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Original Article

Danshen injections protect the renal function of streptozotocin-induced diabetic rats by suppression of inflammatory factors TLR4 and MCP-1

Yong Zhang[1], Liya Lin[2], Jiaming Wen[1]

[1]*Department of Urology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China;* [2]*Department of Basic Medicine Sciences, School of Medicine, Zhejiang University, Hangzhou 310058, China*

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Abstract: Diabetic nephropathy (DN) is one of the leading causes of end-stage renal disease (ESRD) in diabetic patients. Danshen, a Traditional Chinese Medicine, has shown renal protective effects in clinic treatment in China. However, the underlying mechanisms concerning the renal protection it provides remain elusive. The present study was designed to evaluate the protection abilities of Danshen injections on renal function and inflammatory factors in streptozotocin (STZ)-induced diabetic rats. After six weeks of Danshen injection treatments, serum creatinine, blood urea nitrogen, and macroscopic lesions were assessed. Inflammation factors, toll-like receptors 4 (TLR4) and monocyte chemotactic protein-1 (MCP-1), were measured via immunohistochemistry (IHC) analysis. Present results demonstrated that hyperglycemia significantly increased expression of TLR4 and MCP-1, injuring renal structure and function. Interestingly, Danshen injections ameliorated serum creatinine (SCr) and blood urea nitrogen levels. Furthermore, hematoxylin-eosin staining (HE) and Masson's staining assays revealed that Danshen injections improved pathological structural changes and fibrosis in diabetic kidneys. Immunohistochemical assay results demonstrated that Danshen injections inhibited TLR4 and MCP-1 expression. To the best of our knowledge, the current study is the first to demonstrate that Danshen injections may rescue renal structure and function partly via inhibition of inflammatory factors TLR4 and MCP-1 in STZ-induced diabetic rats. Present findings may provide an alternative strategy for treatment of DN in the future.

Keywords: Diabetic nephropathy, Danshen injection, toll-like receptors 4 (TLR4), monocyte chemotactic protein-1 (MCP-1), fibrosis

Introduction

Diabetes mellitus (DM) has become a serious medical problem, worldwide. There are about 1.1 million DM patients in the Netherlands. Approximately 10% of these patients have type 1, while 90% have type 2 DM. The estimated current total economic burden of DM was euro 6.8 billion in 2016. Direct costs of complications totaled euro 1.3 billion [1]. In China, there is also an increasing trend in total medical costs (from 2,383 to 2,780 USD) and diabetes-related costs (from 1,655 to 1,857 USD) for diabetic patients [2]. Diabetic nephropathy (DN) is a major complication of diabetes mellitus. It has been reported that 50% of patients with type 1 DM and about 30% of patients with type 2 DM will develop DN [3]. Kidney biopsies are strongly recommended for patients with type 2 diabetes and atypical renal presentation for DN [4, 5]. Moreover, it has been well-documented that DN accounts for about 15% of end-stage renal disease (ESRD), becoming a leading cause of ESRD worldwide [6]. The cumulative risk of ESRD was 2.2% after 20 years and 7.0% after 30 years from diabetes diagnosis. This highlights the importance of modern treatment of diabetic nephropathy [7, 8]. Recent evidence has reported a close link between innate immunity activation in tissues and diabetic complications, including DN [9, 10]. *In vitro* and *in vivo* studies have demonstrated that hyperglycemia causes the innate immune system-driven inflammatory processes, resulting in cell senescence and tissue fibrosis in diabetic kidneys [11].

The innate immune system includes several different classes of pattern recognition receptors. Toll-like receptors (TLRs) are a class of receptors included in this system [12]. TLR4, a potential therapeutic target for diabetic nephropathy, may induce inflammation, podocyte and tubular epithelial cell injuries, and interstitial fibrosis [13]. Monocyte chemotactic protein-1 (MCP-1) is a member of the chemokine family. It is involved in the initiation of inflammation [14]. Accumulating evidence has demonstrated that TLR4 expression in glomerular mesangial cells and renal tubular epithelial cells may increase in response to diabetes and accelerate secretion of pro-inflammatory cytokines, including MCP-1. Genetic deficiencies of TLR4 ameliorate renal inflammation, fibrosis, and podocytopathy, playing important roles in DN [15].

