Therapeutic Potential of Naja Naja Atra Venom in A Rat Model of Diabetic Nephropathy[*]

DAI Gui Li[1,△], HE Jing Kang[1,△], XIE Yan[1,#], HAN Rong[2], QIN Zheng Hong[2], and ZHU Lu Jia[2]

1. The First Affiliated Hospital of SooChow University, Suzhou 215006, Jiangsu, China; 2. Department of Pharmacology and Laboratory of Aging and Nervous Diseases, Soochow University School of Medicine, Suzhou 215123, Jiangsu, China

Abstract

Objective   To study the protective effects of naja naja atra venom (NNAV) in a rat model of diabetic nephropathy (DN).

Methods   The rat diabetes model was induced by intraperitoneal injection of streptozotocin (STZ). Thirty-two model rats were randomly divided into one DN group (*n*=8) and three treatment groups (*n*=8 each) that received NNAV at doses of 30, 90, or 270 μg/(kg·day) via oral gavage, another eight rats as normal controls. After 12 weeks, all rats were sacrificed and the changes in serum and urine biological index levels were determined by colorimetric assay. Microalbumin (mALB), N-acetyl-β- glucosaminidase (NAG) and cystatin C (CysC) concentrations were measured by ELISA. Renal tissues were sliced for pathological and immunohistochemical observations.

Results   Comparied with the DN group, serum glucose was decreased by 31.04%, total cholesterol 21.96%, triglyceride 23.78%, serum creatinine 19.83%, blood urea nitrogen 31.28%, urinary protein excretion 45.42%, mALB 10.42%, NAG 20.65%, CysC 19.57%, whereas albumin increased by 5.55%, high-density lipoprotein–cholesterol 59.09%, creatinine clearance 19.05% in the treatment group by NNAV administration at dose of 90 μg/(kg·day). NNAV also reduced the levels of malondialdehyde in serum (22.56%) and kidney tissue (9.79%), and increased superoxide dismutase concentration in serum (15%) and decreased it in renal tissue (8.85%). In addition, under light microscopy kidney structure was improved and glomerular hypertrophy decreased by 8.29%. As shown by immunohistochemistry, NNAV inhibited transforming growth factor-β1 by 6.70% and nuclear actor-κB by 5.15%.

Conclusion   NNAV improves biological indexes in DN, and it may exert renoprotective effects in rats with STZ-induced diabetes.

Key words: Diabetic nephropathy; Naja naja atra venom; Renal function

INTRODUCTION

Diabetic nephropathy (DN), as a microvascular disease, represents a major long-term complication of diabetes mellitus, and is also the leading cause of chronic kidney disorders with the renal failure at the end stage. In addition, DN is a silent epidemic worldwide, and its incidence has increased continuously in the past three decades[1]. DN is

therefore becoming a major challenge to health care systems due to its increasing morbidity and mortality, and is imposing enormous mental, physical and social burdens. The seemingly endless cost for DN is becoming unaffordable for both developed and developing countries. Hence, a host of current researches are focused on pathogenesis of DN and new drugs for its treatment.

Snake venoms contain cardiotoxic, neurotoxic or cytotoxic toxins, nerve growth factors, lectins, disintegrins and many enzymes, which mediate a variety of pharmacological activities. Russell's viper venom can reduce spontaneous motility, hypothermia, hypnosis, analgesia and muscle relaxation[2]. Venoms from snakes, such as king cobra[3] and Crotalus durissus[4] have been shown to have antinociceptive effects. Batroxobin is an enzyme derived from the venom of Bothrops atrox, which plays a role in the anticoagulant activity and the blood coagulation system[5]. Some animal experiments have shown that the activity of toxic compounds is inhibited by blood protein factors, including metalloproteinase or phospholipase A2 inhibitors[6].

The present study is aimed to investigate the role of venom isolated from naja naja atra (NO.NC070501, Shangdong, China), in improving renal function and in delaying the pathogenesis of DN. No researches on the effect of this snake venom on kidneys have been reported so far. A rat model of DN, which is most widely used in DN researches[7], was established and diabetes was induced by injection of streptozotocin (STZ).

