Taxus chinensis ameliorates diabetic nephropathy through down-regulating TGF-β1/Smad pathway

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[ABSTRACT] Diabetic nephropathy (DN) is one of the common microvascular complications of diabetes mellitus. Renal fibrosis is closely related to the deterioration of renal function. The present study aimed to investigate protective effect of Taxus chinensis on high-fat diet/streptozotocin-induced DN in rats and explore the underlying mechanism of action. The rat DN model was established via feeding high fat diet for 4 weeks and subsequently injecting streptozotocin (30 mg·kg⁻¹ body weight) intraperitoneally. The rats with blood glucose levels higher than 16.8 mmol·L⁻¹ were selected for experiments. The DN rats were treated with Taxus chinensis orally (0.32, 0.64, and 1.28 g·kg⁻¹) once a day for 8 weeks. Taxus chinensis significantly improved the renal damage, which was indicated by the decreases in 24-h urinary albumin excretion rate, blood serum creatinine, and blood urea nitrogen. Histopathological examination confirmed the protective effect of Taxus chinensis. The thickness of glomerular basement membrane was reduced, and proliferation of mesangial cells and podocytes cells and increase in mesangial matrix were attenuated. Further experiments showed that Taxus chinensis treatment down-regulated the expression of TGF-β1 and α-SMA, inhibited phosphorylation of Smad2 and Smad3. These results demonstrated that Taxus chinensis alleviated renal injuries in DN rats, which may be associated with suppressing TGF-β1/Smad signaling pathway.

[KEY WORDS] Diabetic nephropathy; Taxus chinensis; TGF-β1/Smad signaling pathway


Introduction

Diabetic nephropathy (DN) is the most common complication of diabetes and the main cause of end-stage renal disease, which leads to high morbidity and mortality in diabetic patients [1]. DN is featured by increases in kidney size and glomerular volume, resulting in renal functional decline including albuminuria, glomerulosclerosis, and tubular interstitial fibrosis. Development of renal fibrosis is closely related to deterioration of kidney function [2], which is mainly caused by accumulation of glomerular extracellular matrix (ECM) and mesangial expansion [3]. Hyperglycemia increases expression of ECM constituents such as collagen, fibronectin, and laminin. These products boost the accumulation of ECM. TGF-β is a critical mediator to stimulate the ECM formation in DN, which has been verified in clinic and experimental animal models [4]. TGF-β1 mRNA and protein levels are increased by hyperglycemia. Treatment with neutralizing antibody to TGF-β attenuates the production of collagen and fibronectin in streptozotocin (STZ)-induced diabetic mice [5] and improves renal function in type 2 diabetic db/db mice. These data strongly suggest that TGF-β1 is a promising therapeutic target for DN.

Smad signal transduction plays a central role in TGF-β1-stimulated accumulation of ECM. Classic TGF-β1/Smads signaling is considered as a key pathway for development of renal fibrosis [6]. TGF-β receptor (TβR) binding stimulates phosphorylation of Smad2 and Smad3, and then phospho-Smad2/3 combines with Smad4 to form the hetero-oligomeric complexes. The complexes are translocated into the nucleus
from the cytoplasm and act as a transcriptional regulator of other gene expression related fibrosis [7].

*Taxus chinensis* is a valuable plant, which belongs to the Taxaceae family. There are many kinds of soluble bioactive substances in Taxaceae plants, such as polysaccharides and flavones [8]. The crude polysaccharides (TCPs) from the Taxus cuspidate dose-dependently significantly increase body weight, decrease blood glucose, and reverse the decrease in SOD in STZ-induced diabetic mice [9]. Sequoyitol significantly decreases the levels of fasting blood glucose (FBG), serum insulin, blood urea nitrogen (BUN) and serum creatinine (Scr), and increases the insulin level in diabetic rats [10-11]. These results suggest that *Taxus chinensis* has a therapeutic potential for the treatment of diabetic complications. However, whether *Taxus chinensis* attenuates diabetic kidney disease is still unclear. The present study was designed to investigate the beneficial effects of *Taxus chinensis* on high-fat diet/STZ-induced DN and explore the underlying mechanism of action.

**Materials and Methods**

**Preparation of Taxus chinensis extract**

*Taxus chinensis* was purchased from Shanghai Zhong-De Chinese Materia Medica Co., Ltd. (Shanghai, China). After 30 min soaking in the water, *Taxus chinensis* was boiled under water heat-reflux for 1 h twice. Then the water extract was concentrated and stored for the experiment using.

**Animals**

Male Sprague-Dawley (SD) rats weighing 160–180 g were purchased from Slaccas-Shanghai Lab Animal Ltd. (SPF II Certificate; No. SCXK2013-0016). They were kept under the normal house conditions (12 h lighting cycle and 25 °C temperature) and had free access to chow and tap water. All the rats received humane care in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Procedures involving animals and their care were approved by the Animal Ethical Committee of School of Pharmacy, Fudan University ( Permit Number: 2013-3).