Danshen is a Traditional Chinese Medicine. It is the dried root of the plant *S. miltiorrhizae Bunge*. Danshen injections are the aqueous extracts of Danshen. They have been widely used throughout clinics in Eastern Asia to treat strokes, heart disease, and chronic kidney disease [16-18]. The main active components in Danshen injections are phenolic acids, including salvianolic acid A, salvianolic acid B, danshensu, rosmarinic acid, and lithospermic acid B [19, 20]. Recent studies have revealed that Danshen could suppress LPS-induced inflammation, partially due to blocking TLR4 dimerization. At present, homoplantaginin, a main flavonoid from Danshen, protects endothelial cells from ameliorating endothelial inflammation via suppressing toll-like receptor-4 and NLRP3 pathways [21]. Furthermore, a previous study found that Danshen could improve glomerulus structure and function in STZ-induced diabetic rats [22]. However, the underlying mechanisms concerning the renal protection it provides remain unknown [23].

Therefore, the present study investigated the protective effects of Danshen injections in diabetic kidneys. Renal function and pathological changes were analyzed after Danshen injection treatment. Furthermore, the effects of Danshen injections on modulating expression levels of TLR4 and MCP-1 in diabetic kidneys were investigated. The present study may provide a novel view of Danshen injection therapy for DN via inhibition inflammation under diabetic conditions.

Materials and methods

Animal model induction and Danshen injection treatment

Thirty SD male rats (Experiment Animal Center of Zhejiang University) were divided into three groups (n = 10), including the control group, diabetic group, and Danshen injection-treated group. All rats were fasted for ten hours. The diabetic group and Danshen injection-treated group were then intraperitoneally injected with 65 mg/kg streptozotocin (STZ) (Sigma Chemical Company, St. Louis, MO, USA). The remaining 10 rats, as the control group, were injected the same volume of 0.9% saline. Forty-eight hours after injection, rats with blood glucose > 16.7 mmol/L and urine glucose > (+) were considered as successful diabetic model rats. Rats of the Danshen injection-treated groups were intraperitoneally injected with Danshen injections, at a dose of 1 ml/kg, once a day for six weeks. Rats in the other two groups were injected with the same volume of 0.9% saline. The current study was approved by Committee for the Care and Use of Laboratory Animals at Zhejiang University (Hangzhou, China).

Six weeks after Danshen injection treatment, the rats were anesthetized with an intraperitoneal injection of 60 mg/kg sodium pentobarbital. Blood samples were collected for serum creatinine (SCr) and blood urea nitrogen analysis. Next, the thoracic cavities of the rats were opened. They were perfused intracardially with 100 mL normal saline and 300-400 mL fixative 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the kidneys of each rat were taken out for further analysis.

Haematoxylin and eosin staining and immunohistochemistry assays

The kidneys were fixed in the same fixative for 4 hours, then placed in 30% phosphate buffered sucrose until the tissue sank. Twelve μm-thick sections were cut on freezing microtome through transverse planes for H&E staining, Masson's staining, and diaminobenzidine (DAB) immunohistochemical staining.

The kidney sections were rinsed in 0.01 M phosphate-buffered saline (PBS) and mounted onto 0.02% poly-l-lysine-coated slides. The ABC

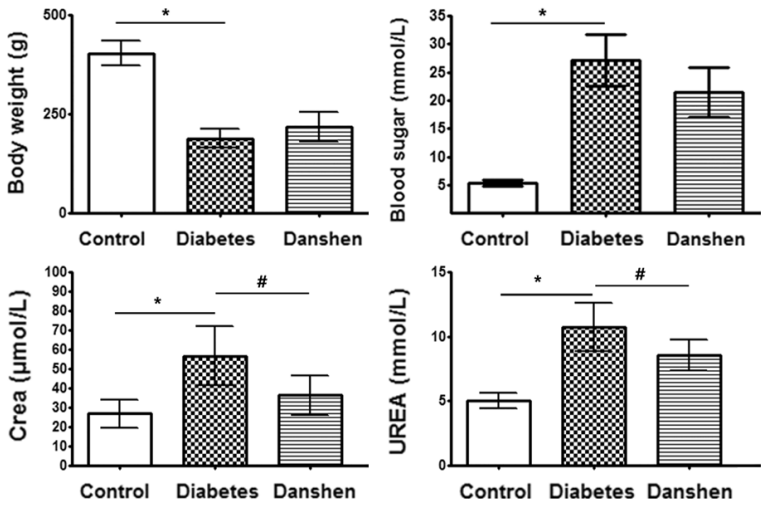

Figure 1. Body weight, blood glucose, serum creatinine, and blood urea nitrogen levels in three groups six weeks after Danshen injection treatment. Values are presented as mean ± standard deviation. ($^{*}P < 0.05$, *vs* control group; $^{\#}P < 0.05$, *vs* the diabetes group).

system was used with DAB as the chromagen. Briefly, tissue sections were first washed in PBS. They were then incubated with 1% bovine serum albumin (BSA) for 30 minutes. Tissues were then incubated overnight at 4 °C in the medium of PBS with TLR4 and MCP-1 (Boster Biotechnology Company, Wuhan, China) antibody (1:100) plus 1% BSA. Control sections were incubated in PBS plus 1% BSA. The next day, the sections were incubated in a biotinylated goat-anti-mouse secondary antibody (diluted to 1:200 in PBS, Boster Biotechnology Company, Wuhan, China) and, subsequently, in an avidin-horseradish peroxidase (HRP) solution. Immunolabeling was visualized with 0.05% DAB plus 0.3% H_2O_2 in PBS. The sections were then dehydrated with ethanol and xylene before using cover slips.

