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Original Article

Huangqi-Danshen decoction alleviates diabetic nephropathy in *db/db* mice by inhibiting PINK1/Parkin-mediated mitophagy

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Abstract: Huangqi-Danshen decoction (HDD) is composed of Astragali Radix (Huang-qi) and Salviae Miltiorrhizae Radix et Rhizoma (Dan-shen), both of which are the most commonly used herbs for the clinical treatment of diabetic nephropathy (DN) in traditional Chinese medicine and show good efficacy. However, the underlying mechanism of this effect is unclear. The aim of this study was to evaluate the effect and potential mechanism of HDD in the treatment of DN in a type 2 diabetic animal model, *db/db* mice. HDD extract was administered orally to *db/db* mice at a dose of 6.8 g/kg/day for 12 weeks. At the end of the study, serum, urine, and kidney samples were collected for biochemical and pathological examination. The expression of proteins associated with mitochondrial fission and mitophagy was determined by quantitative real-time PCR, Western blotting, and immunohistochemical analysis. The results showed that treatment with HDD substantially reduced urinary albumin excretion and improved renal injury in *db/db* mice. Moreover, mitochondrial fission was increased in the kidneys of the *db/db* mice, as evidenced by enhanced expression of dynamin-related protein 1 and mitochondrial morphological changes. Furthermore, PTEN-induced putative kinase 1 (PINK1)/Parkin-mediated mitophagy was activated in the *db/db* mice, which manifested as increased protein expression and obvious autophagic vacuole encapsulating mitochondria. HDD treatment significantly reversed the enhanced mitochondrial fission and PINK1/Parkin-mediated mitophagy in the *db/db* mice. In conclusion, this work suggested that HDD could protect against type 2 diabetes-induced kidney injury possibly by inhibiting PINK1/Parkin-mediated mitophagy.

Keywords: Diabetic nephropathy, traditional Chinese medicine, Huangqi-Danshen decoction, PINK1/Parkin, mitophagy, *db/db* mice

Introduction

Diabetic nephropathy (DN) is one of the most severe chronic microvascular complications of diabetes mellitus (DM) [1]. The results from the Global Burden of Disease 2017 Study showed that the age-standardized prevalence of DN in men and women was 15.48/1000 and 16.50/1000, respectively [2]. DN affects approximately 40% of people with diabetes and is the leading cause of chronic kidney disease (CKD) worldwide [3]. The current standard of treatment for DN involves early detection, glycaemic control and stringent blood pressure management with preferential use of renin-angiotensin system blockade [4, 5]. In China and other Asian countries, traditional Chinese medicine (TCM) has been widely used to treat diabetes and its complications for a long time [6, 7]. Huangqi-Danshen decoction (HDD) is composed of Astragali Radix (Huang-qi) and Salviae Miltiorrhizae Radix et Rhizoma (Dan-shen), both of which are the most commonly used herbs for the clinical treatment of DN [8]. Our previous studies have reported that HDD could retard the progression of CKD in rats [9, 10]. However, the efficacy and potential mechanisms of HDD in DN remain unknown.

The kidney is the organ with the second highest mitochondrial content and oxygen consumption after the heart [11]. Accumulating evidence indicates that mitochondrial dysfunction contributes to the development and progression of

DN [12, 13]. Mitochondrial function depends on their quality control mechanism, and an essential characteristic of this quality control is the selective elimination of dysfunctional mitochondria by mitophagy [14, 15]. In mammalian cells, the main orchestrators of mitophagy are (PTEN)-induced putative kinase 1 (PINK1) and the ubiquitin ligase Parkin [16, 17]. A growing body of evidence has indicated that altered mitophagy may be important in the development and progression of DN [18-21]. In the present study, we investigated the role of HDD in delaying DN and explored the potential mechanism related to PINK1/Parkin-mediated mitophagy in a *db/db* mouse model of type 2 diabetes.

Materials and methods

Preparation of HDD extract

HDD consists of Astragali Radix [roots of *Astragalus membranaceus* (Fisch). Bge. var. *mongholicus* (Bge). Hsiao] and Salviae Miltiorrhizae Radix et Rhizoma (roots and rhizomes of *Salvia miltiorrhiza* Bge) at a ratio of 2:1 (W/W) based on the dry weight of the product. Astragali Radix and Salviae Miltiorrhizae Radix et Rhizoma were weighed and boiled twice in 8x ddH_2O (w/v) for 1 h per time. The extraction liquid was centrifuged, and the supernatant was dried by a freeze dryer and stored at -80°C. Before the treatment, the powder was redissolved with Milli-Q water and vortexed at room temperature to obtain the HDD extract. The quality control of the HDD extract was conducted via high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis as previously described [9, 10].

