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^{1}H NMR-based metabolomics approach to investigating the renal protective effects of Genipin in diabetic rats

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[ABSTRACT] Diabetic nephropathy is one of the various complications of diabetes mellitus, affecting patients for lifetime. Earlier studies have revealed that genipin can not only improve diabetes, but also induce cytotoxicity. Therefore, it is not clear which effect of genipin on kidneys occurs, when it is used in the treatment of diabetes. In the present study, we performed nuclear magnetic resonance (NMR)-based metabolomics analysis of urine and kidney tissue samples obtained from diabetic rats to explore the change of endogenous metabolites associated with diabetes and concomitant kidney disease. Nine significant differential metabolites that were closely related to renal function were screened. They were mainly related to three metabolic pathways: synthesis and degradation of ketone bodies, glycine, serine and threonine metabolism, and butanoate metabolism, which are involved in methylamine metabolism, energy metabolism and amino acid metabolism. In addition, after the intervention of genipin, the metabolic levels of all the metabolites tended to be normal, indicating a protective effect of genipin on kidneys. Our results may be helpful for understanding the antidiabetic effect of genipin.

[KEY WORDS] Diabetes; Diabetic nephropathy; Genipin; Protection; Metabolomics

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from defects of insulin secretion and insulin action, or both [1]. With a huge financial burden in many countries, DM is a serious global health problem [2]. It is estimated that by 2035, the number of patients with diabetes will increase from 382 million to 592 million [3].

Diabetic nephropathy (DN) is a severe microvascular complication of diabetes mellitus, affecting patients for lifetime [4]. What's more, nearly half of all DM patients will develop into end-stage renal disease, resulting in large medical costs and lost productivity. In addition, pulmonary dysfunction [5], hyperlipidemia [6], cardiovascular disease [7], and even heart failure [8] have been reported to be positively associated with DN progression. The characteristics of multi targets of Chinese herbal medicine make it suitable for the treatment of diabetic nephropathy. For example, *Glycyrrhiza uralensis* (gan-cao), *Carum carvi* (zang-hui-xiang) and *Allium sativum* (da-suan) have been reported to have therapeutic effects on DN [9-11]. Because of DN is a common complication of diabetes, it is an advantage to discover a natural product in the plant that can treat both diabetes and DN.

Gardenia jasminoides has been used over hundreds of years in traditional Chinese medicine to alleviate symptoms of diabetes [12]. Genipin, derived from the fruit of *gardenia jasminoides*, has been reported to have anti-inflammatory [13], anti-oxidative [14], anticancer [15], immunosuppression [16], anti-depression [17] and antithrombotic effects [18]. In published literature, genipin has been reported to stimulate insulin secretion by pancreatic β-cells [19]. Besides, it is also corroborated that genipin inhibits the expression of UCP2 and ameliorates the injury of podocyte in DN mice [12]. However, fur-

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ther studies confirm that genipin can increase cytotoxic effects *in vivo*/*in vitro* [20] and the mortality result from 200 mg·kg^{-1} of oral genipin in rats is about 77.8% [21].

In our previous studies, we have investigated the antidiabetic activities of genipin in diabetic rats induced by alloxan and explored its possible mechanism of action [22]. Detailed analysis of the altered metabolite levels indicates that genipin significantly ameliorates the disturbance in glucose metabolism, tricarboxylic acid cycle, lipid metabolism and amino acid metabolism. However, according to other reports [12, 20], genipin can not only ameliorated podocyte injury in DN mice but also increase cytotoxic effects. Therefore, what is the main effect of genipin on kidney when it is used in the treatment of diabetes caught our attention. We performed NMR metabolomics analysis of urine and kidney tissue extracts obtained from diabetic rats to cope with this problem in the present study.

Metabolomics is a rapidly emerging area of ‘‘-omics’’ research in which endogenous metabolites of bio-fluids or tissues are comprehensively assessed, followed by systematic identification and accurate quantification [23]. With the development of effective analytical technologies and methods, metabolomics approaches are gaining widespread applications in diagnosis and evaluation of diseases such as DM [24], identification of potential biomarkers, and affording global and crucial insights into the pathogenesis of diseases, thanks to its advantages in evaluating systemic responses to any subtle metabolic perturbation [25]. Nuclear magnetic resonance (NMR) spectroscopy is one of the means of metabolomics. Its advantages include the nondestructive nature of the analysis, the robust and reproducible measurements and the minimal preparation requirement [26]. NMR metabolomics has been applied in several diabetes researches [27-28].

