Original Paper

Huangqi Decoction Ameliorates Streptozotocin-Induced Rat Diabetic Nephropathy through Antioxidant and Regulation of the TGF- β /MAPK/PPAR- γ Signaling

Haiyan Han^{a,c} Aili Cao^b Li Wang^b Hengjiang Guo^b Yingjun Zang^a Zezheng Li^a
Xuemei Zhang^d Wen Peng^{a,b}

^aDepartment of Nephrology, ^bLaboratory of Renal Disease, Putuo Hospital, Shanghai, University of Traditional Chinese Medicine, ^cDepartment of Nephrology, Shanghai Seventh People's Hospital, ^dDepartment of Pharmacology, School of Pharmacy, Fudan University, Shanghai, China

Key Words

Huangqi Decoction • Diabetic nephropathy • Antioxidant

Abstract

Background/Aims: Huangqi Decoction (HQD) has been traditionally used to treat diabetes mellitus in China. The present study was carried out to assess the protective effect of HQD on diabetic nephropathy (DN) using the streptozotocin-induced (STZ) diabetic rats. **Methods:** Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg) in male Wistar rats. 40 diabetic rats were divided into 5 groups: vehicle-treated (DN group), 0.45, 0.15, 0.05 g/kg HQD-treated diabetic group (HQD group) and 1 mg/kg rosiglitazone-treated diabetic group (RGZ group). 16 normal rats were randomly divided into 2 groups: vehicle-treated normal control group (NC) and 0.45 g/kg HQD-treated normal control group (NC+0.45 g/kg HQD). At the end of 8-week experiment, we measured changes of renal pathological morphology, function, antioxidant enzyme levels and the activation of TGF- β /PPAR- γ /MAPK signaling pathway. **Results:** After HQD treatment, renal function, including blood urea nitrogen (BUN), 24-h albuminuria and blood glucose level were improved significantly; meanwhile, impaired kidney redox balance was diminished in diabetic rats. The activation of TGF- β , phospho-JNK, phospho-p44/42, p47 and p42 phox was blocked and the decrease in PPAR- γ in diabetic rats was attenuated by treatment with HQD in a dose-dependent manner. **Conclusion:** These results suggest that HQD shows therapeutic efficacy in DN characterized by renal dysfunction and pathological changes through hypoglycemic and antioxidant effects.

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H. Han and A. Cao contributed equally to this work.

Professor Wen Peng
and Professor Xuemei Zhang

Dept Nephrol, Lab Renal Disease, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, 164 Lan Xi Road, Shanghai 200062 (China) and Dept Pharmacol, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203 (China)
E-Mail pengwen_01@vip.sina.com or xuemeizhang@fudan.edu.cn

Introduction

The total prevalence of diabetes mellitus in China population was 11.6 % and about one-thirds developed into diabetic nephropathy (DN), one of the most important complications of diabetic mellitus and with high risk of progressive kidney disease [1, 2]. Sustained hyperglycemia, results from lack of insulin secretion in type 1 diabetes or reduced sensitivity of tissues to insulin in type 2 diabetes, is the leading cause of DN and induces glomerular dysfunction and renal damage [3]. Since the progression to end-stage renal disease is irreversible, it is necessary to find methods to delay renal damage progress. Tremendous progress has been made in understanding the pathophysiology of DN. However, effective therapies are still lacking for DN patients [4, 5].

Enhanced oxidative stress and the activation of transforming growth factor- β (TGF- β) signaling pathway are the main molecular pathways mediated during DN [6]. The balance between antioxidant defense system, including dicarboxylic aldehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, upstream of the reactive oxygen species (ROS), determine the degree of oxidative stress [7, 8]. ROS further up-regulate the expression of TGF- β through the activation of a signal transduction cascade involving mitogen-activated protein kinase (MAPK) and nuclear receptor peroxisome proliferator-activated receptor- γ (PPAR- γ) [9, 10]. Recently, antioxidant treatments have begun to be investigated as potential DN therapeutics [11, 12].