**Induction of diabetes in rats**

After one-week acclimatization, the rats were fed with high-fat diet which was prepared by mixing cane sugar (20%), lard (10%), cholesterol (2.5%), and bile salts (1%) with the standard rodent chow (Suzhou Shuangshi animal feed Technology Co., Ltd., Suzhou, China) [12]. After 4 weeks, the overnight fasting rats were intraperitoneally injected freshly prepared streptozotocin (30 mg·kg$^{-1}$, dissolved in 0.1 mol·L$^{-1}$ of cold citrate buffer, pH 4.4) to induce diabetes. After one week, postprandial blood glucose levels were measured by blood glucose test strips (Roche, Mannheim, Germany). The rats with blood glucose levels higher than 16.7 mmol·L$^{-1}$ were defined as diabetic rats and chosen for experiments. Age-matched normal rats were given an equal volume of buffer solution as the control.

**Treatment with Taxus chinensis**

The diabetic rats were randomly allocated into 4 groups of 9 animals each, including one non-treated diabetic group (model group) and three *Taxus chinensis* treated diabetic groups (0.32, 0.64, and 1.28 g·kg$^{-1}$). The water extract of *Taxus chinensis* was orally administered to the diabetic rats once daily for 8 weeks. Normal non-diabetic rats received normal saline orally as control group ($n = 7$).

At the end of the treatments, the rats were placed in individual metabolic cages for 24 h to collect urine and record the urine volume. Then each rat was weighed and anesthetized with intraperitoneal injection of pentobarbital sodium (30 mg·kg$^{-1}$ body weight). Blood samples were harvested via aortaventralis and centrifuged at 3 000 r·min$^{-1}$ for 10 min at 4 °C to obtain the serum. Both kidneys of each rat were rapidly dissected out and weighed. Kidney index (kidney weight/body weight) × 100% was calculated. The right kidney was fixed in 4% paraformaldehyde for pathological examination and immunohistochemistry analysis. The renal cortices of the left kidney were snap frozen in liquid nitrogen and stored at −80 °C for molecular assessments.

**Biochemical assays in serum and urine samples**

Serum glucose, serum creatinine (Scr), blood urea nitrogen (BUN) and urinary albumin excretion rate (UAER) were measured using assay kits, following manufacturers’ instructions (Nanjing Jian Cheng Co., Nanjing, China).

**Pathological examination**

Kidneys fixed in 4% paraformaldehyde were embedded in paraffin. The specimens were processed to obtain 3-µm-thick longitudinal paraffin sections. The tissue sections were stained with hematoxylin and eosin (H&E) for histological examination using light microscopy.

**Immunohistochemistry**

Immunohistochemistry was performed by the streptavidin-biotin-peroxidase complex (SABC) method on paraffin sections [13-14]. The sections were deparaffinized, rehydrated, and blocked of endogenous peroxidase activity. After being blocked with 5% Bovine Serum Albumin (BSA), the sections were incubated with rabbit monoclonal anti-TGF-β1 and anti-α-SMA antibody overnight. Subsequently, the sections were incubated with second antibody and SABC reagent. Staining was visualized by incubation with 3, 3′-diaminobenzidine-tetrahydrochloride. The sections were counterstained with hematoxylin and examined under a light microscope.

**Western blotting assay**

Kidney tissues were homogenized in lysis buffer and incubated on ice for 30 min. After centrifugation at 10 000 g for 15 min at 4 °C, the supernatants were collected and total protein was measured using the bicinchoninic acid assay. Samples were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were blocked with 5% skim milk for 1 h. The blots were probed with primary antibodies against TGF-β1, α-SMA, P-Smad2, P-Smad3, and GAPDH at 4 °C overnight. Then, membranes were incubated with peroxidase-labeled secondary antibodies for 3 h at room
Effects of Taxus chinensis on blood glucose level

During the experiment, there were nine rats died, who were 2 of model group, 2 of 0.32 g·kg\(^{-1}\), 2 of 0.64 g·kg\(^{-1}\), and 3 of 1.28 g·kg\(^{-1}\) Taxus chinensis treatment groups, respectively. At the end of 8-week treatment, the number of animal is 6-7 in the 6 groups. The body weights of all diabetes model rats were significantly lower than that of the normal control rats (P < 0.01). Taxus chinensis treatment (0.32, 0.64, and 1.28 g·kg\(^{-1}\)) attenuated body weight loss and increased the average body weight, compared with the vehicle-treated diabetes rats (P < 0.01; Fig. 1).