Immunohistochemistry sections were analyzed using TLR4 and MCP-1 positive cells in the kidney per vision field of each rat in three groups. A Nikon microscope (Nikon E600, Nikon Company, Japan) with magnifications of 400× was used.

Statistical analysis

Positive cells for TLR4 and MCP-1 staining, in each visual field under the microscope at 400× magnification, were counted. Data are presented as mean ± SD. Differences were evaluated by analysis of one-way analysis of variance (ANOVA). $P < 0.05$ indicates statistical significance.

Results

Body weight, blood glucose, serum creatinine, and blood urea nitrogen analysis

Initially, body weight, blood glucose, serum creatinine, and blood urea nitrogen levels showed no significant differences ($P > 0.05$) in all groups before STZ injections. Six weeks after diabetes establishment, the diabetic rats had significantly higher blood glucose, serum creatinine, and blood urea nitrogen, as well as lower body weights, compared to the control group ($P < 0.05$). Furthermore, Danshen injection-treated rats showed improved serum creatinine and blood urea nitrogen levels. The other rats still maintained high blood glucose levels, compared to control rats ($P < 0.05$, **Figure 1**).

H&E and Masson's staining assays

Six weeks after diabetes establishment, kidneys of the diabetic rats displayed increasing extracellular matrix, glomerular balloon diffusion, and membrane expansion. The structure of the glomerulus and renal tubules were clear. The distribution of the extracellular matrix was normal in control rat kidneys. Furthermore, the glomerular of the diabetic group apparently infiltrated with inflammation cells. Results of Masson's staining demonstrated that hyperglycemia induced glomerular basement membrane thickening and glomerular enlargement. Treatment with Danshen injections significantly improved these pathological changes, including increasing extracellular matrix, glomerular balloon diffusion, membrane expansion, glomerular enlargement, and infiltration of inflammation cells (**Figure 2**).

Immunohistochemistry analysis

TLR4 and MCP-1 immunoreactivity was visualized via DAB staining. They showed buffy granules in the cytoplasm. Quantitative analysis for the number of TLR4 positive cells per vision

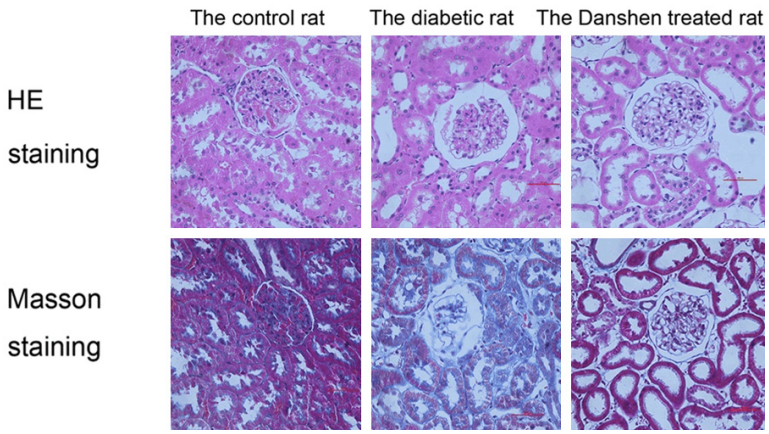

Figure 2. Haematoxylin and eosin staining and Masson's staining of kidneys in the three groups, ×400.