Huangqi decoction (HQD), comprised of *Astragalus*, *Poria*, *Trichosanthes*, *Ophiopogon*, *Schisandra*, *Licorice* and *Rehmannia*, has been used for treatment diabetes mellitus thousands of years ago in China. HQD effectively inhibits unilateral ureteral obstruction kidney damage in mice through downregulating the TGF- β / Smad signaling pathway [13]. HQD improves dysfunction initiated by homocysteine through antioxidant mechanisms [14]. Astragaloside IV, the main active ingredients of HQD, attenuates podocyte apoptosis mediated by endoplasmic reticulum stress in DN [15]. Accordingly, HQD possesses antioxidant activities or could improve diabetic complications. Here, we aimed to evaluate the effect of HQD on streptozotocin (STZ)-induced DN thus exploring whether the antioxidant mechanism is involved in the beneficial effects of HQD.

Materials and Methods

HQD preparation and animal treatment

Astragalus, *Poria*, *Trichosanthes*, *Ophiopogon*, *Schisandra*, *licorice*, and *Rehmannia* were provided by Shanghai Huayu Chinese Herbs Co. Ltd. (Shanghai, China). HQD was extracted and the composition of the complete HQD was analyzed by LC-MS as previously reported [14]. Fifty six male Wistar rats (180 g) were purchased from Shanghai laboratory animal center (Shanghai, China) and housed for 2 weeks of acclimation in a temperature controlled room under 12/12 h light/dark cycle and had free access to food and water. Then animals were induced to diabetes by intraperitoneal injection of freshly prepared STZ (Sigma-Aldrich, St Louis, MO, USA, dissolved in 0.01 M citrate buffer, pH 4.5) at a single dose of 60 mg/kg. 1 week after STZ injection, fasting blood glucose was measured to verify the development of diabetes. Rats with blood glucose > 14 mM were randomly separated into 5 groups (n=8 for each group) and treated respectively with vehicle (0.5% carboxymethyl cellulose), 0.05, 0.15, 0.45 g/kg HQD, 1 mg/kg rosiglitazone by daily gavage for 8 weeks. NC rats without STZ treatment were randomly divided into 2 groups and administered respectively with vehicle (n=8) and 0.45 g/kg HQD (n=8) as controls. This work was carried out in accordance with the approved guidelines for the use of experimental animals in Putuo Hospital, Shanghai University of Traditional Chinese Medicine.

Measurement of metabolic and biochemical parameters

Body weight, water intake, food intake and fasting glucose were measured at 4-week intervals. Blood glucose level was monitored with Omron HEA-230 Glucometer (Omron Corporation, Kyoto, Japan) by using

one drop of tail blood. Blood samples were drawn from orbit for serum BUN and creatinine detection at 8 weeks after drug treatment. Kidneys were immediately harvested for protein or histological analysis in the end of experiment. Urinary albumin and blood urea nitrogen were measured with commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and determined according to the manufacturer's instructions.

Measurement of oxidative stress

Serum and kidney MDA, SOD and GSH-PX levels were assayed according to the instructions of the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Renal histology and immunohistochemistry

The kidneys were fixed in 4% paraformaldehyde (PFA), dehydrated, embedded in paraffin and cut into 4 μ m-thick sections. Renal sections were stained with hematoxylin and eosin (HE), Masson's trichrome or periodic acid-Schiff (PAS). Semiquantitative scoring of glomerular sclerosis was performed using a five-grade method described previously [16]. Twenty to thirty glomeruli randomly selected from per rats were scored from six rats in each group. For immunohistochemistry, paraffin-embedded sections were stained with primary antibodies against TGF- β (ab64715, Abcam, MA, USA), and PPAR- γ (ab66343, Abcam, MA, USA) overnight at 4 °C. The integrated optical density was measured by computer analysis with ImagePro Plus 6.0 (Media Cybernetics, MD, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

The apoptotic cells in glomeruli were evaluated by TUNEL staining with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. TUNEL-positive cells were semiquantified by randomly counting at least 30 glomeruli in each rat.