Effects of Taxus chinensis on renal functions

At the end of 8-week treatment, serum creatinine (Scr), blood urea nitrogen (BUN), urinary albumin excretion rate (UAER) and kidney index were measured as the markers of renal functions. The results showed these markers were strikingly elevated in diabetic nephropathy model rats, compared with the normal control group (Figs. 3A–3D). Taxus chinensis treatment (0.32, 0.64 and 1.28 g·kg\(^{-1}\)) significantly decreased these markers of renal functions, compared to the vehicle-treated diabetic rats (P < 0.01; Figs. 3A–3D). Taxus chinensis exhibited pronouncedly therapeutic effects on renal functions.

Discussion

In recent decades, relieving complications has been considered the most important care for diabetic patients \[^{15-16}\]. DN is one of diabetic microvascular complications, which is associated with end-stage renal disease \[^{17}\]. Therefore, searching for drugs to treat DN has been a research focus. In the present study, a high-fat diet/STZ-induced diabetic model in rats was used to evaluate the effects of Taxus chinensis on DN. We found that the diabetic rats displayed hyperglycemia and renal dysfunction, such as abnormal

**Fig. 2** Effects of Taxus chinensis on blood glucose levels in high-fat diet/STZ-induced diabetic rats. The diabetic rats were treated with Taxus chinensis (0.32, 0.64 and 1.28 g·kg\(^{-1}\)) or vehicle for 8 weeks. The data were expressed as means ± SD (n = 6–7); \(^{##}P < 0.01\) vs normal control group; \(^{*}P < 0.01\) vs vehicle-treated model group.

**Fig. 1** Effects of Taxus chinensis on body weights in high-fat diet/STZ-induced diabetic rats. The diabetic rats were treated with Taxus chinensis (0.32, 0.64, and 1.28 g·kg\(^{-1}\)) or vehicle for 8 weeks. The data were expressed as means ± SD (n = 6–7); \(^{##}P < 0.01\) vs normal control group; \(^{*}P < 0.01\) vs vehicle-treated model group.

**Fig. 3** Effects of Taxus chinensis on renal histological changes in diabetic rats. The diabetic rats were treated with Taxus chinensis (0.32, 0.64, and 1.28 g·kg\(^{-1}\)) or vehicle for 8 weeks. The data were expressed as means ± SD (n = 6–7); \(^{##}P < 0.01\) vs normal control group; \(^{*}P < 0.01\) vs vehicle-treated model group.

**Fig. 4** Effects of Taxus chinensis on TGF-β1/Smads signaling pathway in diabetic rats. The diabetic rats were treated with Taxus chinensis (0.32, 0.64, and 1.28 g·kg\(^{-1}\)) or vehicle for 8 weeks. The data were expressed as means ± SD (n = 6–7); \(^{##}P < 0.01\) vs normal control group; \(^{*}P < 0.01\) vs vehicle-treated model group.

**Fig. 5** Effects of Taxus chinensis on renal histological changes in diabetic rats. The diabetic rats were treated with Taxus chinensis (0.32, 0.64, and 1.28 g·kg\(^{-1}\)) or vehicle for 8 weeks. The data were expressed as means ± SD (n = 6–7); \(^{##}P < 0.01\) vs normal control group; \(^{*}P < 0.01\) vs vehicle-treated model group.

**Fig. 6** Effects of Taxus chinensis on renal histological changes in diabetic rats. The diabetic rats were treated with Taxus chinensis (0.32, 0.64, and 1.28 g·kg\(^{-1}\)) or vehicle for 8 weeks. The data were expressed as means ± SD (n = 6–7); \(^{##}P < 0.01\) vs normal control group; \(^{*}P < 0.01\) vs vehicle-treated model group.
renal structure and the elevated levels of Scr, BUN, UAER, and kidney index. The expressions of TGF-β1 and α-SMA were up-regulated and p-Smad2/3 were activated in the diabetic rats. These results suggested that the high-fat diet/SZ-induced rat diabetic model is a suitable animal model to study the DN treatment.

Fig. 3 Effects of *Taxus chinensis* on renal functions in high-fat diet/STZ-induced diabetic nephropathy rats. (A) serum creatinine; (B) blood urea nitrogen (BUN); (C) urinary albumin excretion rate (UAER); and (D) kidney index. The diabetic rats were treated with *Taxus chinensis* (0.32, 0.64 and 1.28 g·kg⁻¹) or vehicle for 8 weeks. The data were expressed as means ± SD (n = 6–7); **P < 0.01 vs normal control group; ***P < 0.01 vs vehicle-treated model group.

Fig. 4 Effects of *Taxus chinensis* on renal histological changes in high-fat diet/STZ-induced diabetic rats (H&E 400 ×). (A) normal control group; (B) vehicle-treated model group; (C) *Taxus chinensis* 0.32 g·kg⁻¹ treated group; (D) *Taxus chinensis* 0.64 g·kg⁻¹ treated group; and (E) *Taxus chinensis* 1.28 g·kg⁻¹ treated group.