MATERIALS AND METHODS

Experimental Design

All animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by institutional review boards. Male Wistar rats (180-220 g) were used (No. 2007-0005 SLRC Laboratory Animals, Shanghai, China). All animals were kept in wire-bottomed cages, with free access to tap water and standard laboratory animal chow, exposed to a 12/12-hlight/dark cycle, and housed at 22-25 °C and 50%-60% humidity. After several days of adaptation, diabetes was induced in rats by a single intraperitoneal injection of STZ (55 mg/kg) diluted in citrate-citrate sodium buffer, pH 4.5. At the same

time, an equal volume of citrate-citrate sodium buffer alone was injected into normal control rats. After 3 days, induction of diabetes was confirmed by measuring blood glucose concentration (≥16.7 mmol/L). Diabetic rats were then randomly divided into four groups (n=8 each): one diabetes nephropathy control group, and the other three treatment groups receiving naja naja atra venom at 30, 90, or 270 μg/(kg·day) via oral gavage. In the course of the experiment, 12 h (21:00-09:00) urine samples were collected from all rats every 2 w to determine urinary protein excretion. The 12 h urine collection was equivalent to 24 h collection in previous researches[8]. Serum glucose was measured in tail vein blood of rats at 0, 4, 8, and 12 w after overnight fasting. At 12 w, rats were weighed and sacrificed by intraperitoneal injection of ether (50 mg/kg). Blood samples were obtained from the abdominal aorta for the test of biochemical indexes. Serum was immediately separated by centrifugation at 6000 rpm for 20 min and stored at -80 °C until measurement after completion of the study. The kidneys were removed from each rat and weighed. Right kidneys were sliced transversely and fixed in 10% formalin for subsequent light microscopic evaluation. Left kidneys were frozen at -80 °C after rinsing with PBS. At the end of the experiment, we calculated the kidney coefficient: kidney weight/body weight.

Serum and Urine Levels

The biological indexes such as serum glucose, albumin, total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), triglyceride, creatinine (Cr), blood urea nitrogen (BUN), malondialdehyde (MDA) and superoxide dismutase (SOD), and urinary protein excretion were determined using commercial reagents via quantitative colorimetric assay at ultraviolet visible spectrophotometer (Instrument No.18-1650-01-1325, Japan). Serum glucose was measured by Glutest EII (Rongsheng Biotech Co., Ltd. Shanghai, China), serum Cr concentration was measured by the picric acid colorimetric method and BUN levels were determined with the urease indophenol method (Njjcbio Nanjing Chin). Urine samples were collected using metabolic cages, and proteinuria was determined by modified Coomassie blue G dye-binding assay (Njjcbio Nanjing China). Serum HDL-C was measured after precipitation by phosphotungstic acid with a magnesium chloride kit (BHKT Clinical Reagent Co., Ltd. Beijing, China). TC

was measured by the cholesterol oxidase method. Triglyceride measurement was done using the triglyceride oxidase colorimetric method (Rongsheng Biotech Co., Ltd. Shanghai, China). Serum albumin concentration was determined with the bromocresol green colorimetric assay (Njjcbio Nanjing China). Cr clearance (Ccr) was calculated as follows: Ccr [(mL/(min·kg)]=[urinary Cr (mg/dL) × urinary volume (mL)/serum Cr (mg/dL)] × [1000/body weight (g)] × [1/720 (min). The levels of microalbumin (mALB) and N-acetyl-β-glucosaminidase (NAG) in urine and Cystatin C (CysC) in serum were quantified by mouse-specific, double-antibody sandwich ELISA (Yfswbio Shanghai, China). The stop solution changed the color from blue to yellow and the intensity of the color was measured at 450 nm using a spectrophotometer (SN:239698; Bio Tek Instruments U.S.A). The concentration of these parameters in the samples was then determined by comparing OD to the standard curve.

Determinations of MDA and SOD in Serum and Kidney Tissue

Kidney tissue homogenate (0.1 g) was used to determine tissue protein concentrations and then MDA and SOD. Serum concentrations were determined directly. MDA levels were estimated by the TBA method. Total SOD activity was determined according to xanthine/XO system[9].

Light Microscopy and Immunohistochemistry

Renal tissues were fixed in 10% formalin and embedded in paraffin. Tissue slices were cut at 3-μm thickness, dewaxed, and stained with hematoxylin-eosin, and then examined by light microscopy. Glomeruli were randomly selected in the cortex (a total of 30 glomeruli for each group) and examined under high magnification (400×) (Nikon Dxm 1200 and Nikon Eclipse TE2000-U, Indonesia). The glomeruli were used for measuring and calculating the glomerulis average area, which was accomplished by manually tracing the glomerular outline and analyzing it using Image-Pro Plus 5.0 software. Kidney slices were processed for immunohistochemistry using the streptavidin biotin method as described previously[10]. Wax sections (3 μm) were incubated with 3% H_2O_2 for 15 min to dewax, and immersed in PBS three times for 5 min each to eliminate endogenous peroxidase activity. Sections were incubated with a polyclonal antibody (Code No:BA0610/0290; Boster, Wuhan, China) at

