



Protective effect of the n-butanol *Toona sinensis* seed extract on diabetic nephropathy rat kidneys

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ABSTRACT. The objective of this study was to observe the protective effect of the n-butyl alcohol phase of *Toona sinensis* seed extract on the kidneys of diabetic nephropathy (DN) rats and its preliminary mechanism. Male wistar rats were administered a normal or high-fat diet for 1 month. DN rats were divided into a model group and a petroleum ether phase of *T. sinensis* seed extract intervention group. The intervention group was administered 5 mg·100 g⁻¹·day⁻¹ extract. After treatment for 10 weeks, the rats were sacrificed and blood samples and the renal cortex were collected. Biochemical indicators in the serum and renal indices were assessed. Pathological changes of the renal tissues were also determined. Changes in the renal structure and protein levels were detected. Compared with the normal group, the blood glucose, urinary albumin, renal index, and oxidative stress index were sharply increased in the model group. The protein levels of TGF-β1, collagen IV, and connective tissue growth factor (CTGF) were increased. Compared with the model group, the n-butyl alcohol phase of *T. sinensis* seed extract significantly reduced the blood glucose, urinary albumin, renal index, oxidative stress index, serum creatinine,

and urea nitrogen levels. The renal pathology abnormality was improved in DN rats. The protein levels of TGF- β 1, collagen IV, and CTGF were increased. The expression of TGF- β 1, collagen IV, and CTGF decreased. In conclusion, the n-butyl alcohol phase of *T. sinensis* seed extract has protective effects on DN rats via the inhibition of oxidative stress and protein expression of TGF- β 1, collagen IV, and CTGF.

Key words: N-butyl alcohol phase; *Toona sinensis* seed extracts; Diabetic nephropathy; Oxidative stress

INTRODUCTION

Diabetic nephropathy (DN) is glomerulosclerosis caused by the abnormal metabolism of diabetes accompanied by proteinuria, and its disease mechanism is a popular research topic (Satirapoj, 2012). Excessive production of reactive oxygen species is the common upstream event of the polyol pathway flux, protein kinase C, advanced glycation end-products, and the hexosamine biosynthesis pathway. Oxidative stress is an independent factor in diabetes mellitus (DM) and angiopathy, and is considered a common mechanism of the disease.

Shandong is one of the main production areas of *Toona sinensis* seeds. *T. sinensis* seeds are infused in water or decocted with water to treat DN in Weifang, Linyi, and Liaocheng. *T. sinensis* seeds have many effects such as decreasing blood glucose, antioxidation, anticoagulation, and protective effects on neurons (Li and Chen, 2009, 2010; Xing and Chen, 2010; Jin and Chen, 2011; Du et al., 2011; Zhao et al., 2011). Here, we studied the effects of n-butyl alcohol extract (NBAE) of *T. sinensis* seeds on DN rats with oxidative stress and the roles of oxidative stress in the disease mechanism of DN.

MATERIAL AND METHODS

Animals

Wistar rats were purchased from the Medical Experimental Animals Center of Shandong Lu Kang. The certification number was SCXK Lu 20130001.

Drugs and reagents

T. sinensis seeds (Jinan Shengke Technology Company) were identified as *T. sinensis* (A. Juss.) Roem. fruits by Dr. Xu Chongmei of the biopharmacy department of Weifang Medical Academy. The NBAE of the *T. sinensis* seeds was prepared by the Pharmacy Department Laboratory of Weifang Medical Academy. Streptozotocin (STZ, Sigma), detection kits (Nanjing Jiancheng Bioengineer Research Laboratory), and antibodies were purchased from Sigma-Aldrich and Santa Cruz.

Instruments

The blood glucose meter was obtained from Roche. The enzyme mark instrument was from Bio-Rad. The automated biochemical analyzer was obtained from Rili. The microscope was from Olympus. The transmission electron microscope used was a Rili (H-7650).

Methods

Model and treatment groups

Male Wistar rats weighing 180-200 g were fed a normal diet for 1 week. Then, 10 rats were randomly selected as the control group and given normal food. Twenty-five rats were used as model rats and were administered only food with high fat and glucose (Shanghai Silaike Experimental Animal Company) for 4 weeks. Then, rats in the model group were administered 60 mg/kg STZ, and their blood glucose levels were within 16.7-25.0 mM (Yang and Li, 1993; Tang et al., 2010; Hu et al., 2011). The rats were fed until their urine protein content was over 20 µg/mL. Nine of the DM rats were used in the model group and 10 in the NBAE of *T. sinensis* seed group, in which rats were treated with 5 mg·100 g⁻¹·day⁻¹ for 10 weeks. The other groups were treated with the same amount of normal saline.

