the body weight decrease. DM rats showed decreased levels of SOD and GSH-Px and increased levels of IL-6, TNF-α, hsCRP, and MDA, whereas all these changes were significantly reversed by ZJPE administration. ZJPE alleviated BRB breakdown. Furthermore, ZJPE also alleviated the retinal electrophysiology changes and impaired morphology of the retina and lowered the high levels of TNF-α, IL-1β, ICAM-1, VEGF, AGEs, and AR in the retina.
Conclusions: ZJPE treatment attenuated the progression of DR in STZ-induced diabetic rats.

1. Introduction

Diabetic retinopathy (DR) is known to be the most common and serious complication of diabetes mellitus (DM), and it is a top cause of acquired blindness in developed countries, greatly affecting the quality of life and the survival of diabetic patients [1,2]. It has been reported that nearly all type 1 diabetes patients and about 60% type 2 diabetes patients have retinopathy after 20 years [3]. Although the precise mechanisms of DR remain unclear, numerous clinical and laboratory investigations have identified inflammation and hyperglycemia-induced oxidative stress as important mechanisms in the pathogenesis of DR [4,5].

In recent years, using herbal medicines in the treatment of chronic disease has received the attention of researchers all over the world due to an increase in a wide range of biological activity and herbal medicines' lack of side effects [6]. Zhujing pill (ZJP) is a combination of crude extracts from three medicinal plants, including Cuscuta Semen, Plantaginis Semen, and Radix Rehmanniae Praeparata. Cuscutae Semen (Tu-Si-Zi) is the dried ripe seed of *Cuscuta Chinensis* Lam. It has been recorded in Chinese pharmacopeia as useful for toning the liver and kidney and improving eyesight medicine. Plantaginis Semen (Che-Qian-Zi), derived from the dried ripe seed of *Plantago Asiatica* L.and *Plantago Depressa* Willd., is a widely used traditional drug for improving blurred vision or internal oculopathy [7]. Besides, pharmacological investigation has revealed that phenolic antioxidants extract from Plantaginis Semen has potential benefits in the prevention and progression of DR [8]. Radix Rehmanniae Praeparata (Shu-Di-Huang) is the processed root product of *Rehmannia Glutinosa* Libosch. The experimental evidence also suggests that catalpol from Radix Rehmanniae Praeparata exerts an anti-diabetic effect on diabetic rats [9]. In clinical practice for

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https://doi.org/10.1016/j.biopha.2017.12.071
Received 1 September 2017; Received in revised form 29 November 2017; Accepted 15 December 2017

thousands of years in China, ZJP has been widely used in juvenile myopia, central serous chorioretinopathy, age-related macular degeneration, primary retinitis pigmentosa and retinal light damage [10].

It has recently been predicted that except for in the cases of oxidative stress and inflammatory components, multiple biochemical pathways have attributed to the development of DR, including hyperglycemia and increased activity of aldose reductase (AR) and protein kinase C(PKC) [11], as well as promoting increased glycation end products (AGEs) [12]. To further explore the protective effects of ZJP and the possible mechanisms behind these effects, we undertook the present study to examine the effects of ZJP on retinal structure and function, and to evaluate the influence of ZJP on biomarker levels in serum and retina, such as pro-inflammatory cytokines, reactive oxygen species (ROS) markers and the expression of relevant proteins in streptodotoxin (STZ)-induced DM rats. Our findings might provide new insights about the inhibitory effects of ZJP on the development of DR.

2. Materials and methods

2.1. Preparation of ZJPE

ZJP consists of the following three herbs: 56% of dried seed of Cuscuta Semen, 11% of dried seed of Plantaginis Semen, and 33% of root of Radix Rehmanniae Praeparata. These herbs were supplied by Medicinal Material Company (Shaanxi province, China) and authenticated by one of the authors, Prof. Jianwei Dou (School of Medicine, Xi'an Jiaotong University). Voucher specimens were preserved in the School of Pharmacy of Xi'an Jiaotong University.

The preparation of ZJPE was conducted as follows: Cuscuta Semen (1000 g), Plantaginis Semen (200 g), and Radix Rehmanniae Praeparata (600 g) were immersed together for 2 h and boiled in a 10-fold volume of distilled water for 1.5 h twice, followed by alcohol precipitation, and then the supernatant was kept. Finally, the supernatant was filtered, mixed, and concentrated to relative density. The concentrated extract was dried by vacuum concentration to achieve the ZJPE (yield: 12.45%). This method was recommended in the *Chinese Pharmacopoeia* (2015 edition).