1:100 dilution, followed by biotinylated secondary antibody against mouse IgG for 1 h after rinsing with PBS for 30 min, and then with horseradish peroxidase (HRP)-conjugated streptavidin solution for 1 h. HRP labeling was detected using a peroxide substrate solution with 0.8 mmol/L diaminobenzidine and 0.01% H_2O_2 (Code No.GK500705/10; Gene Tech Co. Ltd., Shanghai, China). Renal tissues were examined at a high magnification (400×) to observe the expression of nuclear factor (NF)-κB and transforming growth factor (TGF)-β1 to conduct half quantitative analysis and to calculate mean density.

Statistical Analysis

The results are presented as the means±S.E.M. The effect of naja naja atra venom on each parameter was examined using one-way analysis of variance. Individual differences among groups were analyzed by Dunnett's test, and significance was accepted at $P<0.05$.

RESULTS

Body and Kidney Weight Changes

The changes in body and kidney weights are shown in Table 1. After oral administration of naja naja atra venom at 30 and 90 μg/kg body weight/day for 12 w, body weights increased in comparison with diabetic nephropathy rats (P=0.005, 0.003). However, after oral administration of naja naja atra venom at 270 μg/kg body weight/day for 12 w, changes of body weight showed no statistical significance (P=0.154). Furthermore, the 7.95-fold body weight gain in normal rats was observed, as compare with that in the diabetic nephropathy control group and the body weight gains in three treatment groups were 2.98 (30 μg), 2.26 (90 μg), 1.39 (270 μg)-fold respectively. The kidney coefficients in the diabetic nephropathy control group were larger than the normal value, 2.21 fold for the right kidney and 2.35 fold for the left kidney. However, kidney coefficients were obviously reduced by the treatment of naja naja atra venom. (Data shown in Table 1, $P<0.05$)

Serum Constituents

The effects of naja naja atra venom on serum glucose, TC, triglyceride, HDL-C and albumin are shown in Table 2. The levels of serum glucose decreased by 22.91%, 31.04%, and 27.89%, after administration of 30, 90, or 270 μg/(kg·day) naja

naja atra venom for 12 w. Serum glucose level in the DN control rats was 30.73 mmol/L, which was significantly higher (3.91-fold) than that in the normal group. The serum albumin level in the DN control rats was 30.45 g/L, which was significantly lower than that in the normal rats. After treatment with naja naja atra venom at doses of 90 and 270 μg/(kg·day), these levels were significantly increased by 5.5% and 5.7% respectively. Serum TC, triglyceride and HDL-C (indexes of lipid peroxidation) in DN rats were significantly higher than those in the normal rats (by 1.98-, 2.15-, and 2.27-fold, respectively, P=0.000, 0.000, and 0.002). naja naja atra venom at 90

μg/(kg·day) decreased TC and triglyceride significantly (P=0.035, 0.025). In contrast, naja naja atra venom treatment at 30 and 270 μg/(kg·day) had no significant effect (P>0.05). Naja naja atra venom at 30, 90, or 270 μg/(kg·day) led to 1.68-(68.18%), increases the HDL-C level by 1.59-(59.09%) and 1.48-(48.48%) folds, respectively. There was no significant difference among three treatment groups. Figure 1 shows the changes in blood glucose during 12 w. At 12 w, the level of hyperglycemia was markedly reduced by Naja naja atra venom administration, whereas at 4 and 8 w, there was no such change, with only a slight tendency towards a decrease in hyperglycemia.

Table 1. The Changes of Body Weight and Kidney Weight (Data showing means±S.E.M)

Items	Naja naja atra venom			DN Control	Normal
	30 μg/kg body wt/d	90 μg/kg body wt/d	270 μg/kg body wt/d		
Body Weight (g)					
Initial	203.12±1.39[###]	203.25±1.58[###]	203.75±1.41[###]	203.12±1.87[###]	223.12±1.36[***]
Final	261.2±11.53[**###]	255.12±7.41[**###]	231.00±7.41[###]	222.62±6.24[###]	378.38±10.63[***]
Gain(12 w)	58.13±11.86[**###]	51.88±6.49[**###]	27.25±7.10[###]	19.50±6.30[###]	155.25±10.20[***]
Kidney Coefficient					
Right	0.53±0.021[**###]	0.54±0.019[**###]	0.57±0.022[*###]	0.62±0.009[###]	0.28±0.005[***]
Left	0.49±0.025[**###]	0.51±0.020[**###]	0.54±0.024[*###]	0.61±0.007[###]	0.26±0.005[***]

Note. [*]P<0.05, [**]P<0.01, [***]P<0.001 versus DN control values; [###]P<0.001 versus normal values. Figures in Parentheses are 95% CI.