Animals

All animal experiments were conducted with protocols approved by the Ethics Committee of Shenzhen Traditional Chinese Medicine Hospital, Guangzhou University of Chinese Medicine, and all efforts were made to minimize animal suffering. Male diabetic *db/db* mice and nondiabetic littermate control *db/m* mice at the age of 8 weeks were obtained from the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). After one week of acclimatization, 12 *db/db* mice were randomly divided into two groups: the model group (*db/db*, n = 6) and the HDD treatment group (*db/db*+HDD, n = 6). Six *db/m* mice served as a control group (*db/m*, n = 6). The *db/db*+HDD group was administered HDD extract (6.8 g/kg/day) by gastric irrigation for 12 weeks. All mice had free access to food and water during the experiments. At the end of the study, urine samples were collected in metabolic cages, all mice were anesthetized, and blood samples were obtained by eye enucleation. The mice were euthanized by cervical dislocation without regaining consciousness. The kidneys were rapidly harvested, weighed, and processed for histological examination, PCR, Western blotting, and immunohistochemical analysis.

Biochemical analysis

Blood glucose levels were determined by using GlucoDr™ Plus (Allmedicus, Anyang, Gyeonggi-Do, Korea). Serum creatinine (Scr) and blood urea nitrogen (BUN) were measured by a creatinine serum detection kit and a BUN detection kit (StressMarq Biosciences, British Columbia, Canada), respectively, following the manufacturer's instructions. Urine creatinine was measured by a QuantiChrom™ Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA). The urinary albumin concentrations were measured using a mouse albumin ELISA Kit (Bethyl Laboratories, Montgomery, TX, USA). Urinary albumin to creatinine ratio (ACR) was calculated by dividing urinary albumin by urine creatinine.

Histological analysis

Periodic-acid-Schiff (PAS) staining was performed to detect the structures of the paraffin-embedded kidney sections. One hundred and twenty glomeruli from six mice in each group were measured for glomerular tuft area using Nikon NIS-Elements BR software version 4.10.00 (Nikon, Japan) in a blinded manner.

Transmission electron microscopy (TEM)

Kidney cortexes were fixed in cold 2.5% glutaraldehyde, treated with osmium tetroxide and stained with uranyl acetate. After being dehydrated in gradient acetone, the kidney tissues were embedded in epoxy resin. Ultrathin sections were cut (EM UC7, Leica, Wetzlar, Germany) and visualized by using a transmission electron microscopy (HT7700, Hitachi, Tokyo, Japan).

Table 1. Primer sequences for quantitative real-time PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Drp-1 (Mouse)	ACCGGGAATGACCAAAGTACC	TGGGATTACTGATGAACCGAAGA
PINK1 (Mouse)	CACACTGTTCCTCGTTATGAAGA	CTTGAGATCCCGATGGGCAAT
Parkin (Mouse)	TCTTCCAGTGTAACCACCGTC	GGCAGGGAGTAGCCAAGTT
GAPDH (Mouse)	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC

Quantitative real-time PCR

Total RNA was extracted from the renal cortex using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Equal amount of total RNA (1 μg) was reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit (Perfect Real Time, Takara, Japan) according to the manufacturer's instructions. The primer sequences used in this study were shown in **Table 1**. Quantitative real-time PCR was performed in triplicates on an Applied Biosystem 7500 quantitative PCR system (Applied Biosystems, Foster City, CA, USA). The Ct values obtained from different samples were compared using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal reference gene.

Western blotting

Proteins were extracted from kidneys using RIPA buffer (Cell Signaling Technology, Beverly, MA, USA) containing a protease inhibitor cocktail. The protein concentration was measured by the Bradford method. Equal amounts of protein lysates were loaded on and electrophoresed through 10% SDS-PAGE gels and were then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% nonfat milk for 1 h at room temperature and then incubated with primary antibodies against dynamin-related protein 1 (Drp-1, 1:1000) (Cell Signaling Technology, Beverly, MA, USA), PINK1 (1:500) (Gene Tex, San Antonio, TX, USA), Parkin (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (1:5000) (Proteintech, Wuhan, China), and β-actin (1:5000) (Sigma-Aldrich, St Louis, MO, USA) at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000) (Life Technologies, Carlsbad, CA, USA). The blots were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) and a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Densitometric analysis was performed by using Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry

Immunohistochemistry was performed on the formalin-fixed paraffin sections using sodium citrate (pH 6.0) for antigen retrieval. The slides were immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity and then blocked with 10% goat serum for 1 h at 37°C. Primary antibodies against Drp-1 (1:100), PINK1 (1:100), and Parkin (1:50) were added, followed by treatment with SignalStain Boost Detection Reagent (Cell Signaling Technology). The sections were developed with SignalStain diaminobenzidine (DAB) substrate (Cell Signaling Technology) to produce a brown product. The integrated optical density (IOD) values of the positive staining for Drp-1, PINK1, and Parkin were measured by using ImagePro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). Five microscopic fields (200×) of each mouse and three mice in each group were used for quantification in a blinded manner.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM) or a box-and-whisker diagram. The significance of the differences among groups was examined by one-way ANOVA followed by *post hoc* analysis with Tukey's test. SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of HDD on the physiological parameters of the db/db mice

Compared with the *db/m* mice, the *db/db* mice exhibited higher blood glucose, higher urinary ACR, a higher body weight and kidney weight, a lower kidney weight to body weight ratio, and a higher Scr level (**Figure 1A-F**). Strikingly, the high blood glucose and enhanced urinary ACR levels were significantly reversed after HDD

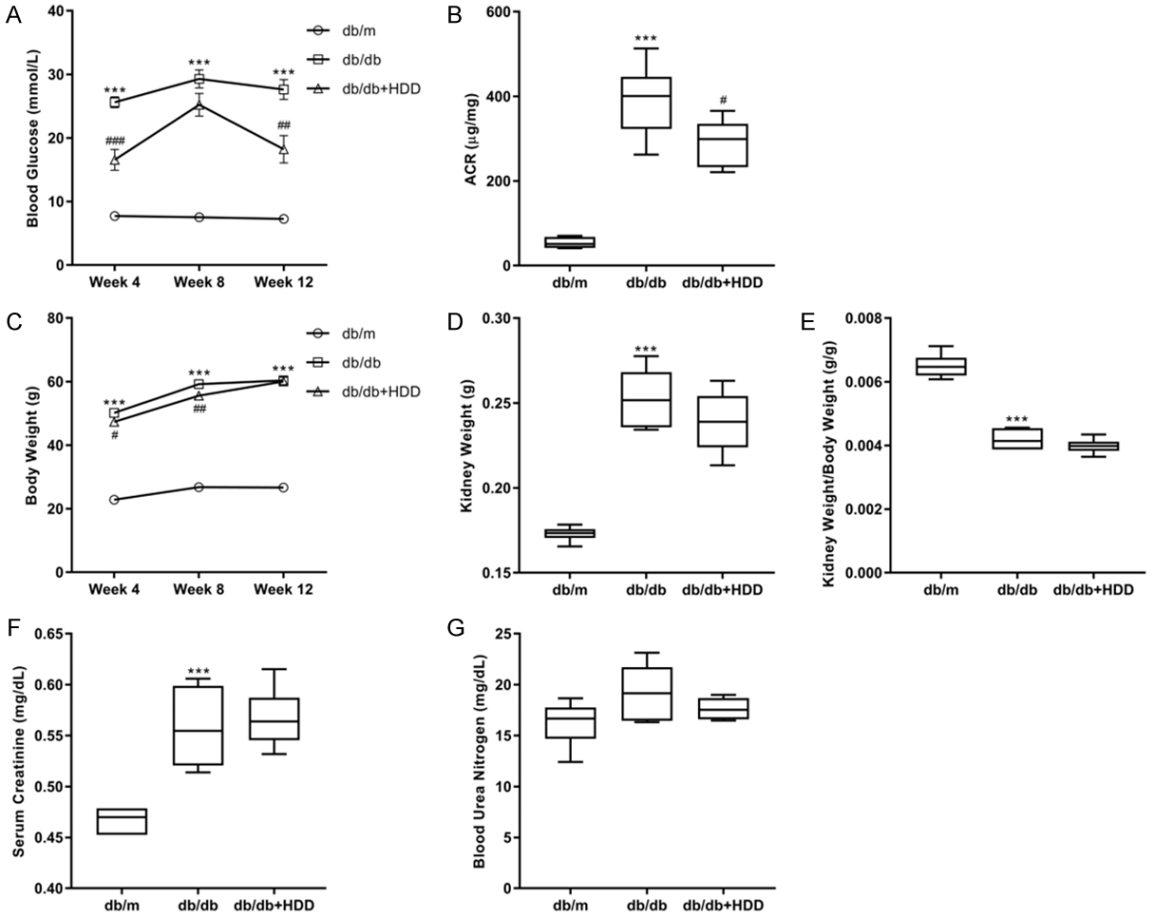

Figure 1. Effect of HDD on the physiological parameters of the *db/db* mice. A. Blood glucose levels of each group at week 4, week 8, and week 12. B. Urinary albumin to creatinine ratio (ACR) level increased in the *db/db* mice and was reversed after HDD treatment. C. Body weight of each group at week 4, week 8, and week 12. D. Kidney weight was higher in the *db/db* mice at the end of the study. E. Kidney weight to body weight ratio was lower in the *db/db* mice at the end of the study. F. Serum creatinine (Scr) level was higher in the *db/db* mice at the end of the study. G. Blood urea nitrogen (BUN) levels were not significantly different among the three groups at the end of the study. n = 6 mice per group ($^{***}P < 0.001$ compared with the *db/m* group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the *db/db* group).

treatment (**Figure 1A**, **1B**). A slight reduction in the body weight, kidney weight, kidney weight to body weight ratio, and Scr levels was observed in the *db/db* mice treated with HDD (**Figure 1C-F**). No significant difference in the BUN levels was observed among the three groups (**Figure 1G**).

HDD ameliorated renal injury in the db/db mice

In PAS staining, the kidneys from the *db/db* mice demonstrated obvious features of DN, including glomerular hypertrophy and increases in mesangial cells and mesangial matrix. These histological lesions were significantly attenuated in the mice treated with HDD (**Figure 2**). Furthermore, transmission electron microscopy revealed extensive fusion of the podocyte foot processes in the *db/db* mice, which was obviously improved after HDD treatment (**Figure 3**). These results indicated that HDD ameliorated renal injury in the *db/db* mice.

HDD suppressed mitochondrial fission in the kidneys of the db/db mice

For mitophagy to occur, mitochondria must undergo fission to fragment into spheroids that can be encapsulated within autophagic vesicles [22]. Therefore, mitochondrial fission was investigated in the present experimental setting. PCR and Western blot results showed that the expression of Drp-1, a master regulator of

