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Effects of combinations of Xiexin decoction constituents on diabetic nephropathy in rats

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ABSTRACT

Ethnopharmacological relevance: Xiexin decoction (XXD) has been used as a treatment for diabetes mellitus for more than 1300 years. XXD constituents with protective effects against diabetic nephropathy (DN) include Rhizoma Coptidis alkaloids (RA), Radix et Rhizoma Rhei polysaccharides (RP), and Radix Scutellaria flavones (RF). The aim of the study is to investigate the effects of combinations of RA, RP, and RF on DN and their mechanisms of action.

Materials and Methods: *In vitro*, high glucose-induced rat mesangial cells were treated with RA, RP, RF, and combinations thereof. Cell proliferation and levels of inflammatory factors were measured. *In vivo*, high-fat diet and streptozotocin-induced diabetic rats were treated with different combinations of RA, RP, and RF once per day for 12 weeks. Blood and urine biochemical parameters, renal tissue morphology, and inflammation were investigated.

Results: *In vitro*, the combination of the three groups of components inhibited mesangial cell proliferation and reduced the levels of monocyte chemotactic protein-1 (MCP-1) and collagen IV. The effects of the three constituent groups in combination were stronger than those of each group alone or combinations of two groups. In diabetic rats, combinations of the three groups of herb components ameliorated blood glucose, urinary albumin excretion and decreased renal mesangial matrix expansion and basement membrane thickening. In addition, the combinations reduced renal tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) protein levels, down-regulated the expression of nuclear factor κB (NF-κB) and transforming growth factor beta 1 (TGF-β1), and up-regulated the expression of inhibitor of nuclear factor κB (IκB) protein. Among the three groups of herb components, RA produced the strongest effects, followed by RP, and then by RF.

Conclusions: The combination of the three groups of herb components produced anti-DN effects through inhibition of inflammation mediated by NF-κB. Among the three groups of herb components, RA produced the strongest effect while RP and RF produced weaker effects.

1. Introduction

Diabetic nephropathy (DN) is a primary microvascular complication of diabetes and a major cause of end-stage renal disease (ESRD) (Rossing, 2006). Medications administered clinically, including anti-diabetic drugs, anti-hypertensive drugs, and inhibitors of the renin–angiotensin system, can delay the occurrence and development of DN (Van Buren and Toto, 2013). However, the number of patients who has progressed from diabetes to ESRD has been increasing steadily in recent years (Heerspink and de Zeeuw, 2011). Therefore, it is important to develop new drugs to effectively prevent the occurrence and development of DN.

Traditional Chinese medicine (TCM) generally involves the application of compounds composed of several medicinal herbs and has been used in the treatment of diabetes for thousands of years (Tong et al., 2012). Recently, an increasing number of experimental studies have revealed that TCM compounds have preventive and curative effects on diabetes and its complications (Shi et al., 2011; Wen et al., 2012). Network pharmacology and polypharmacology research have revealed that multiple components in TCM compounds act on multiple targets within the diabetes disease network, thereby producing an overall systemic

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effect (Gu et al., 2011). These studies have shown that TCM compounds have potential in the treatment of diabetes and its complications.

Xiexin decoction (XXD) is a formula composed of Radix et Rhizoma Rhei, Radix Scutellariae, and Rhizoma Coptidis. It has been used as a remedy for the treatment of diabetic mellitus (called xiaoke disease in TCM) since the Tong dynasty (6th century C.E.) (Sun, 1999), and is still used to treat diabetic patients in China and Japan (Zuo et al., 2008). We have previously reported that XXD provided resistance against DN (Wu et al., 2013). Multiple components of XXD contribute to its protective effects on the kidneys in diabetics, including Rhizoma Coptidis alkaloids (RA), Radix Scutellaria flavones (RF), Rheum anthraquinones and so on (Wu et al., 2013). However, some studies have shown that the long-term use of Rheum anthraquinones produced toxicity (Zhang et al., 2004), and that emodin and rhein may have carcinogenic and mutagenic effects (Zhu et al., 2011). These reports have limited the long-term application of Rheum anthraquinones and slowed XXD research and development. Some studies have shown that Radix et Rhizoma Rhei polysaccharides (RP) produce hypoglycemic effects (Li, 2007). Furthermore, RP showed protective effects on glomeruli in a high glucose-induced glomerular mesangial cell model that were independent from hypoglycemic and hypolipidemic effects (Y. Liu et al., 2012).

Several issues that are of significance in the development of modern therapeutic vectors from XXD were investigated in this study. Using *in vivo* and *in vitro* models, we studied the anti-DN effects of RA, RP, and RF, the corresponding mechanisms of action, and the effects of combinations of these compounds. It is hoped that these results will promote further research and development of XXD.

