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Early intervention with *Didang* decoction delays macrovascular lesions in diabetic rats through regulating AMP-activated protein kinase signaling pathway

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[ABSTRACT] The study aimed to investigate the intervening role of *Didang* decoction (DDD) at different times in macrovascular endothelial defense function, focusing on its effects on the AMP-activated protein kinase (AMPK) signaling pathway. The effects of DDD on mitochondrial energy metabolism were also investigated in rat aortic endothelial cells (RAECs). Type 2 diabetes were induced in rats by streptozotocin (STZ) combined with high fat diet. Rats were randomly divided into non-intervention group, metformin group, simvastatin group, and early-, middle-, late-stage DDD groups. Normal rats were used as control. All the rats received 12 weeks of intervention or control treatment. Western blots were used to detect the expression of AMP-activated protein kinase α1 (AMPKα1) and peroxisome proliferator-activated receptor 1α (PGC-1α). Changes in the intracellular AMP and ATP levels were detected with ELISA. Real-time-PCR was used to detect the mRNA level of caspase-3, endothelial nitric oxide synthase (eNOS), and Bcl-2. Compared to the diabetic non-intervention group, a significant increase in the expression of AMPKα1 and PGC-1α were observed in the early-stage, middle-stage DDD groups and simvastatin group ($P < 0.05$). The levels of Bcl-2, eNOS, and ATP were significantly increased ($P < 0.05$), while the level of AMP and caspase-3 were decreased ($P < 0.05$) in the early-stage DDD group and simvastatin group. Early intervention with DDD enhances mitochondrial energy metabolism by regulating the AMPK signaling pathway and therefore may play a role in strengthening the defense function of large vascular endothelial cells and postpone the development of macrovascular diseases in diabetes.

[KEY WORDS] *Didang* decoction; Caspase-3; AMPK; Endothelial cells; PGC-1α

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Introduction

Diabetic macrovascular diseases are the most common arterial diseases observed in patients with type 2 diabetes mellitus [1]. Large prospective clinical studies show that, even with good blood glucose control, many diabetic patients will still develop serious macrovascular diseases [2]. Therefore, strengthening the defense function of the macrovascular endothelium is critical. This reflects the preventive theory of traditional Chinese medicine (TCM), which is originated from the Inner Canon of *Huangdi*: Saint does not treat the diseases, but prevent them.

Didang decoction, which is composed of leech, gadfly, peach and rhubarb, is recorded in the Treatise on Febrile Diseases and Synopsis of Golden Chamber [3]. It aids in breaking stagnancy and in eliminating blood stasis, according to the TCM theory. Our previous experiments have found that the early intervention with DDD reduces the expression of MCP-1, CD68, ICAM-1, VCAM-1, TGF-β_1, and PKC-β_2, and therefore inhibits the inflammation-associated diabetic vascular diseases [4]. AMPK is the energy sensor of cell. The impaired endothelial progenitor cells along with the decreased AMPK phosphorylation has been found in diabetic patients [5]. It is well known that the invasion of pathogenic factors can be

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avoided if the defense system is strong. The accumulation of pathogenic factors indicates the impairment of vascular defense system. In the present study, we studied the effects of DDD intervention conduced at different times on mitochondrial energy metabolism mediated by the AMPK signaling pathway in aorta endothelium in diabetic rats, and explored the role of DDD in increasing the defense ability of endothelium to counteract the impairment of high glucose and high lipids in diabetes.

Materials and Methods

Animals

Male Sprague-Dawley rats (4 weeks; weighing 98–103 g) were obtained from the Tianjin Center for Experimental Animals (qualified number: SCXK Jin 2014-0001). All animal experiments were performed according to the guidelines of the Belgian Regulations for Animal Care and were approved by the Tianjin authorities for animal research.

Drugs and reagents

The gruff of the *Didang* decoction were purchased from the Pharmacy Service in The First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine (Tianjin, China). All the single herbs were weighed, including rhubarb (6 g), leech (10 g), peach (10 g), and gadfly (10 g). They were decocted for 1 h thrice. In accordance with the traditional preparation process of water decoction, the decoction was subjected to a decompression and concentration to form a 1 $g \cdot mL^{-1}$ of crude drug and was preserved at 4 °C. The contents of rhein and emodin were tested by high performance liquid chromatograph with the following method: the separation was performed on a Waters SunFireTMC18 column (250 mm × 4.6 mm, 5 μm) at 30 °C; the mobile phase was methanol –0.1% phosphoric acid (90 : 10) with a flow rate of 1.0 $mL \cdot min^{-1}$; the detection wavelength was set at 254 nm. The mass concentrations of rhein and emodin in DDD were 1.1 and 0.73 $\mu g \cdot mL^{-1}$, respectively.