Sample collection

After the last treatment, the 24-h urine of the rats was collected, centrifuged, and stored at 4°C. Rats were weighed and then injected with 10% chloral hydrate (0.4 mL/100 g). Blood was collected, centrifuged to obtain the serum, and stored at -20°C. The kidneys were washed with 4°C normal saline via infusion from the heart until the color was white. Two kidneys were obtained and weighed after removal of the capsule. A portion was used to make an electron microscope specimen, and the other parts were fixed with paraformaldehyde.

Detection of biochemical index

The rat fasting blood glucose, serum creatinine (Scr), blood urea nitrogen (BUN), and urinary creatinine (Ucr) were detected. Kits were used to measure urine protein, serum total antioxidant capacity (T-AOC), superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GSH-PX), and catalase (CAT) contents.

Kidney pathological parameters

The specimens were fixed with paraformaldehyde, embedded using paraffin, sectioned, and stained using hematoxylin and eosin (H&E), periodic acid-silver methemamine (PASM), periodic acid-Schiff (PAS), and Masson's trichrome. The pathological changes in the rat kidneys were observed under a microscope.

Immunohistochemistry

Five visual fields from each immunohistochemistry sample were imaged under 400X magnification. The I-solutions software was used to quantify and analyze the positive results, and then the average positive strength was obtained.

Electron microscope observation of kidney

Kidney specimens were fixed, stained, and observed using electron microscopy.

Statistical analysis

SPSS 15.0 was used to analyze the data, and data are reported as means \pm SD. The quantitative data were analyzed by a test of normality, and the differences between groups were analyzed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of n-butyl alcohol phase of *T. sinensis* seed extract on the weight and serum glucose of DN rats

The weight decreased in the DN group compared with the control group ($P < 0.01$). Additionally, the kidney index (kidney weight/body weight) and blood glucose increased ($P < 0.01$). Compared with the DN group, the weight of the NBAE group significantly increased ($P < 0.05$), and its kidney index and blood glucose decreased ($P < 0.05$; Table 1).

Table 1. Body weight, index of renal hypertrophy, and the renal function-related parameters in different groups at the end of treatment.

	Normal group	Model group	NBAE group
Body weight (BW) (g/rat)	612.7 \pm 24.21	410.5 \pm 39.12*	455.3 \pm 41.58*#
KW/BW ratio (%)	3.28 \pm 0.59	5.02 \pm 0.71*	4.18 \pm 0.56*#
Serum glucose (mM)	6.72 \pm 1.05	20.91 \pm 3.87*	11.56 \pm 2.79*#
HbA1c (%)	4.15 \pm 0.36	14.07 \pm 1.28*	12.55 \pm 1.05*#
Urine volume (mL/100 g/24 h)	5.94 \pm 0.78	34.25 \pm 7.26*	19.23 \pm 3.21*#
Urine protein (mg/24 h)	10.93 \pm 1.06	28.93 \pm 2.76*	16.03 \pm 1.55*#
Ucr (mM)	12.65 \pm 1.73	2.86 \pm 0.49*	6.35 \pm 0.93*#
Scr (μ M)	42.58 \pm 4.16	69.75 \pm 5.64*	57.83 \pm 5.02*#
BUN (mM)	6.71 \pm 0.98	12.26 \pm 1.35*	8.51 \pm 1.25*#

NBAE = n-butyl alcohol extract; KW = kidney weight; HbA1c = hemoglobin A1c; Ucr = urinary creatinine; Scr = serum creatinine; BUN = blood urea nitrogen. * $P < 0.01$ compared to the normal group. # $P < 0.05$ compared to the model group.

Improvement in kidney function in DN rats

Compared with the control group, the 24-h urine amount, urine protein, HbA1c, Scr, and BUN were improved in DN rats ($P < 0.01$), while the Ucr decreased ($P < 0.01$). Compared with the DN group, the 24-h urine amount, urine protein, HbA1c, Scr, and BUN decreased ($P < 0.01$ or 0.05), while the Ucr increased ($P < 0.01$). The results are shown in Table 1.

Effects of the n-butyl alcohol phase of *T. sinensis* seed extract on oxidative stress parameters of DN rats

Compared with the control group, the activities of T-AOC, SOD, GSH-Px, and CAT were all decreased in the DN group ($P < 0.01$), while the MDA level improved ($P < 0.01$). Compared with the DN group, the activities of T-AOC, SOD, GSH-Px, and CAT increased in the NBAE group ($P < 0.01$ or 0.05), while the MDA level decreased ($P < 0.01$). Results are shown in Table 2.

