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Lonicerae Japonicae Flos attenuates diabetic retinopathy by inhibiting retinal angiogenesis

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ABSTRACT

Ethnopharmacological relevance: Lonicerae Japonicae Flos (Jin-Yin-Hua) is a well-known traditional Chinese medicine used for clearing away heat and toxic material.

Aim of the study: This study aims to observe the attenuation of aqueous extract of Lonicerae Japonicae Flos (FL) against streptozotocin (STZ)-induced diabetic retinopathy (DR) and its engaged mechanism.

Materials and methods: STZ-induced proliferative DR (PDR) for 5 month in C57BL/6 mice was used in this study. Retinal vessels were observed by immunofluorescence staining with cluster of differentiation 31 (CD31) and histopathological evaluation. Enzyme-linked immunosorbent assay (ELISA) was used to detect serum vascular endothelial growth factor (VEGF) content. Cell proliferation was detected by 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) assay in choroid-retinal endothelial RF/6A cells. VEGF-induced tube formation in RF/6A cells was observed. The contents of chlorogenic acid (CGA), caffeic acid (CA), and luteolin in FL were detected by high-performance liquid chromatography (HPLC).

Results: Histopathological evaluation demonstrated that retinal vessels were increased in STZ-induced PDR mice, whereas FL decreased such increase. The results of CD31 staining also showed that FL decreased the increased number of retinal vessels in STZ-induced PDR mice. In addition, FL reduced the increased serum VEGF content in STZ-induced PDR mice. FL reduced VEGF-induced RF/6A cell proliferation in the concentration-dependent manner, but had no obvious effect on RF/6A cell viability without VEGF stimulation. VEGF-induced tube formation in RF/6A cells was inhibited by different concentrations of FL. CGA, CA and luteolin all inhibited VEGF-induced tube formation in RF/6A cells, and the lowest effective concentration of CGA and CA was both 0.625 μM, but of luteolin was 5 μM. Furthermore, the results of HPLC demonstrated that the amount of CGA was the highest in FL.

Conclusions: FL ameliorates STZ-induced PDR by inhibiting retinal angiogenesis. Phenolic acid CGA is the main compound contributing to the inhibition of FL on retinal angiogenesis.

1. Introduction

Lonicerae Japonicae Flos (Jin-Yin-Hua), derived from the dried flower buds of *Lonicera japonica* Thunb. (Caprifoliaceae), is traditionally used for clearing away heat and toxic materials. It is commonly used for acute fever, headache, inflammation, and infection (Shang et al., 2011; Jiang et al., 2014a). Due to its clod nature, Lonicerae Japonicae Flos is the commonly used ingredient for various health-care products, such as tea or drinking sold in market in China or other Asian countries, which provide relief of summer heat and benefits for eyesight. Chlorogenic acid (CGA) is the main phenolic compound contained in Lonicerae Japonicae Flos, and it is also used as the chemical marker for evaluating the

Abbreviations: FL, aqueous extract of Lonicerae Japonicae Flos; STZ, streptozotocin; DR, diabetic retinopathy; PDR, proliferative DR; CD31, cluster of differentiation 31; ELISA, enzyme-linked immunosorbent assay; VEGF, vascular endothelial growth factor; MTT, 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide; CGA, chlorogenic acid; CA, caffeic acid; HPLC, high-performance liquid chromatography; DM, diabetes mellitus; NPDR, non-proliferative DR; FITC, fluorescein isothiocyanate; SEM, standard error means; LSD, least significant difference; BRB, blood-retinal barrier; HUVECs, human umbilical vein endothelial cells; HRMECs, human retinal microvascular endothelial cells; ROP, retinopathy of prematurity; H&E, hematoxylin-eosin

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quality of Lonicerae Japonicae Flos indexed in Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010).

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes mellitus (DM). Diabetes can be generally divided into two types: type 1 (insulin-dependent) and type 2 (insulin-independent), and patients of both types will have hyperglycemia. A previous study reported that about one-third of the diabetic patients have signs of DR and about one-tenth of them finally will have vision-threatening retinopathy (Saaddine et al., 2008). According to the international clinical diabetic retinopathy disease severity scale, DR is generally classified into non-proliferative DR (NPDR) and proliferative DR (PDR) (Wilkinson et al., 2003). NPDR is the early stage of DR, and it can further develop into PDR, which is the later stage of DR. In PDR stage, retinal angiogenesis occurs, which will further lead to vitreous hemorrhage, traction retinal detachment and finally vision loss (Kohner, 1993; Cheung et al., 2010). DR seriously affects the quality of life and the survival of diabetic patients. There is study showed that nearly 60% of studied diabetic patients were reported progress to PDR and even severe vision loss in 10 years (Wong et al., 2009). However, there is still no effective and safety drug for the treatment of DR in clinic.