In the present study, we adopted the ^{1}H NMR based metabolomics technology to analyze the endogenous metabolites in rat kidney and urine. The significant differences in metabolism in the diabetic rats after oral administration of genipin were investigated to further understand the protective effect of genipin on the kidneys at our experimental dose level.

Materials and Methods

Animals and reagents

Male Sprague Dawley rats (weighing 180–200 g) purchased from Beijing Vital River Laboratories Co. (SCXK (Jing) 2011-2012) were kept in a barrier system. Before the experiments were conducted, the animals were acclimated to the new environment for one week. They were housed in rat cages under controlled conditions of light (12h/12h hight/dark cycle, lights on at 8: 00 a.m.), temperature (24 ± 1 °C), and humidity (45% ± 15%), with free access to food and tap water. All the procedures in the present study were approved by Animal Ethics Committee of Shanxi University (SXU-2014-05-001) and were in strict accordance with the National Institutes of Health (NIH) guidelines considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care.

Genipin, extracted and hydrolyzed from the fruit of *Gardenia jasminoides,* with purity of greater than 98%, was supplied by Lin-chuan-zhi-xin Bio-Technology Co., Ltd. in Fuzhou, Jiangxi, China. Metformin hydrochloride capsules (Hebei TianCheng Pharmaceutical Co., Ltd. in Cangzhou, Hebei, China; Lot No. 71310162) were commercially obtained from the Xinhe Drug Store in Taiyuan, Shanxi, China. Alloxan was purchased from Sigma (St Louis, MO, USA).

Experimental design and sample collection

To prepare the diabetic model, the rats were randomly selected and injected intraperitoneally with alloxan monohydrate after fasted for 12 h. Alloxan monohydrate were freshly dissolved in physiological saline and the rats were given at a dosage of 120 mg·kg^{-1} body weight (the concentration is 24 mg·kg^{-1}, and the volume of injection is 1 mL/200 g body weight) for three days, whereas the normal control animals (NC, n = 6) were injected with the same volume of physiological saline. Three days after the last injection, the blood glucose concentration was measured and the rats with a fasting blood glucose (FBG) level higher than 11.00 mmol·L^{-1} were defined as diabetic rats. The diabetic rats were weighed and randomly divided into six groups (n = 6): The positive control group (diabetic but not treated, DM), genipin high dose group (100 mg·kg^{-1} body weight, GH), genipin middle dose group (50 mg·kg^{-1} body weight, GM), genipin low dose group (25 mg·kg^{-1} body weight, GL) and metformin hydrochloride group (125 mg·kg^{-1} body weight, positive treatment group, YV). The intake of food and tap water were strictly controlled daily. Genipin and metformin hydrochloride were dissolved in saline to the required concentration and administered to the diabetic rats *via* gastric intubation for two weeks. The urine samples of all the rats were collected for 24 h in individual urine collection cages and then centrifuged and stored at –80 °C until analysis. Each rat was decapitated after isoflurane anesthesia treatment and the cortical parts of kidney tissues were removed, weighed, immediately immersed in liquid nitrogen, and stored for metabolic analysis.

Preparation of samples and acquisition of ^{1}H NMR spectra

Urine samples were thawed and 500 μL of aliquot were mixed with 200 μL of phosphate buffer (pH 7.4) to minimize variations in pH value. The mixture was centrifuged to remove precipitates, and then 550 μL of the supernatants were transferred to 5-mm NMR tubes. Kidney tissues (about 200 mg) were extracted with 900 μL of methanol/water (2/1) using a tissue lyzer (QIAGEN TissueLyser II, Germany) at 20 Hz for 90 s. The homogenate was then sonicated in an ice bath to further break cells. After centrifugation (13 000 r·min^{-1}, 15 min, 4 °C), the supernatants were lyophilized in a vacuum to remove the methanol. Each extract was reconstituted in 600 μL of phosphate buffer, and after centrifugation at 4 °C and 13 000 r·min^{-1} for 20 min, 550 μL of the supernatants were transferred into 5-mm NMR tubes for ^{1}H NMR analysis.

^{1}H NMR spectra of urine and kidney samples were re-