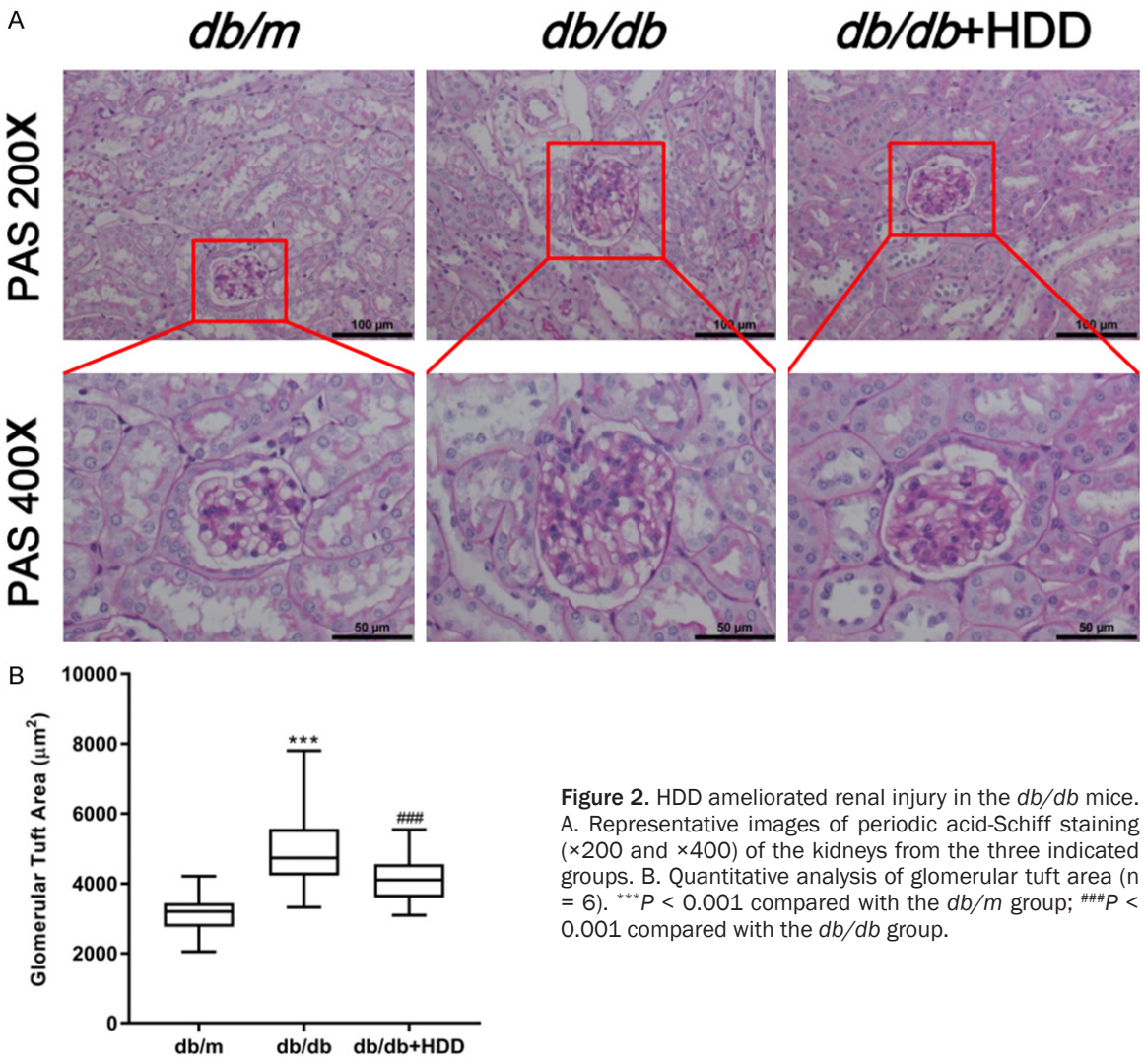

Figure 2. HDD ameliorated renal injury in the *db/db* mice. A. Representative images of periodic acid-Schiff staining (×200 and ×400) of the kidneys from the three indicated groups. B. Quantitative analysis of glomerular tuft area (n = 6). $^{***}P < 0.001$ compared with the *db/m* group; $^{###}P < 0.001$ compared with the *db/db* group.

mitochondrial fission, was significantly upregulated in the kidneys of the *db/db* mice and was markedly reduced after HDD treatment (**Figure 4A-C**). By immunohistochemistry, we confirmed that the enhanced protein level of Drp-1 was decreased in the *db/db*+HDD group (**Figure 4D** and **4E**). Moreover, transmission electron microscopy showed that mitochondria fragmented into small, punctuated suborganelles in the *db/db* mice, and this effect was partially reversed in the *db/db*+HDD group (**Figure 4F**). These findings indicated that HDD suppressed mitochondrial fission in the *db/db* mice.

HDD inhibited PINK1/Parkin-mediated mitophagy in the kidneys of the db/db mice

By PCR and Western blot analysis, the mRNA and protein expression of PINK1 and Parkin were significantly upregulated in the *db/db* mice, and were reduced after HDD treatment (**Figure 5A-E**). Immunohistochemistry analysis further confirmed that administration of HDD inhibited the enhanced expression of PINK1 and Parkin in the *db/db* mice (**Figure 5F-H**). Moreover, transmission electron microscopy showed obvious autophagic vacuole encapsulating mitochondria in the *db/db* group but not in the *db/db*+HDD group (**Figure 5I**). These data indicated that HDD inhibited PINK1/Parkin-mediated mitophagy in the *db/db* mice.

Discussion

The *db/db* mouse model of type 2 diabetes is considered to be a suitable experimental model for studying the development and progression of DN since it exhibits typical characteristics