Metformin (0.5 g/film), simvastatin (20 mg/film), and STZ were purchased from Tianjin *Metabolic Diseases Hospital* (Tianjin, China). The polymerase chain reaction kit (PCR), the ultrapure RNA extraction kit and the HiFi- MMLVcDNA first chain kit were provided by Beijing Comwin Biotech Co., Ltd. (Beijing, China). The primer sequences and ELISA kit were obtained from Tianjin chemical reagent factory (Tianjin, China).

Apparatus

The blood glucose meter and blood glucose test strips were purchased from Bayer (Tianjin, China). The insulin radioimmunoassay kit, the microscope and automatic image analyzer and the fluorescence quantitative PCR instrument were from Tianjin Jiuding Medical Bioengineering Co., Ltd. (Tianjin, China). The slicing machine and the desktop vulgar high-speed centrifuge were obtained from Huabei experimental instrument Co., Ltd. (Tianjin, China). The electrophoresis apparatus was purchased from Beijing Liuyi Instrument Factory (Beijing, China). The HPIAS-2000 image analysis software was provided by Tongji Qianping image engineering company (Wuhan, China).

Animal model

Fifteen rats were randomly selected as control rats and received a regular chow diet, while the remaining rats received a high fat diet (20% sucrose, 1% cholesterol, 10% grease, 5% custard powder, 0.2% sodium cholate, and 63.8% standard chow, which were purchased from Tianjin Experimental Animal Center Laboratory (Tianjin, China)). All the rats were given free access to food and tap water at 22 °C, with a 12 h/12 h light/dark cycle. After 8 weeks, the fasting blood glucose and serum insulin were detected. Diabetes was induced on 12th week by a caudal vein injection of STZ after the rats had been fasted for 24 h. Type 2 diabetes was considered successfully induced in rats if their random blood glucose > 16.9 $mmol \cdot L^{-1}$ one week after the STZ injection (success rate was approximately 80%).

Intervention and sample collection

All the diabetic rats were divided into non-intervention group, metformin group, simvastatin group, early-stage DDD intervention group, middle-stage DDD intervention group and late-stage DDD intervention group. The rats in control group and diabetic non-intervention group received equal volumes of sterile drinking water daily by gavage. DDD (3.24 $g \cdot kg^{-1}$) was initiated 4 weeks before diabetes were induced in the early-DDD group, right after diabetes were induced in the middle-stage DDD group or 4 weeks after diabetes were induced in the late-stage DDD group, respectively. The rats in the metformin group received 0.36 $mg \cdot kg^{-1}$ of metformin by gavage right after diabetes were induced, and the rats in the simvastatin group received 2.67 $mg \cdot kg^{-1}$ of simvastatin by gavage right after diabetes were induced. All the rats were sacrificed by cervical dislocation at week 24, and the thoracic aorta tissues were taken out quickly. Part of the tissue was fixed in 4% paraformaldehyde solution at 4 °C, while the remaining tissue was transferred to different cryopreserved tubes and preserved at –80 °C until analysis.

Western blotting analysis

SDS alkaline lysis method was used to crack the aortic tissues for the extraction of the total protein. Coomassie Brilliant Blue method was used to detect the protein concentration in each sample. After 10% separation gel and 4% concentrate become solidified in the glass tank, the comb was removed.Then the protein and protein gel electrophoresis sample buffer (5 ×) were added, blending gently for 10 minutes at 95 °C. And it was transferred to the gel pores for the electrophoresis, adjusting the voltage to 80 v. After that, the proteins were transferred to a polyvinylidene difluoride membrane and the membrane was incubated with the primary antibody (dilution 1 : 200) of AMPKα1 or PGC-1α and then the secondary antibody conjugated with horseradish peroxidase (dilution 1: 3 000). The membranes were then transferred into a black box for full exposure, and finally the gray