Protein extraction and Western blot Analysis

Renal tissues from each group were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) complemented with protease inhibitor cocktail and phosphatase inhibitor (Sigma-Aldrich, St Louis, MO, USA). Total protein concentration from the supernatant was determined by the bicinchoninic acid (BCA) protein assay (Boster Biotechnology, Wuhan, China). Protein samples (60 μ g) were separated by electrophoresis on 12% SDS-PAGE gels, electrotransferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% bovine serum albumin (BSA) for 1 h and incubated with p47phox (sc-14015, Santa Cruz, CA, USA); p22phox (sc-20781, Santa Cruz, CA, USA); TGF- β (ab64715, Abcam, MA, USA); PPAR- γ (ab66343, Abcam, MA, USA); phospho-p44/42 (4376S, CST, MA, USA), p44/42 (4695S, CST, MA, USA), phospho-JNK (sc-6254, Santa Cruz, CA, USA), JNK (sc-571, Santa Cruz, CA, USA), phospho-p38 (9211S, CST, MA, USA), p38 (9212S, CST, MA, USA) and β -actin (4324866, BD, New Jersey, USA) at 4 °C overnight and then incubated with the appropriated HRP-conjugated secondary antibodies for 2 h at room temperature. Density of the corresponding bands was measured by Bio-Rad VersDoc imaging system using chemiluminescence detection reagents (Millipore, Billerica, MA, USA). The data were analyzed with Bio-Rad Quantity One software (Bio-Rad, CA, USA). Protein expression was normalized with β -actin and with total proteins for phosphorylated proteins.

Statistical analysis

Data are expressed as the means $\pm$ SEM, with n representing the number of animals. Statistical analysis was conducted with GraphPad Prism software 5 software (GraphPad Software Inc., San Diego, CA, USA). Unpaired two-tailed t test or one-way ANOVA followed by the Newman-Keuls multiple comparisons test was used for statistical comparisons among experimental groups, with a value of P < 0.05 being considered statistically significant.

Results

HQD prevented the development of DN in STZ-induced diabetic rats

Body weight, food intake, water intake and fasting blood glucose were recorded during the 8-week period. After treatment for 8 weeks, HQD and RGZ could attenuate the weight loss of the DN rats. Meanwhile, diabetic rats took more water and food, and the hyperglycemia

Fig. 1. HQD prevented the development of diabetes in STZ-induced diabetic rats. (A) Body weight, (B) water intake, (C) food intake, and (D) blood glucose. $**P<0.001$, compared with the normal control (NC) group; $#P<0.05$, $##P<0.001$, compared with the diabetic nephropathy (NC) group ($n=8$).

persisted during the experiment period. HQD at medium (0.15 g/kg) or high dose (0.45 g/kg) and RGZ significantly dampened the increase in water and food intake and blood glucose after 4-week treatment in diabetic rats, while low dose group did not change these parameters during 8 weeks (Fig. 1).

The damaged renal function in the diabetic rats was also manifested by the elevation of BUN and urinary albumin (Fig. 2). At 8 weeks after HQD administration, HQD treatment reduced 24-h urine albumin and BUN in a dose-dependent manner (Fig. 2A&B). In addition, RGZ treatment of the diabetic rats also prevented the increase of urinary albumin (Fig. 2B). Therefore HQD improved renal function in the STZ-induced diabetic rats.