Table 2. Several Parameters in Serum and Urine (Data showing means±S.E.M)

Items	Naja naja atra venom			DN Control	Normal
	30 μg/kg body wt/d	90 μg/kg body wt/d	270 μg/kg body wt/d		
Serum					
Glucose (mmol/L)	23.69±2.19[*###]	21.19±3.23[*###]	22.16±2.67[**###]	30.73±0.94[###]	7.86±0.32[***]
Albumin (g/dL)	30.95±0.21[###]	32.14±0.35[**##]	32.21±0.23[***##]	30.45±0.26[###]	34.24±0.55[***]
TC (mmol/L)	1.69±0.19[##]	1.35±0.09[*##]	1.41±0.10[##]	1.73±0.13[###]	0.87±0.08[***]
Triglyceride (mmol/L)	1.69±0.15[##]	1.41±0.11[*#]	1.52±0.33[#]	1.85±0.14[###]	0.86±0.07[***]
HDL-C (mg/dL)	1.11±0.13[*###]	1.05±0.06[**###]	0.98±0.09[*###]	0.66±0.08[##]	0.29±0.05[**]
Urea Nitrogen (mmol/L)	14.46±1.11[*##]	13.05±0.62[**#]	14.83±0.98[*##]	18.99±1.29[###]	9.40±0.36[***]
Creatinine (umol/L)	61.21±1.43[***]	63.15±1.54[***]	63.57±2.81[**]	78.77±2.24[##]	6.45±1.64[***]
CysC (ng/mL)	231.05±8.82[*##]	228.55±8.53[*#]	229.66±6.16[*##]	284.17±2.25	196.56±6.66[**]
Urine					
Protein Excretion (g/L)	24.23±2.21[*###]	24.42±3.43[*###]	26.95±1.93[*###]	44.74±6.75[###]	8.27±0.42[***]
NAG (U/L)	19.89±2.06[##]	19.14±1.09[**##]	19.57±1.66[##]	24.12±0.72[###]	12.62±1.00[***]
Malb (ng/mL)	29.89±0.67[*###]	28.88±0.86[**###]	29.93±0.87[*###]	32.24±0.57[###]	16.39±0.66[***]
Ccr (mL/kg body wt/min)	20.08±1.62[##]	21.05±0.95[*##]	20.65±1.10[##]	17.04±1.49[###]	26.35±1.26[***]

Note. [*]P<0.05, [**]P<0.01, [***]P<0.001 versus DN control values; [#]P<0.05, [##]P<0.01, [###]P<0.001 versus normal values. Figures in Parentheses are 95% CI.
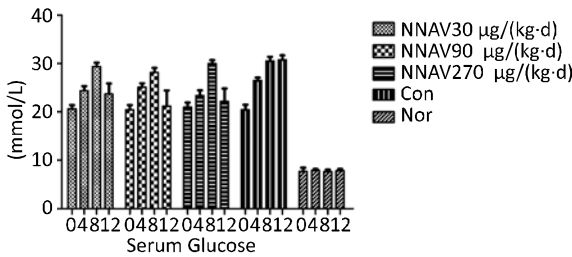
Figure 1. The variation tendency of serum glucose during 12 w via oral naja naja atra venom(NNAV) treatment.

Renal Function Parameters

Changes of renal function after naja naja atra venom treatment are shown in Table 2. BUN, serum Cr and urinary total protein excretion were significantly higher in the diabetic nephropathy control group than those in the normal group (2.02-, 1.22-, and 5.41-fold higher, respectively, P=0.000), whereas Cr was lower (35.33% decrease) and the concentration of serum Cr was close to normal. Urinary protein excretion was inhibited by 12 w treatment with all doses of naja naja atra venom in comparison with that in the diabetic nephropathy control group (P<0.05), and there was no significant difference among the three treatment groups. Ccr was increased by 23.53%, especially in the 90 μg/(kg·day) Naja naja atra venom group. Serum CysC and urinary NAG and mALB concentrations were increased significantly (1.44-, 1.91-, and 1.96-fold, respectively) in the DN rats. The CysC and mALB concentrations decreased significantly by all doses of naja naja atra venom (P<0.05). In contrast, urinary NAG concentrations were reduced by 90 and 270 μg/(kg·day) naja naja atra venom from 24.12 to 19.14 U/L (20.65% decrease, P=0.032) and 19.57 U/L (18.86% decrease, P=0.022), respectively. A dose of 30 μg/(kg·day) naja naja atra venom showed a tendency