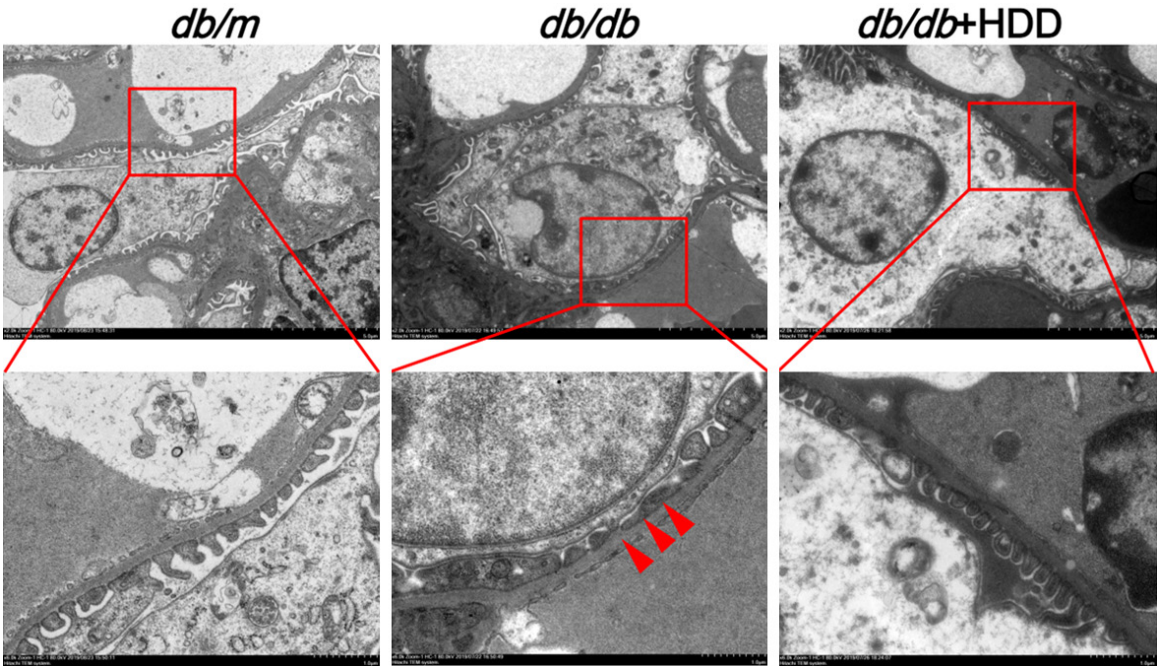

Figure 3. HDD attenuated podocyte foot process fusion in the *db/db* mice. Red triangle indicates fusion of podocyte foot processes in the *db/db* mice, which was obviously improved after HDD treatment.