HQD attenuated the pathological changes in STZ-induced diabetic rats

It is apparent that STZ-induced rats had significantly higher kidney to body weight ratios compared with the controls. Treatment with 0.45 g/kg dose of HQD or RGZ

produced significant decrease in kidney to body weight ratios (Fig. 3E). In order to examine potential renal cortical morphological changes in STZ-induced diabetic rats, we performed HE, PAS and Masson's trichrome staining. As shown in Fig. 3 A-C, in diabetic rats, there was a significant increase in nephropathy compared with control group, the glomerular surface area increased, mesangial matrix markedly expanded, collagen deposition elevated in the glomeruli. HQD treatment markedly attenuated glomerular hypertrophy, mesangial matrix expansion, glomerulosclerosis and collagen deposition in DN rats (Fig. 3A-C, E-G).

Podocyte is the component of glomerular filtration barrier and its injury is the onset of DN [17]. The number of apoptotic cells within the glomeruli significantly increased in DN-vehicle rats compared with NC-vehicle rats, which was partially rescued by HQD administration in a dose-dependent manner, as evidenced by TUNEL staining (Fig. 3D&H), implying that HQD ameliorated the renal pathological changes from kidney injuries in the diabetic rats.

Fig. 2. HQD treatment protected kidney function in STZ-induced diabetic rats. (A) BUN, (B) 24-h urine albumin. $**P<0.001$, compared with NC group; $#P<0.05$, $##P<0.001$, compared with NC group ($n=8$).

Fig. 3. HQD treatment attenuated the pathological changes in the diabetic kidney. (A) HE staining, (B) PAS staining, (C) Masson staining, (D) TUNNEL staining, (E) kidney weight/body weight ratio (mg/g) (F) glomerular sclerosis index, and (G) quantification of Masson-stained areas. ** $P < 0.001$, compared with NC group; ## $P < 0.001$, compared with NC group (n=8).

Fig. 4. Effect of HQD on oxidative stress parameters in the diabetic kidney. (A) Renal MDA, (B) SOD and (C) GSH-PX activities. (D) Serum MDA, (E) SOD and (F) GSH-PX activities. * $P < 0.05$, ** $P < 0.001$, compared with NC group; # $P < 0.05$, ## $P < 0.001$, compared with NC group (n=8).

Effect of HQD on oxidative stress in the kidneys of STZ-induced diabetic rats

Oxidative stress is associated with the pathogenesis of DN. Excessive lipid deposition increases oxidative stress and thus developing into metabolic syndrome [18]. Intracellular MDA (end product of lipid peroxidation) and activities of antioxidant enzymes, including SOD and GSH-PX, express the state of oxidative stress within the cell. Activities of serum and kidney MDA, SOD and GSH-PX were shown in Fig. 4. The untreated diabetic rats showed significantly reduced serum and renal SOD, GSH-PX and increased MDA activities compared to the non-diabetic control rats. Treatment with 0.45 or 0.15 g/kg dose of HQD produced significant increase in SOD and GSH-PX activities, and decrease in MDA activity. Meanwhile, RGZ showed antioxidant effect on diabetic rats as well.

Fig. 5. Effect of HQD on TGF- β expression in the diabetic kidney. (A) TGF- β immunostaining, (B) quantification of integrated optical density, (C) immunoblots and (D) quantification of TGF- β . ** P <0.001, compared with NC group; # P <0.05, ## P <0.001, compared with NC group (n=8).

Fig. 6. Effect of HQD on PPAR- γ expression in the diabetic kidney. (A) PPAR- γ immunostaining, (B) quantification of integrated optical density, (C) immunoblots and (D) quantification of PPAR- γ . ** P <0.001, compared with NC group; # P <0.05, ## P <0.001, compared with NC group (n=8).

Effect of HQD on TGF- β expression in the kidneys of STZ-treated diabetic rats

TGF- β plays an important role in the diabetic glomerular pathology and is activated under high-glucose conditions [6]. In this study, the TGF- β expression was assessed using IHC staining and western blot. A robust higher expression of TGF- β 1 was observed in the kidneys of diabetic rats compared to normal control; this abnormal upregulation was also reduced by treatment with HQD in a dose-dependent manner. (Fig. 5A&B). Western blot analysis revealed the same result of higher expression of TGF- β 1 observed in the diabetic rat kidney (Fig. 5C&D) and reduced expression after treatment with HQD. RGZ showed similar effects as HQD.