to slightly decrease the urinary NAG levels. Figure 2 shows the changes in urinary protein excretion over 12 w.
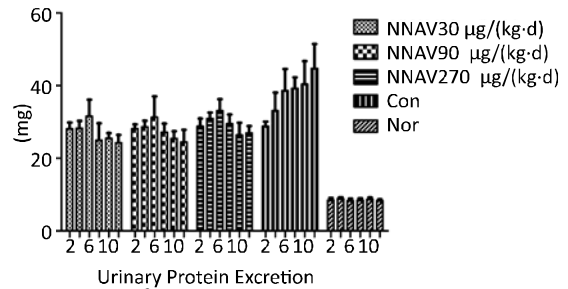

Figure 2. The variation tendency of urinary protein excretion via oral Naja naja atra venom (NNAV) treatment during 12 w.

MDA and SOD

Table 3 shows the serum and kidney tissue levels of MDA and SOD. Serum and kidney levels of MDA markedly increased in the DN rats by 1.72- and 1.66-fold, respectively. The serum levels decreased by 90 μg/(kg·day) naja naja atra venom (5.54 to 4.29 nmol/mL, 22.56% decrease, P=0.011) and 270 μg/(kg·day) naja naja atra venom (5.54 to 4.41 nmol/mL, 20.39% decrease, P=0.022). On the other hand, renal MDA levels increased by 17.15%, 9.79%, and 28.38%, by 30, 90, and 270 μg/(kg·day) naja naja atra venom, respectively (P<0.01). Serum SOD concentration in the DN rats was lower than that in the normal rats (17.79% decrease), however, kidney levels displayed an increasing trend (13.04% increase). Serum concentrations of SOD were significantly increased by 16.49% (P=0.011) and 15.46% (P=0.008) by 30 and 90 μg/(kg·day) naja naja atra venom, respectively, and the kidney levels were inhibited significantly by naja naja atra venom, at all doses (P<0.05).

Table 3. MDA and SOD Levels (Data showing means±S.E.M)

Items	Naja naja atra venom			DN Control	Normal
	30 μg/kg body wt/d	90 μg/kg body wt/d	270 μg/kg body wt/d		
Serum					
MDA (nmol/mL)	5.02±0.22##	4.29±0.28*#	4.41±0.29*#	5.54±0.32###	3.23±0.35***
SOD (U/mL)	1.13±0.042*	1.12±0.038**	1.05±0.039#	0.97±0.0319##	1.18±0.055**
SOD/MDA(U/nmol)	0.23±0.018*###	0.27±0.016***	0.24±0.018**	0.18±0.008###	0.42±0.083***
Kidney					
MDA (nmol/mgprot)	18.01±0.91**###	19.61±0.82**###	15.57±0.63***##	21.74±0.58###	13.11±0.51***
SOD (U/mgprot)	260.72±4.44*	260.89±5.91*	260.20±5.38*	286.21±8.88#	253.18±9.05*
SOD/MDA (U/nmol)	14.79±0.92##	13.91±0.70##	16.94±0.92*	13.25±0.62###	20.08±1.21***

Note. *P<0.05, **P<0.01, ***P<0.001 versus DN control values; #P<0.05, ##P<0.01, ###P<0.001 versus normal values. Figures in Parentheses are 95% CI.