Table 2. Antioxidant indices in experimental rats at the end of treatment.

	Normal group	Model group	NBAE group
T-AOC (U/mL)	10.98 ± 2.25	5.49 ± 1.46*	8.71 ± 1.56**
SOD (U/mL)	153.46 ± 9.35	118.79 ± 7.34*	139.53 ± 8.92**
MDA (nM)	5.51 ± 0.98	12.14 ± 1.13*	7.12 ± 0.75**
GSH-Px (U/mL)	528.17 ± 6.95	221.81 ± 11.25*	240.02 ± 16.97**
CAT (U/mL)	4.52 ± 0.63	1.94 ± 0.48*	3.49 ± 0.56**

NBAE = n-butyl alcohol extract; T-AOC = serum total antioxidant capacity; SOD = superoxide dismutase, MDA = malondialdehyde, GSH-Px = glutathione peroxidase; CAT = catalase. *P < 0.01 compared to the normal group. #P < 0.05 and **P < 0.01 compared to the model group.

Improvement in pathological injury of STZ diabetic rats by oxidative stress parameters

The pathological changes in the kidneys were observed under the microscope after H&E, PASM, PAS, and Masson's trichrome staining. A proportional area of fibrosis and glomerulosclerosis index were calculated in sections stained with PASM, PAS, and Masson's trichrome. There were no significant pathological changes in the control group. Compared with the control group, the DN group showed glomerular hypertrophy, capillary narrowing, Bowman capsule narrowing, extracellular matrix increasing in the mesangium region, and glycogen and collagen deposition. The pathological injuries to the kidney were more developed in the NBAE group compared to the STZ group (P < 0.05). Results are shown in Figure 1.

Electron microscope observations

Under the electron microscope, the kidney structure was normal in the control group. The glomerular basement membrane was uniform and without thickening, and the distribution of the epithelial foot processes was uniform. In the DN group, the glomerular basement membrane of the rat kidney exhibited significant thickening and was not uniform, having the shape of a hump. Part of the foot processes significantly merged with secondary foot processes, and there was some vascular endothelial cell proliferation. In the NBAE group, the rat kidney showed segmental thickening and foot processes appeared to significantly merge, with different degrees of development. The results are shown in Figure 2.

Detection of expression of TGF-β1, collagen IV, and connective tissue growth factor (CTGF) in rat kidney tissues by immunohistochemistry

Oxidative stress could induce the secretion of cytokines and synthesis and deposition of extracellular matrix by activating TGF-β1, collagen IV, and CTGF signal pathways, leading to glomerulosclerosis. There were no significant differences in TGF-β1, collagen IV, and CTGF levels in the control group. Compared with the control group, the TGF-β1, collagen IV, and CTGF levels were significantly increased in the DN group. Compared with the DN group, TGF-β1, collagen IV, and CTGF levels were significantly decreased in the NBAE group (P < 0.05). The results are shown in Figure 3.