HQD up-regulated the expression of PPAR- γ in the kidneys of DN

PPAR- γ is located in all three types of glomerular cells and benefits all kinds of kidney cells [19, 20]. Here, PPAR- γ expression was dramatically downregulated in DN

Fig. 7. Effect of HQD on p47 and p22 phox protein expression in the diabetic kidney. (A) Immunoblots and (B) quantification of p47 and p22 phox. ** P <0.001, compared with NC group; # P <0.05, ## P <0.001, compared with NC group (n=8).

Fig. 8. Effect of MAPK signaling in the diabetic kidney. (A) Immunoblots and (B-D) quantification of phospho-p42/44, phospho-JNK and phospho-p38. ** $P < 0.001$, compared with NC group; # $P < 0.05$, ## $P < 0.001$, compared with NC group (n=8).

group compared with NC group, both of which were rescued by HQD in a dose-dependent manner (Fig. 6A&B). Western blot analysis revealed the same result of decreased expression of PPAR- γ observed in the diabetic rat kidney (Fig. 6C&D) and enhanced expression after treatment with HQD. RGZ showed similar results as HQD.

Effect of HQD on the expression of the p47 and p22 phox in the kidneys of STZ-treated diabetic rats

As shown in Fig. 7, rats in the diabetic group exhibited significantly increased kidney p47 phox and p22 phox expression levels relative to those observed in the normal control group. However, the group treated with 0.45 or 0.15 g/kg dose of HQD or RGZ exhibited markedly decreased kidney p47 phox and p22 phox protein expression relative to those observed in non-treated diabetic rats. Thus HQD could suppress the expression of the NADPH oxidase subunit p47 phox and p22 phox during DN.

Effect of HQD on the expression of MAPK signaling pathway in the kidneys of DN

MAPK signaling pathway, known to stimulate TGF- β production, also play a critical in the development of DN [21]. In this work, rats in the DN group exhibited dramatically elevated protein expression of phospho-p42/44, phospho-JNK, and phospho-p38 compared with NC group. However, the group treated with 0.45 g/kg dose of HQD showed a significant decrease in the elevation of phospho-JNK expression compared with the diabetes group. In addition, the group treated with 0.45 or 0.15 g/kg dose of HQD showed a significant decrease in the elevation of phospho-p42/44 expression. Meanwhile, RGZ treatment decreased the expression of phospho-p42/44, phospho-JNK and phospho-p38 (Fig. 8).

Discussion

HQD, a classic traditional Chinese medical formula, has been demonstrated to exert protective effects on diabetic complications through antioxidative mechanism [14]. In our study, we demonstrated that HQD had potential to treat DN by improving kidney function, blood glucose level through attenuating oxidative damage. It decreased the expression of NADPH oxidase, inhibited the production of TGF- β , phospho-JNK, phospho-p42/44, and increased PPAR- γ , thereby delaying DN progression.

DN, with hallmarks of proteinuria and glomerulosclerosis, is a complication of diabetes that develops in about 30% of patients with type 1 diabetes [22]. Studies have shown that high glucose concentrations are related to increased ROS production and inhibits proximal tubular function and induces podocytes apoptosis [23]. In our study, STZ-treated

rats developed elevated blood glucose, albuminuria, serum BUN, glomerular hypertrophy, cell apoptosis, mesangial matrix expansion and glomerulosclerosis similar to human DN, indicating the impaired renal function and the development of DN. Interestingly, HQD treatment markedly attenuated these parameters, implying HQD effectively prevented the progression of DN.