Renal Histology and Immunohistochemistry

Microscopic examination revealed marked differences in tubulointerstitial histology between the treatment and diabetic nephropathy control groups. Rats treated with naja naja atra venom had minimal renal lesions compared with those in the diabetic nephropathy control group (Figure 4). Naja naja atra venom alleviated proximal and distal tubule steatosis degeneration. In addition, glomerular hypertrophy in the diabetic nephropathy group increased by 1.36-fold as compared with that in the normal rats. Naja naja atra venom at 30, 90, and 270 μg/(kg·day) for 12 w decreased glomerular hypertrophy by 8.15%, 8.29%, and 6.75%, respectively (Figure 3). Immunohistochemical analysis showed that TGF-β1-and NF-κB-positive areas in glomeruli and renal tubules were significantly more frequent in diabetic nephropathy rats than in normal ones (1.22-and 1.09-fold, respectively; $P<0.001$). However, expression of NF-κB and TGF-β1 was suppressed by naja naja atra venom administration ($P<0.05$) (Figure 3).
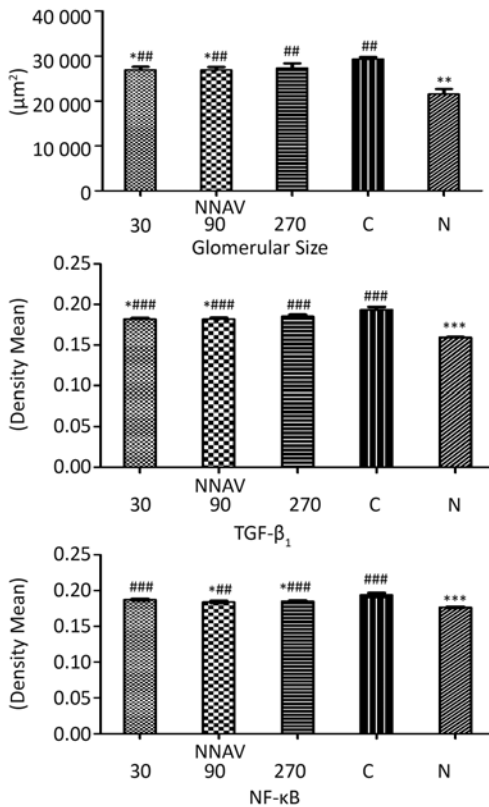

Figure 3. Show the glomerular size and the expression of TGF-β1 and NF-κB Glomerular size and TGF-β1, NF-κB in the renal cortex of normal rats (N) and in diabetic nephrcpathy rats treated with Naja naja atra venom at 30 μg/(kg·d) (NNAV 30), 90 μg/(kg·d) (NNAV 90), 270 μg/(kg·d) (NNAV 270), or water (diabetic control, C) for 12 w. Values represent the means±S.E.M. ($^{*}P<0.05,^{**}P<0.01,^{***}P<0.001$ versus DN control values; $^{##}P<0.01$, $^{###}P<0.001$ versus normal values).
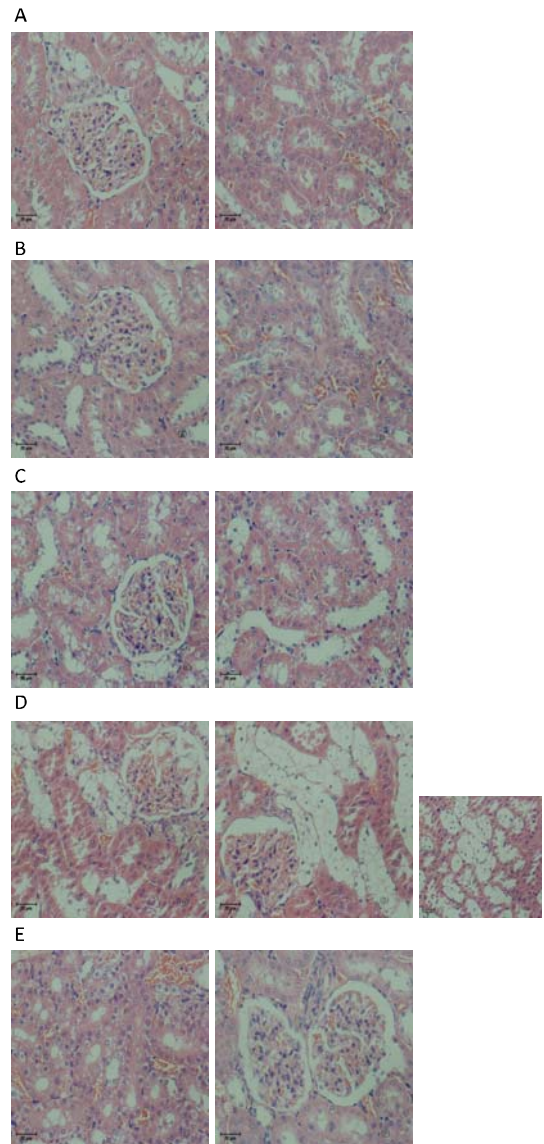

Figure 4. Renal Histology Morphologic. Photomicrographs of the diabetic nephropathy rats treated with naja naja atra venom at 30 μg/(kg·d) (A), 90 μg/(kg·d) (B), 270 μg/(kg·d) (C), diabetic control (D), normal rats(E). Scale bar, 20 μm.

DISCUSSION

In the present study, STZ-injected rats displayed typical characteristics of diabetes mellitus, such as hyperglycemia, polyuria, growth retardation, proteinuria, renal dysfunction, oxidative stress, and lipid peroxidation. The changes in markers of DN in our study were consistent with some previous studies[9,11-13]. We also demonstrated in our study that Naja naja atra venom could influence these parameters in the DN rats, especially at a dose of 90 μg/(kg·day), although there were no significant differences among the three dose-groups.