In Chinese Medicine theory, the blood heat and Yin deficiency in the process of diabetes is the main reason for leading to subsequent retinal hemorrhage and abnormal neovascularization during the development of DR. Thus, traditional Chinese medicines for clearing heat or nourishing Yin may be helpful for treating DR. As a well-known heat-clearing drug, Lonicerae Japonicae Flos is commonly used combined with other drugs and included in numerous Chinese medical formulas for improving eyesight, such as He-Ying-Qing-Re Formula, which has already been reported to can ameliorate DR (Wang et al., 2015).

In the present study, the amelioration of streptozotocin (STZ)-induced PDR in mice induced by aqueous extract of Lonicerae Japonicae Flos (FL) was observed, and the inhibition of FL on retinal angiogenesis *in vivo* and *in vitro* was detected. Meanwhile, the main active compounds in FL for inhibiting retinal angiogenesis were evaluated *in vitro*.

2. Materials and methods

2.1. Chemical compounds and reagents

Lonicerae Japonicae Flos was purchased from Shanghai Kangqiao Herbal Pieces Co. Ltd. The voucher sample was deposited in the Institute of Chinese Material Medical, Shanghai University of Traditional Chinese Medicine. CGA, CA, and luteolin with 98% purity were purchased from Shanghai Hitsanns Co. Ltd. (Shanghai, China) or Internet Aladdin Reagent Database (Shanghai, China). Cluster of differentiation 31 (CD31) antibody and fluorescein isothiocyanate (FITC) conjugated anti-Rat IgG were purchased from BD Biosciences (Franklin Lakes, NJ). Enzyme-linked immunosorbent assay (ELISA) kit for VEGF was obtained from R&D (Minneapolis, MN). Human recombinant VEGF (isoform 165) was purchased from PeproTech (Rocky Hill, NJ). Matrigel was purchased from BD Biosciences (San Diego, CA). Streptozotocin (STZ) and other reagents unless indicated were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Preparation of FL powder

FL was prepared from dried Lonicerae Japonicae Flos as described in the previous reported study (Jiang et al., 2014b). Dried Lonicerae Japonicae Flos (100 g) was heated under reflux with 1000 ml of distilled water for 4 h. The extracts were filtered, and the residue was re-extracted under the same conditions. Those above extracts were combined and concentrated to 100 ml under reduced pressure, and further lyophilized to fine powder for long-term storage.

2.3. Experimental animals

The C57BL/6 mice (20 ± 2 g) were purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). The animals were maintained under controlled temperature (22 ± 1 °C), humidity (50%), and lighting (12 h light/12 h dark). The animals were fed with a standard laboratory diet and given free access to tap water. All animals were received humane care according to the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine.

2.4. Treatment of animals

Thirty mice were intraperitoneally injected (i.p.) with STZ (55 mg/kg) for 5 consecutive days, while the other ten mice were intraperitoneally injected (i.p.) with physiological saline and served as control animals. The concentration of serum glucose was measured 7 days after the last injection, and the mice with high glucose concentration (>16.5 mmol/L) were considered as diabetic mice. In this experiment, the glucose concentration of 27 mice was >16.5 mmol/L, and those mice were randomly divided into three groups: DR model (n=9), DR+FL (100 mg/kg) (n=8), and DR+FL (200 mg/kg) (n=10), respectively. At 4 months after the injection of STZ, the mice were orally administered with FL (100, 200 mg/kg per day) consecutively for 1 month. At 5 months after the injection of STZ, the mice were anesthetized by sodium pentobarbital (30 mg/kg, i.p.), the blood samples were taken from the abdominal aorta, and the eyes were removed immediately. The body weight was monitored and the concentration of blood glucose was determined by Glucometer® (Accu-Check® Performa Nano, Roche Diagnostics, Germany) during the whole experimental process.

2.5. Histopathological evaluation

Retinas were isolated from mice, and then fixed in 4% paraformaldehyde solution. Retinal tissues were subsequently sectioned (5 μm), stained with haematoxylin and eosin (H&E), and then pictured under the microscopy (Nikon, Japan).

2.6. Retinal immunofluorescence staining with CD31

Retinas were stained with CD31 as described in our previous published papers (Gong et al., 2013, 2014), and pictured under the fluorescence microscopy (IX81, Olympus, Japan). The retinal vessels were counted as in the previously reported method (Huang, 2006; Gong et al., 2013, 2014).

2.7. ELISA analysis

The whole blood was centrifuged at 3000 rpm, 4 °C for 15 min, and serum was collected for ELISA analysis according to the manufacturer's instructions.

2.8. Cell culture

RF/6A cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI1640 media supplemented with 10% [v/v] heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.