Hyperglycemia-induced excess superoxide, resulted from the formation of ROS, has been founded during DN in many studies [6, 24]. ROS levels are regulated by endogenous antioxidants and antioxidases including GSH and SOD, which indirectly reflects the body's ability to clear ROS. SOD1 and SOD3 was downregulated in diabetic rats [25]. MDA, an end-product of lipid peroxidation, directly reflect the extent of lipid peroxidation [26]. In the present study, HQD treatment restored SOD and GSH-PX activities, and decreased MDA levels compared with non-treated diabetic group, suggesting that the antioxidant effect of HQD. Additionally, NADPH oxidase, source of superoxide, protects mesangial matrix expansion in STZ-treated rats [27]. NADPH oxidase activity was affected accompanied by the increased expression of p47 and p22 phox in the diabetic rat kidneys [7]. Administration of HQD could reverse the increased expression of both p47 and p22 phox, putatively verifying that NADPH oxidase-dependent ROS generation exerts effects on STZ-induced DN. HQD improved DN due to its anti-oxidative effect. Similar antioxidant activity had been observed in endothelial dysfunction study on HQD [14].

Thiazolidinediones (TZDs), the synthetic exogenous ligands of PPAR- γ , were widely used in clinic for the treatment of type-2 diabetes mellitus [28]. RGZ is one of representative TZDs and effectively protected kidneys from diabetic rats. Although the blood glucose level was decreased significantly after 8 weeks RGZ treatment compared with non-treated diabetic rats, the blood glucose level was still sustained at a high level, compared with normal rats. Interestingly, Sarafidis et al., and Yang et al., found that TZDs could improve kidneys from diabetic injury independently of its antihyperglycemia action [29, 30]. PPAR- γ activation ameliorated the mitochondrial dysfunction induced by aldosterone in podocytes [31]. Impaired mitochondria generate excessive ROS and release the proapoptotic proteins including cytochrome c, subsequently leads to intrinsic apoptosis and tissue damage [32]. Here, HQD elevated PPAR- γ protein expression in STZ-induced diabetic rats, implying the regulation of mitochondrial dysfunction.

Decreased NADPH oxidase expression may mediate the down-regulation of TGF- β expression [33]. TGF- β is an important mediator of renal fibrosis, characterized by excessive deposition of extracellular matrix leading to glomerulosclerosis [34, 35]. In our study, we found that HQD could reduce collagen deposition thus inhibiting renal ECM accumulation. Besides, the expression of TGF- β was markedly upregulated in the kidneys of diabetic rats and suppressed in HQD-treated rats.

TGF- β induces renal damage by activating its downstream signaling pathway, in which MAPK are included [6]. MAPK is an important intracellular signal transduction molecule, and it possesses multiple functions in regulating cellular growth, proliferation, differentiation and apoptosis. The MAPK family comprises three major subfamilies, namely P42/44, JNK and p38-MAPK, which are believed to contribute to the pathogenesis and development of diabetic nephropathy [36]. High glucose induces Bcl-2 modifying factor via ROS generation and TGF- β expression, thus leading to renal proximal tubular cell apoptosis in diabetic rats [37]. This phosphorylation of Bcl-2 modifying factor was associated with JNK activation, which would result in the collapse of mitochondrial transmembrane potential and activation of the intrinsic pathway of apoptosis. [38]. Meanwhile, ERK and p38 kinase are the components of TGF- β signaling. Their inhibitors attenuated high glucose-induced fibronectin expression in renal interstitial fibroblasts [39]. Increased renal protein expression of P42/44, JNK and p38 were observed in STZ-induced rats. However, HQD attenuated P42/44 and JNK kinase, suggesting decreased TGF- β signaling.

Conclusion

In conclusion, the current study suggests an important role for HQD in mediating oxidative stress in the diabetic rat kidney. HQD could effectively restore the biochemical, protein expression and histological changes associated with the diabetes-induced renal injury. This is associated with down-regulation of TGF- β , p47 phox, p22 phox, phospho-p42/44, phospho-JNK expression and up-regulation of PPAR- γ expression. We propose that HQD may function as an effective therapeutic agent for DN.

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Disclosure Statement

The authors declare no conflict of interest.

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