The mechanisms of DN are complex, which include hyperglycemia, advanced glycosylation end-products (AGEs), oxidative stress, lipid metabolites and inflammatory mediators, as well as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways. Hyperglycemia is a critical risk factor for the pathogenesis of DN. Chronic hyperglycemia is directly linked to the pathogenesis of diabetic microvascular complications, especially in the kidney[14]. Hyperglycemia damages the endogenous antioxidant defense system, and many channels boost the development of DN, including intracellular reactive oxygen species, oxidative stress, lipid peroxidation, free radicals, inflammatory factors, and urinary protein leakage. In addition, hyperglycemia facilitates generation of AGEs, activation of PKC, and overexpression of various factors[15-16]. Some studies[17] have reported that hyperglycemia acts as a solitary factor in the progression of DN. The present study revealed that serum glucose concentration in the diabetic rats was 3.91-fold higher than that in the normal rats, but Naja naja atra venom inhibited hyperglycemia at all doses, especially at dose of 90 μg/(kg·day). The mechanism of the inhibition of hyperglycemia by naja naja atra venom is unknown. It may be through protection of pancreas islets or through inhibition of oxidative stress and inflammation. In addition, naja naja atra venom increased body weight and reduced renal weight.

Our present research has shown that naja naja atra venom affects renal function, delays urinary protein excretion, and improves serum albumin levels. Urinary mALB are associated with a reduction, as compared with diabetic nephropathy control animals via Naja naja atra venom treatment. Serum Cr and BUN also significantly decreased following naja naja atra venom treatment, and Ccr increased especially at a dose of 90 μg/(kg·day). In addition,

our research showed that the levels of urinary NAG and serum CysC increased markedly in diabetic rats, however, these were unaffected by naja naja atra venom treatment. Previous researches[9] reported that proteinuria led to a very early renal endothelial dysfunction in diabetes, and accelerated the occurrence of tubular cell damage. Therefore, reduction of proteinuria may be beneficial for kidney function and is able to prevent progression of DN towards the end-stage, namely, renal failure. mALB has been widely used as a clinical index of early DN due to its higher sensitivity and accuracy for the estimation of renal diseases. Increase of mALB levels is equal to an albumin excretion rate of 20-200 μg/min[18]. The higher level of BUN and serum Cr could imply progressive renal damage, indicating the alteration of GFR in DN[19]. NAG is located in the glomeruli and proximal tubular cells and is released into the urine when renal parenchymal cells are damaged. Therefore, an increase in urinary NAG excretion may indicate renal as well as tubular cell damage[9]. Donahue et al.[20] have reported that CysC is associated with risk of prediabetes conditions. CysC is a low-molecular-mass plasma protein that is synthesized and secreted constantly by all nucleated cells. Serum CysC has been proposed as a potentially superior index to monitor renal dysfunction and impairment in early DN because of its simplicity, stability and sensitivity. Serum CysC levels are more sensitive and accurate for estimation of Ccr than BUN or serum Cr[13,21]. Besides, CysC can increase the concentration of inflammatory factors[22]. We showed in our study that these indexes were improved by naja naja atra venom, suggesting that naja naja atra venom reduces proteinuria, improves renal function, and delays progression of DN towards the end-stage, namely renal failure.

Oxidative stress may promote the formation of lipid peroxidation products, increase production of oxygen free radicals, and inhibit the endothelial antioxidant defense system by reducing the level of antioxidant enzymes such as SOD. It has been shown previously that increased oxidative stress is related to diabetic renal dysfunction and development of tubulointerstitial injury[9]. MDA is a marker of lipid peroxidation and is detected in rats with DN, and is related to glucose concentration and renal function[23]. Some studies have shown that MDA is a reliable marker of oxidative stress in renal tissue, and increased levels of MDA are involved in the occurrence of oxidative-stress-induced tubular cell damage in diabetic kidneys, and may be possibly