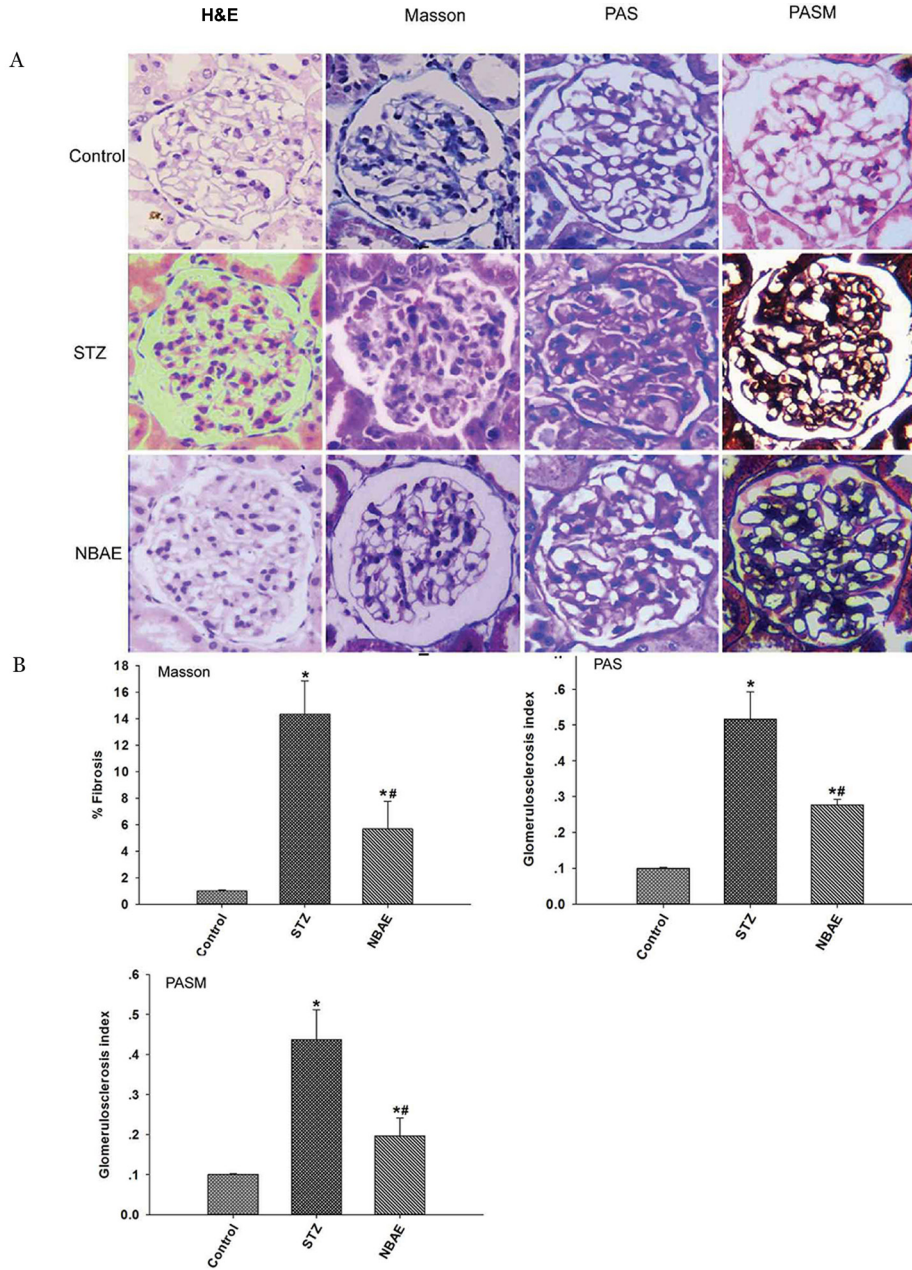


Figure 1. Histopathological changes in the kidneys of diabetic nephropathy (DN) rats with NBAE treatment. **A.** Kidney tissue was stained with H&E, Masson's trichrome, periodic acid-Schiff (PAS), and periodic acid-silver methexamine (PASM). Each image is a representative of eight kidney tissue sections in each group. Original magnification: 400X. **B.** Proportional area of fibrosis and glomerulosclerosis index for various groups as indicated. Masson's trichrome, PAS, and PASM staining were determined semiquantitatively as described above. Data are reported as means \pm SE (N = 8). *P < 0.05 vs control, #P < 0.05 vs STZ.

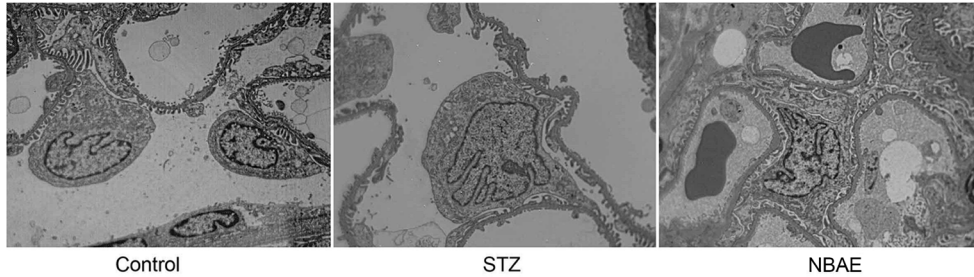


Figure 2. Structural changes observed in the kidneys of DN rats by transmission electron microscope (7000X).