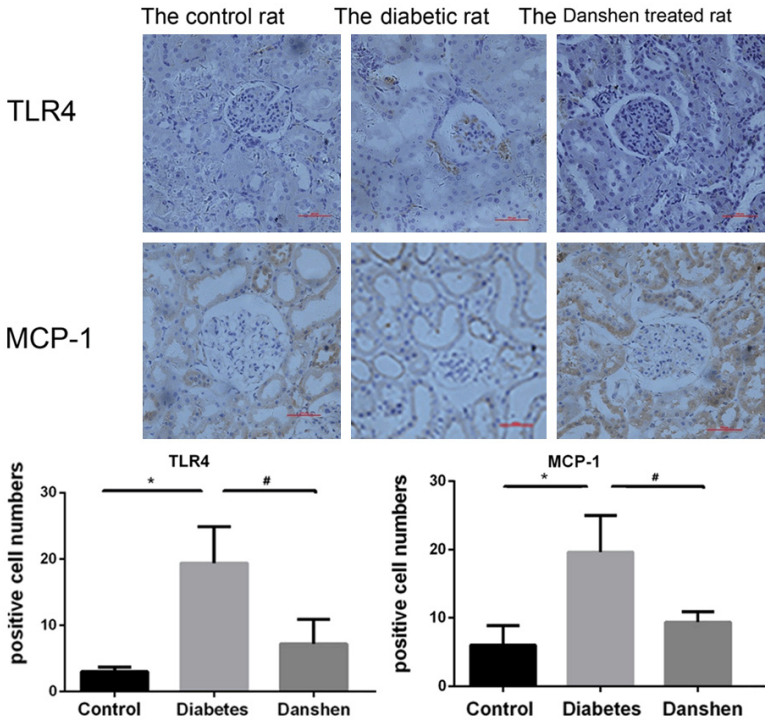

Figure 3. TLR4 and MCP-1 immunohistochemical staining of kidneys in the three groups. Cells that stained positive for TLR4 and MCP-1 showed buff-coloured granules with DAB staining. Values are presented as mean ± standard deviation. ($^{*}P < 0.05$, *vs* the control group; $^{\#}P < 0.05$, *vs* the diabetes group, ×400).

field was significantly increased in rats of the diabetic group, compared to that of normal rats ($^{*}P < 0.05$, **Figure 3**). Similarly, the number of MCP-1 positive cells per vision field was upregulated in diabetic rats, compared with that of normal rats ($^{*}P < 0.05$, **Figure 3**). Danshen injections decreased TLR4 and MCP-1 positive cells in the kidneys, compared with diabetic rats ($^{\#}P < 0.05$, **Figure 3**). Present results suggest that inflammation factors caused by hyperglycemia were significantly improved by Danshen injection treatment. Taken together, results indicate that Danshen injections improved inflammatory response and renal injuries following diabetes.

Discussion

DM is a pandemic metabolic disease featuring chronic hyperglycemia, eliciting dysfunction and failure of various organs, particularly the eyes [24], heart [25], nerves [26], and kidneys [27]. Patients with diabetes are at greater risk of renal dysfunction, with increasing levels of urine protein excretion, SCr, and BUN. They also show pathological changes with basement membrane thickening, glomerular and tubular hypertrophy, glomerulosclerosis, and tubulointerstitial fibrosis [28]. Present results demonstrated that hyperglycemia increase blood SCr and BUN levels. Masson's staining displayed typical renal pathological changes in diabetic rats six weeks after STZ injections, including mesangial matrix accumulation and expansion, as well as focal thickening of the glomerular basement membrane. Interestingly, Danshen injection treatment improved these impaired changes, including blood SCr and BUN levels increasing, glomerular and tubular hypertrophy, and mesangial matrix accumulation, in diabetic rats.

Kidney biopsies from experimental diabetes models or individuals with diabetes are characterized by enhanced macrophage infiltration and pro-inflammatory response under diabetic conditions [29, 30]. TLRs, a class of pattern

recognition receptors of the innate immune system, initiate an inflammatory response in obesity and diabetes mellitus [10, 31]. Some studies have suggested that upregulated TLR4 response in the kidneys could translate the metabolic alterations of diabetes into kidney damage [10, 32]. Activation of TLRs stimulates expression of several inflammatory cytokines and chemokines, including MCP-1. This is associated with progression of diabetic nephropathy [13, 33]. Present results revealed that diabetes could upregulate TLR4 and MCP-1 expression levels in the kidneys, compared with normal control rats. Furthermore, Danshen injection treatment may decrease expression of TLR4 and MCP-1 in diabetic kidneys. This is a fascinating new field of Danshen injection treatment, regarding anti-inflammatory response and renal protection in kidneys of diabetic rats.

In conclusion, present results, for the first time, confirm that Danshen injections suppress hyperglycemia-induced renal impairment and TLR4/MCP-1 expression in kidneys of diabetic rats. Moreover, Danshen injection treatment gradually improved renal function and dramatically improved pathological changes in diabetic kidneys. The present study suggests that Danshen injections improve renal structures and function partly through suppressing the activation of TLR4 and MCP-1 in the kidneys of diabetic rats. Thus, present results may provide an alternative strategy for treatment of DN in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jiaming Wen, Department of Urology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China. E-mail: 2310034@zju.edu.cn

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