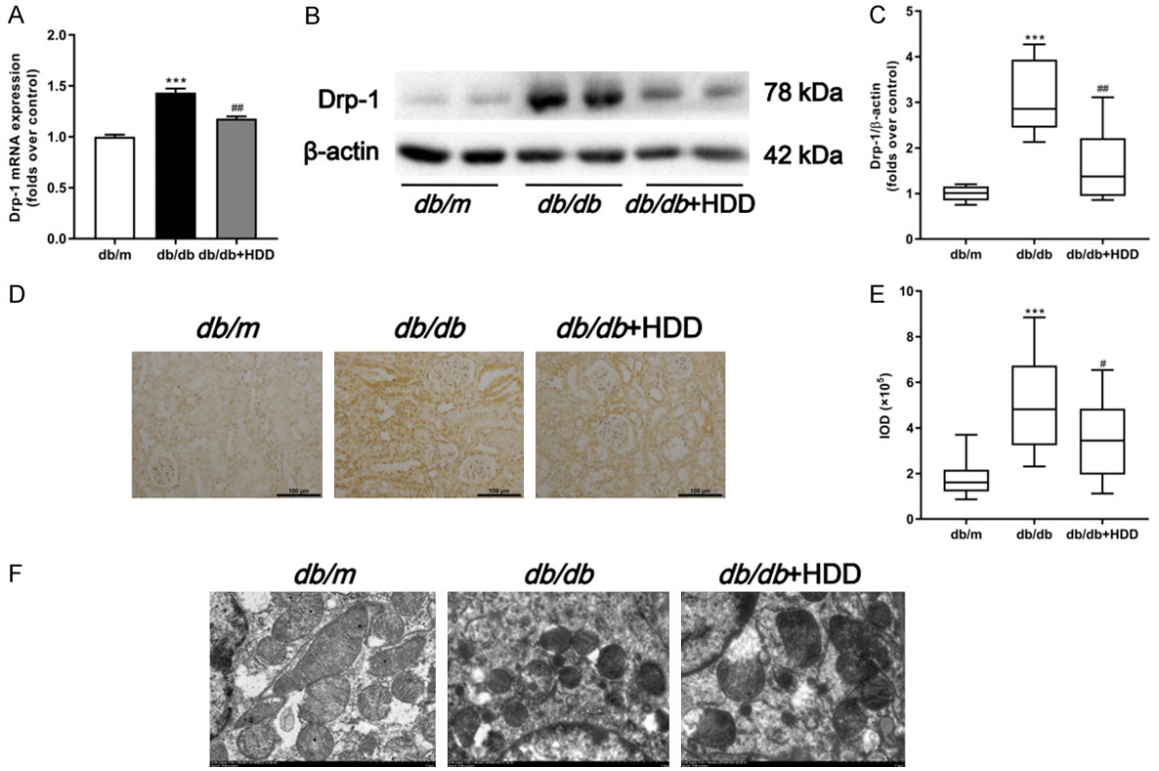

Figure 4. HDD suppressed mitochondrial fission in the kidneys of the *db/db* mice. A. HDD decreased Drp-1 mRNA abundance in the *db/db* mice. B. Representative Western blot image of Drp-1 protein expression. C. Densitometric analysis of Drp-1 protein expression normalized to β-actin content (n = 6). D. Representative immunohistochemistry images of Drp-1. All images are shown at identical magnification, ×200, scale bar = 100 μm. E. Quantitative analysis of Drp-1 positive staining (n = 3). F. Representative transmission electron microscopy images of mitochondria in the kidneys from the three indicated groups. $^{***}P < 0.001$ compared with the *db/m* group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the *db/db* group.