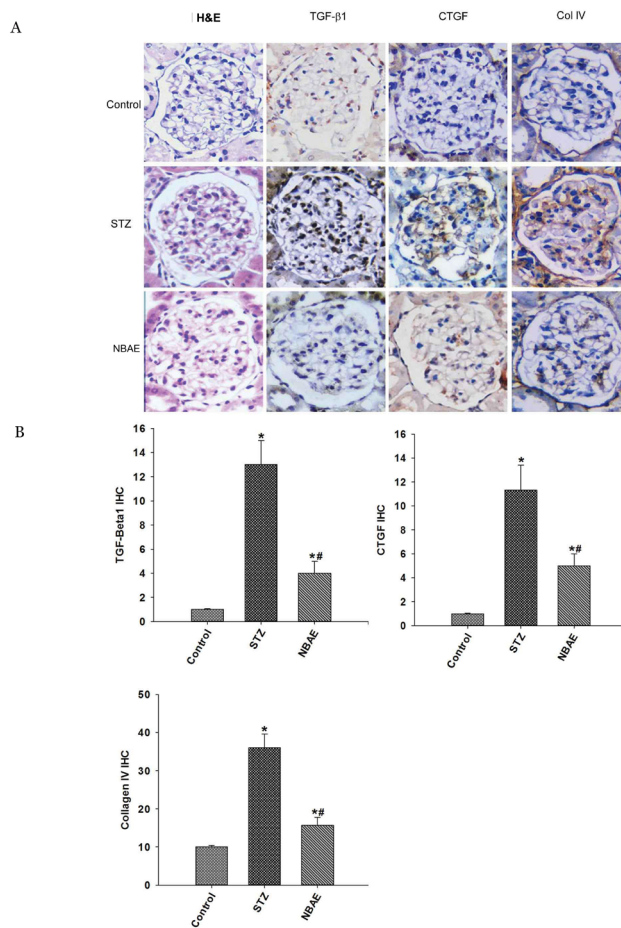


Figure 3. Expression of TGF-β1, connective tissue growth factor (CTGF), and collagen IV (Col IV) in kidneys of diabetic nephropathy (DN) rats with NBAE treatment. **A.** Immunohistochemical photograph of TGF-β1, CTGF, and collagen IV in different groups. Original magnification: 400X. **B.** Semiquantitative analysis of TGF-β1, CTGF, and collagen IV immunostaining in different groups as indicated. Data are reported as means ± SE (N = 8), *P < 0.05 vs control, #P < 0.05 vs STZ.

DISCUSSION

DN is a microvessel complication that occurs as a result of DM, and its disease mechanism is complex (Lv and Wang, 2006; Lin et al., 2014). Oxidative stress could activate the pathological pathway related to DM complications, which is a key link in the DN disease mechanism. Traditional Chinese Medical Science has been successfully used to prevent the incidence and development of DN (Hu and Liang, 2013). Further knowledge of the roles of oxidative stress in the DN disease mechanism could provide the framework for preventing and controlling DN.

Rats were fed a high fat diet initially and then injected with STZ after inducing insulin resistance to destroy the islet tissue, resulting in the rat DM model. Rats were continuously fed high energy food to create a rat DN model with constant high blood glucose and proteinuria.

The pathological changes in the early period of DN in the rats were as follows: glomerular hypertrophy and glomerula with high stress, high infusion, high filtration, and proteinuria (Wu et al., 2014). The NBAE decreased the 24-h urine amount, urine protein, HbA1c, BUN, and Scr in DN rats and regulated the glucose metabolism of DN rats, decreasing the discharging of urine protein and developing the kidney function.

Radicals in the DN rat body could attack the polyunsaturated fatty acids in the membranes and induce the over-oxidation of lipid, producing MDA lipid over-oxidation. Oxide and antioxidant imbalance plays an important role in DN (Susztak et al., 2006). NBAE could improve the activities of SOD, CAT, and GSH-Px and total antioxidant ability affecting the oxidation stress of DN rats.

TGF- β 1 was an important fibrogenic factor and could increase the synthesis of extracellular matrix (ECM). TGF- β 1 could promote cell proliferation, the production of ECM, and tissue fibrosis and mediate the kidney fibrosis process in DN. CTGF could promote the proliferation of fibroblasts, the production and fibrosis of ECM, and the production of collagen by kidney fibroblasts (Zhao et al., 2010; Fang et al., 2013; Liu et al., 2009, 2014). NBAE could relieve kidney disease in DN rats and cause pathological changes.

NBAE decreased oxidation stress and the expression of TGF- β 1, CTGF, and collagen IV. It also reduced the degree of fibrosis and hardening index of the glomerulus. The DN rat kidney was improved, suggesting that NBAE had anti-oxidative properties and significant protective effects in DN rats. This study provided evidence that *Toona sinensis* seeds can be used to prevent DN, and further studies are needed to investigate what specific components play roles in controlling DN and their mechanisms.

Conflicts of interest

The authors declare no conflict of interest.

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