2. Materials and methods

2.1. Materials and reagents

Radix et Rhizoma Rhei (*Rheum palmatum* L.), Rhizoma Coptidis (*Coptis chinensis* Franch), and Radix Scutellaria (*Scutellaria baicalensis* Georgi) were purchased from Shanghai Kang Qiao Herbal Pieces Co. Ltd. (Shanghai, China) and authenticated by Prof. Zhi-Li Zhao of the Department of Botany, Shanghai University of TCM. Streptozotocin and 3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, MO, USA). Dulbecco's modified Eagle's minimum essential medium (DMEM) was obtained from Invitrogen (Carlsbad, California, USA). Fetal bovine serum (FBS) was obtained from FuMeng Gene Co. Ltd. (Shanghai, China). Nuclear factor κBp65 (NF-κBp65), inhibitor of nuclear factor κB subunit a (IκBa), and β-actin antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). ELISA kits for tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), monocyte chemotactic protein-1 (MCP-1), and collagen IV were obtained from R&D Systems (Minneapolis, MN, USA). Trizol, reverse transcriptase, and Fast SYBR Green mix were purchased from Takara (Tokyo, Japan).

2.2. Preparation and quality control of the components of XXD

RA, RP, and RF were prepared as previously described (Xie et al., 2013). HPLC analysis of RA and RF was performed as previously reported (Xie et al., 2013) and shown in Fig. 1. The mass percentages of berberine, coptisine, palmatine, and jatrorrhizine in RA were determined to be 55.8%, 8.5%, 7.6%, and 4.8%, respectively. The mass percentages of baicalin, wogonoside, and baicalein in RF were 89.6%, 0.1%, and 3.0%, respectively. The content of polysaccharides in RP was determined to be 62.3% by the phenol-sulfuric acid-UV method (Xie et al., 2013). The monosaccharide compositions of RP include rhamnose, pectinose, xylopyranose, mannitose, and glucose (Fig. 1), which were detected by GC methods (Zhang et al., 2014).

2.3. Cell culture

The rat mesangial cell line (RMC) HBZY-1 was purchased from the Chinese Center for Type Culture Collection (Wuhan, China) and cultured in normal DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere containing 5% CO_2.

2.4. Influence of components from XXD on cell proliferation

RMC cells at a density of 5×10^4 cells/well were seeded in 96-well plates with DMEM containing 5.5 mM glucose and 10% FBS. After incubation for 24 h, cells were grouped as follows: normal glucose group (5.5 mM glucose, NG), high glucose group (30 mM glucose, HG), and 30 mM glucose with drug intervention groups (30 mM glucose plus 2, 5, or 12.5 μg/ml RA; or 2, 10, or 50 μg/ml RP; or 8, 20, or 50 μg/ml RF). After 24 h of treatment, the cell proliferation of each group was determined by the MTT test, which was performed as described previously (Ma et al., 2009).

2.5. Influence of combinations of berberine, RP, and baicalin on cell proliferation, MCP-1 and collagen IV protein levels

RMC cells were seeded in 96-well plates at a density of 5×10^4 cells/well. After 24 h incubation, cells were grouped as follows: NG, osmotic control group (5.5 mM glucose plus 24.5 mM mannitol), HG, and drug intervention groups (30 mM glucose plus various combinations of berberine, RP, and baicalin with an L_8 (2^7) orthogonal design (Si et al. 2001)). After 24 h of treatment, the cell proliferation of each group was determined by an MTT test performed as described above, and the media was collected for the measurement of MCP-1 and collagen IV.

2.6. Animals

Male Sprague–Dawley rats with weights ranging from 90 to 100 g were purchased from the Shanghai SLAC Laboratory Animal Co. (Shanghai, China). The rats were housed in an air-conditioned room at 22–24 °C under a 12-h dark/light cycle, and were given food and water *ad libitum*. All animal experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals at Shanghai University of TCM.

After a 1-week adaptation, the rats were divided into a normal control (NC) group that was fed a standard diet and a high-fat group that received a high-fat diet. After 4 weeks, rats on the high-fat diet were treated with streptozotocin (40 mg/kg, *i.p.*). All diabetic rats with FBG levels above 16.7 mmol/L were randomly divided into 10 groups: diabetic control group (DM), 10 mg/kg losartan-treated group (Losartan), and groups treated with different combinations of RA, RP, and RF (G1–G8) according to a L_8 (2^7) orthogonal design ((Si et al., 2001; Ma et al., 2009), Table 1). All drugs were administered via intra-gastric gavage (*i.g.*) once per day for 12 weeks.

2.7. Metabolic parameters, urinary albumin excretion, and renal function analysis

After treatments for 12 weeks, the 24 h UAE was measured by radioimmunoassay (Atom High Technology, Beijing, China). FBG was measured using the glucose oxidase method. Next, all animals were anesthetized and blood samples were collected from the abdominal aorta. Serum total cholesterol, creatinine, and urine creatinine levels