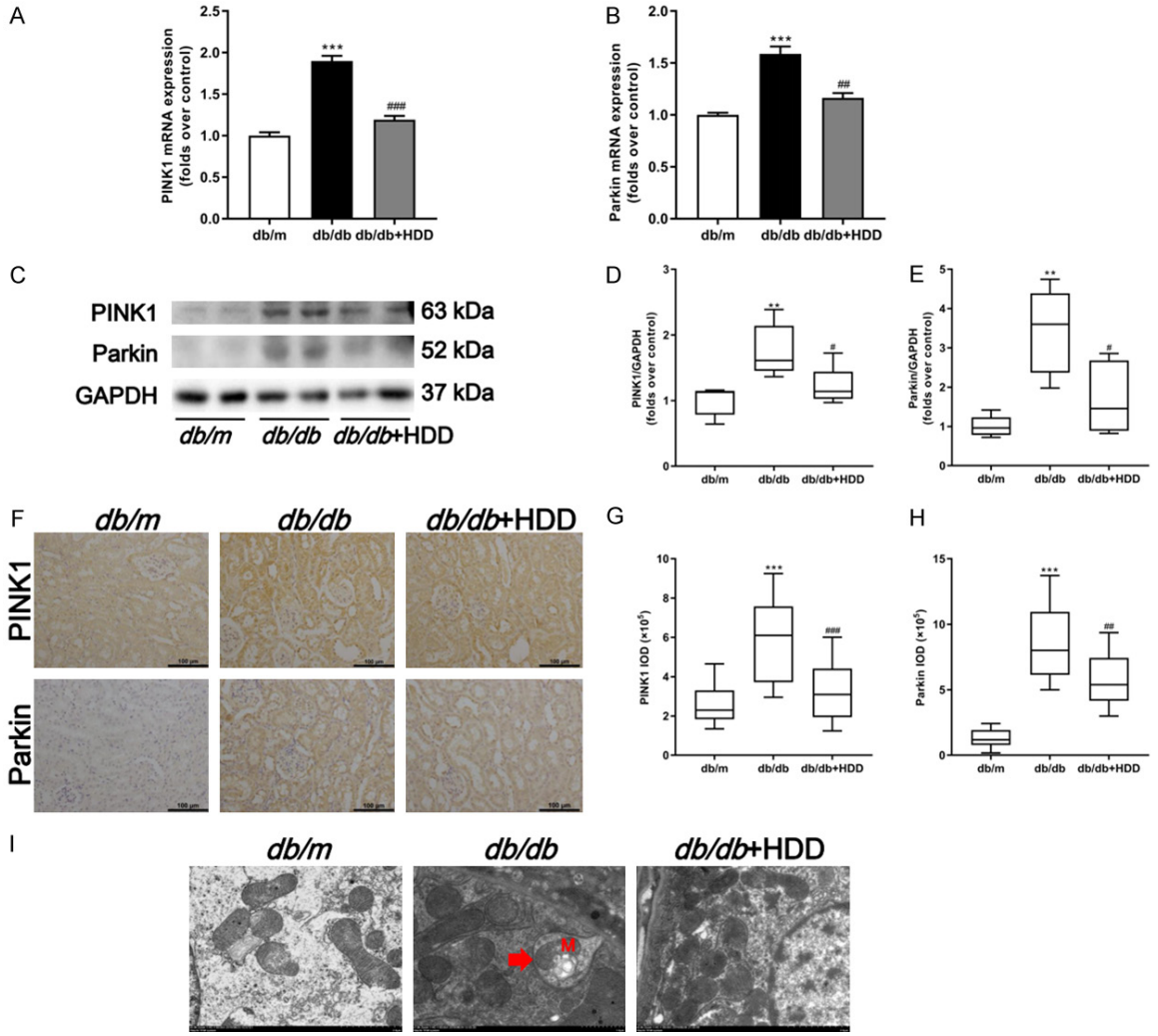

Figure 5. HDD inhibited PINK1/Parkin-mediated mitophagy in the kidneys of the *db/db* mice. A. HDD decreased PINK1 mRNA abundance in the *db/db* mice. B. HDD decreased Parkin mRNA abundance in the *db/db* mice. C. Representative Western blot images of PINK1 and Parkin protein expression. D, E. Densitometric analysis of PINK1 and Parkin protein expression normalized to GAPDH content, respectively (n = 6). F. Representative immunohistochemistry images of PINK1 and Parkin. All images are shown at identical magnification, ×200, scale bar = 100 μm. G, H. Quantitative analysis of PINK1 and Parkin positive staining, respectively (n = 3). I. Representative transmission electron microscopy images of mitophagy in the kidneys from the three indicated groups. Red arrow indicates autophagic vacuole that encapsulates mitochondria. "M" indicates mitochondrion. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the *db/m* group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the *db/db* group.

similar to those of human DN [23, 24]. In the present study, treatment with HDD decreased the urinary excretion of albumin and improved renal injury in the *db/db* mice. PINK1/Parkin-mediated mitophagy was activated in the kidneys of the *db/db* mice and was downregulated in response to HDD treatment.

Increasing evidence indicates that mitochondrial dysfunction contributes to the development and progression of DN [12, 13]. Mitochondria have high plasticity and constantly undergo fission and fusion, biogenesis, and mitophagy. Timely removal of damaged mitochondria via mitophagy is critical for mitochondrial quality control and thus for cellular homeostasis and function [25]. However, similar to autophagy, mitophagy may also be a double-edged sword, indicating that overactivation of mitophagy may be harmful to cells [26, 27]. The state of mitophagy in DN reported in previous studies is not consistent. Smith *et al.* reported that

PINK1 protein was increased in the renal cortex in a rat model of STZ-induced diabetes [28]. Our previous study also found that PINK1/Parkin mediated mitophagy was activated in a *db/db* mouse model [19]. In contrast, defective mitophagy has also been reported to be present in DN in studies that used STZ-induced diabetic mice or *db/db* mice [29, 30]. The possible explanation for these conflicting results includes animal model selection, the experimental cycle, and blood glucose levels. Therefore, how mitophagy is changed in DN and whether this change is beneficial or detrimental to kidney function should be further investigated [31].