High blood glucose level and weight loss are the classic characteristics of diabetes mellitus. It may be a result of protein wasting due to lack of energy from carbohydrates because of dysfunction of islet β-cells [16]. In the present study,
Taxus chinensis significantly improved the blood glucose level and maintained the body weight of diabetic rats during treatment. The results are consistent with other studies\textsuperscript{[10-11]}

A number of studies strongly support that long-term hyperglycemia is the most critical factor inducing the progressive tissue damage and functional deterioration in the development of diabetic complications, especially DN\textsuperscript{[19]}. Therefore, controlling the level of blood glucose is an effective approach to alleviating DN. Our study showed that Taxus chinensis attenuated diabetic syndromes and decreased the blood glucose level of DN rats, which contributed to improve DN. Meanwhile, it was observed that Taxus chinensis ameliorated the elevated levels of Scr, BUN, UAER, and kidney index. The results indicated that Taxus chinensis alleviated the renal structure and function damage. Histological examination confirmed the protective role of Taxus chinensis on kidneys. Thus, further experiments were performed to explore the mechanism of action for Taxus chinensis.

The Classic TGF-\(\beta\)1/Smad pathway is considered as a key signaling pathway involving in the development of renal fibrosis\textsuperscript{[20]}. TGF-\(\beta\)1 is the most potent pro-fibrogenic cytokine and a pivotal mediator of renal fibrosis\textsuperscript{[21-22]}. TGF-\(\beta\)1 mRNA and protein levels were increased in both patients and experimental animal models of diabetic nephropathy\textsuperscript{[23-24]}.

Fig. 5  Effects of Taxus chinensis on TGF-\(\beta\)1 and \(\alpha\)-SMA protein expressions in high-fat diet/streptozotocin-induced diabetic nephropathy rats by immunohistochemical staining (400 \(\times\)). (A) normal control group; (B) vehicle-treated model group; (C) Taxus chinensis 0.32 g·kg\(^{-1}\) treated group; (D) Taxus chinensis 0.64 g·kg\(^{-1}\) treated group; and (E) Taxus chinensis 1.28 g·kg\(^{-1}\) treated group. Diabetic rats were treated with Taxus chinensis or vehicle for 8 weeks
This was also observed in high-fat diet/STZ-induced diabetic rats in the present study. Transgenic mice of TGF-β1 overexpression develop progressive glomerulosclerosis and tubulointerstitial fibrosis, while administration of anti-TGF-β1 antisera ameliorates renal fibrogenesis [25]. Yin et al. have demonstrated that the reduced expression of TGF-β1 using siRNA even reverses the pathological and functional decline in the kidneys of diabetic animals [26]. TGF-β1 is considered as an inducer of ECM accumulation through affecting both the ECM synthesis and the degradation of ECM components. It is reported that TGF-β1 directly inhibits matrix metalloproteinases and induces tissue inhibitors of metalloproteinases, which finally results in a net accumulation of ECM [27]. In the present study, we found that Taxus chinensis treatment down-regulated the level of TGF-β1 protein level in the DN rats. The results suggested that preventive effects of Taxus chinensis on DN might be associated with down-regulation of TGF-β1 signaling pathway.

The canonical TGF-β1 signaling pathway involves activation of Smad2 and Smad3. Smad2 and Smad3 are recruited to the receptor complex and directly phosphorylated by activation of TGF-β type I receptor (TβRI) [28]. Phosphorylated Smad2/3 form an oligomeric complex with Smad4. Then the complex is translocated into the nucleus to regulate the transcription of various TGF-β1 target genes, which contributes to tissue fibrosis [28-31]. In the present study, Taxus chinensis treatment decreased the levels of p-Smad2/3, while no effects in Smad2/3 protein expression in high-fat diet/streptozotocin-induced DN rats. This further suggested that inhibition of Smad2/3 activation may be one of mechanisms of beneficial effects of Taxus chinensis on DN.

The up-regulation of TGF-β1 has been shown to induce the phenotypic transformation of cells associated with epithelial-mesenchymal transition (EMT) [32]. EMT has long been considered to be a very important step to relay fibrogenic signals from tubular epithelial cells to contiguous fibroblasts [33]. Activated fibroblasts are the principal cells responsible for the accumulation of ECM and fibrosis under pathological conditions [34]. In the present study, our results from immunohistochemistry and Western blot analyses showed that Taxus
attenuated the expression of EMT related protein a-SMA, which indicated that the inhibition of EMT could be one of the mechanisms for *Taxus chinensis* to exert its effects on renal damage.

In conclusion, *Taxus chinensis* ameliorated hyperglycemia and improved renal function, which may be associated with the suppression of TGF-β1/Smad pathway.

References