2.2. High-performance liquid chromatography (HPLC) fingerprint of ZJP

The identification of the major compounds in ZJP was performed by using HPLC (Shimadzu LC-20A HPLC system, Japan). ZJPE was dissolved in 60% methonal and filtered through a 0.45 μm filter (Microgen, Laguna Hills, CA, USA). HPLC analysis were performed using a Phenomenex Luna C18 (4.6 mm × 250 mm i.d., 5 μm particle size), with the mobile phase consisting of methanol (A) and 0.2% formic acid in water (B) at a flow rate of 1.0 mL/min. The column temperature was maintained at 35 °C. A linear gradient elution was programed as follows: 5% A (0–20 min), 5–30% A (20–60 min), 30–40% A (60–80 min) and 5% A (80–90 min). Hyperin, geniposidic acid, and verbascoside were regarded as reference substances, which is recommended by the *Chinese Pharmacopoeia* (2015 edition). Pure standards of hyperin (Lot no. 111521-201403), geniposidic acid (Lot no. 111828-201401), and verbascoside (Lot no. 111530-201404) were purchased from the National Institutes for Food and Drug Control. ZJPE mainly contained a constant volume of hyperin (0.8 mg/g), geniposidic acid (0. 8 mg/g), and verbascoside (1.0 mg/g) for quality control.

2.3. Animals and the diabetic model

Male Sprague-Dawley rats (SPF, weighing 140–150 g) were purchased from the Laboratory Animal Center of Xi'an Jiaotong University (Xi'an, China). All animals were housed in a controlled temperature (24 ± 2 °C), humidity (40–70%), and light (12 h light/dark cycle) environment. Water and commercial rat chow were available *ad libitum*.

After 7 days of adaptive breeding, rats were fasted for 12 h (with free access to water), and diabetes was induced by intraperitoneal injection of STZ. (Sigma, USA, CAS: 18883-66-4, diluted in citrate buffer 0.1 mol/L, pH 4.5) at a dose of 60 mg/kg body weight. Blood glucose was tested after 72 h of STZ injection, and the rats with blood glucose over 16.7 mmol/L (Roche glucometer) were confirmed to be in a diabetic state. Finally, 53 diabetic rats were randomly divided into four groups: the diabetes mellitus model group (DM, n = 14), the ZJPE low group (ZJPE-L, n = 13), the ZJPE middle group (ZJPE-M, n = 13), and the ZJPE high group (ZJPE-H, n = 13). Rats received an equivalent volume of citrate buffer (vehicle), which served as the normal control group (NC, n = 13). After successful establishment of the model, intragastric administration of the drugs lasted for 12 weeks. Rats in ZJPE-L, ZJPE-M and ZJPE-H groups were given ZJPE 1.65, 3.33, and 6.66 g/kg body weight, respectively. Rats in the DM and NC groups were given the vehicle. At the end of the study, abdominal aorta blood was collected, and left eyes were removed.

Animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals, and ethical approval was obtained from the Ethics Committee of Xi'an Jiaotong University.

2.4. Measurement of body weight and blood glucose

Body weight was measured weekly. Blood glucose levels were measured at one-week intervals (Roche blood-glucose monitoring system, USA).

2.5. Serum assay

Blood samples were collected from the abdominal aorta of rats 24 h after the last administration and clotted for 1 h. The serum was separated by centrifugation at 2500 r/min for 10 min. Then, serum superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-Px) were measured by using colorimetric assay kits (Nanjing Jiancheng bioengineering institute). The levels of IL-6, TNF-α, and hsCRP were detected by using ELISA kits (Beijing Sino-UK Institute of Biological Technology; Shanghai Westang Bio-Tech Co., Ltd). The examinations were performed according to the manufacturer's instructions.

2.6. Blood-retinal barrier (BRB) breakdown measurement

BRB breakdown was measured with the Evans blue dye injection method, as previously described, with minor modifications [13,14]. Evans blue (Sigma, USA) was dissolved in normal saline (30 mg/mL) and then, stored in the refrigerator. Briefly, rats were injected through the tail vein at a dosage of 45 mg/kg. After Evans blue circulated for 2 h, the chest cavity of each rat was opened and perfused by citrate-buffered paraformaldehyde within 2 min. Eyes were removed, and the retinas were separated under stereoscope immediately after perfusion. Then, the weight of the retinas were measured after a thorough vacuum drying. Evans blue, extracted by incubating each sample in 150 μL formamide at 70 °C for 18 h, was put into each centrifuge tube and centrifuged at 7000 rpm for 1 h at 4 °C. Then the supernatant was collected for the next step. Absorbance was measured by using the supernatant at 620 nm (maximum absorbance value) and 740 nm (minimum absorbance value), respectively. Finally, the concentration of Evans blue in the extracts was normalized by the weight of the dry retina.

2.7. Retinal growth factor and cytokine levels

At the end of treatment, retinal levels of superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-Px) were measured by using colorimetric assay kits. Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), intercellular adhesion molecule 1