Over two thousand years ago, diabetes-related symptoms were called "Xiaoke" disease in traditional Chinese medicine (TCM). Since then, many TCM therapeutic strategies, including Chinese herbal medicine (CHM), acupuncture, moxibustion, and massage for treating diabetes and its complications have been recorded, and abundant experience has been accumulated [6]. CHM is the main form of TCM treatment for DN, and decoction is a common form of CHM [8]. A systematic review and meta-analysis reported that CHM could reduce the albuminuria levels in patients with DN [32]. In a population-based cohort study, Chen *et al.* reported that CHM use was associated with decreased end-stage renal disease and mortality rates among patients with DN [33]. According to the TCM theory, deficiency of Qi with blood stasis (Qi-Xu-Xue-Yu) is the common syndrome pattern of DN. Therefore, tonifying Qi and promoting circulation (Yi-Qi-Huo-Xue) is one of the basic therapeutic principles of TCM in the treatment of DN [8]. Huangqi-Danshen decoction (HDD) is composed of Astragali Radix (Huangqi), which serves to tonify Qi, and Salviae Miltiorrhizae Radix et Rhizoma (Dan-shen), which serves to promote circulation [9]. Huang-qi and Dan-shen are the most and the third most commonly used drugs, respectively, in herbal formulations for DN in clinical trials [8]. Huang-qi has been reported to protect the kidney by inhibiting oxidative stress [34] and rebalancing TGF-β/Smad signaling [35] in a diabetic model. In addition to attenuating oxidative stress [36] and inflammation [37], Dan-shen was recently shown to protect against DN through metabolome regulation and inhibition of Wnt/β-catenin and TGF-β signaling [38]. In the present study, we revealed the regulatory effect of HDD on PINK1/Parkin-mediated mitophagy in a *db/db* mouse model of type 2 diabetes. However, how HDD regulates mitophagy and how mitophagy contributes to DN require further investigation.

In conclusion, HDD significantly alleviated DN in *db/db* mice, which might be associated with the inhibition of PINK1/Parkin-mediated mitophagy.

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Disclosure of conflict of interest

None.

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