used to estimate the severity of renal damage[9]. In the present study, the levels of serum/renal MDA increased, similarly as in previous studies[9,24-25]. In contrast to MDA, SOD is a scavenger of free radicals and it alleviates oxidative stress. Antioxidants and antioxidant enzymes like SOD are controversial: decreases[25-26], increases[9,27] and no change[28] in SOD activity were reported in previous studies. The data from our study showed that the serum SOD concentrations of diabetic rats were lower than those in normal rats, but the renal tissue levels were higher than the normal value (no precise findings can be used to explain this phenomenon). However, naja naja atra venom could ameliorate the concentration of this index correspondingly. In addition, oxidative stress may induce TGF-β1 upregulation[29], which contributes to extracellular matrix accumulation, mesangial expansion, fibrotic processes, and the presence of diabetes complications. Naja naja atra venom slightly inhibited expression of TGF-β1, implying that it might have potential to alleviate fibrotic processes in DN. Sugimoto et al.[19] have reported that serum lipid concentrations are closely related to serum MDA levels in DN. A wealth of evidences suggest that abnormal lipid metabolism and lipid accumulation in kidney play an important role in the development of DN induced by STZ[12,30]. In the present study, the levels of HDL-C were higher in the DN rats than those in the normal group[12], which was different to the findings from previous studies[24-25]. The concentrations of lipid metabolites and serum TC and triglyceride decreased by naja naja atra venom treatment whereas HDL-C levels increased, implying that naja naja atra venom had a positive effect on lipid metabolic abnormalities. Our present study therefore strongly suggests that oxidative stress has a crucial role in the pathophysiology of DN. Our results have shown that Naja naja atra venom enhances oxidation resistance and ameliorates oxidative stress in DN, via inhibition of activation of MDA and changing SOD levels. This suggests that naja naja atra venom is a natural antioxidative agent, which protects against oxidative damage by scavenging excess free radicals, reducing lipid peroxidation, and affecting components of the antioxidative defense system.

There is increasing evidence that inflammation contributes to the pathogenesis of DN, and is related with the associated tubulointerstitial changes. The activation of NF-κB-linked regulatory pathways generally underlies inflammatory processes, which

has been demonstrated in human DN[31]. NF-κB can be activated by various factors including growth factors and oxidative stress. NF-κB is able to facilitate immune and renal damage such as glomerulonephritis, tubulointerstitial disorders, and proteinuria. Reducing NF-κB activation can help to prevent the ongoing structural and functional changes in DN[32-33]. Our results show that the increase in NF-κB in diabetes is inhibited by naja naja atra venom treatment, which is similar to the finding in previous research[34]. Further experiments are required to establish whether naja naja atra venom plays a role in anti-inflammatory activity in DN.

Finally, renal morphology showed renal tubular lesions in DN compared with normal rats, although there was little evidence of glomerulosclerosis. The renal tubule pathological changes in DN rats mainly comprised fatty or vacuolar degeneration. Some studies have reported that tubulointerstitial injury is an important feature of DN, which can predict the renal dysfunction of early DN[35], and therapeutic interventions on renal tubule damage have been discussed in both the experimental and human settings[36]. Naja naja atra venom improved renal tubular lesions and inhibited glomerular hypertrophy, but there were no marked differences among the three treatment groups. This suggests that naja naja atra venom is a potential renoprotective agent.

In conclusion, naja naja atra venom reduces hyperglycemia, decreases urinary protein, improves renal function and structure, restrains oxidative stress and lipid metabolism products, and prevents inflammatory factor infiltration. The mechanism is unknown. The present study provides details about the effect of naja naja atra venom in the early stage of DN, suggesting that it could be a potential renoprotective pharmaceutical for DN patients, though the mechanism of the said effect is not yet clear.

REFERENCE

1. Molitch ME, DeFronzo RA, Franz MJ, et al. Nephropathy in diabetes. Diabetes Care, 2004; 27 Suppl 1,S79-83.
2. Kapoor VK. Natural toxins and their therapeutic potential. Indian J Exp Biol, 2010; 48(3), 228-37.

3. Pu XC, Wong PT, Gopalakrishnakone P. A novel analgesic toxin (hannalgesin) from the venom of king cobra(Ophiophagus hannah). Toxicon, 1995; 33 (11), 1425-31.

4. Picolo G, Cassola AC, Cury Y. Activation of peripheral ATP-sensitive K+ channels mediates the anti-nociceptive effect of Crotalus durissus terrificus snake venom. Eur J Pharmacol, 2003; 469(1-3), 57-64.

5. Lochnit G, Geyer R. Carbohydrate structure analysis of batroxobin, a thrombin-like serine protease from Bothrops moojeni venom. Eur J Biochem, 1995; 228(3), 805-16.

6. Lizano S, Domont G, Perales J. Natural phospholipase A2 myotoxin inhibitor proteins from snakes, mammals and plants. Toxicon, 2003; 42(8), 963-77.

7. Velasquez MT, Kimmel PL, Michaelis OE 4th. Animal models of spontaneous diabetic kidney disease. FASEB J, 1990; 4(11), 2850-9.

8. Markantonis SL, Agathokleous-Kioupaki E. Can two-,four-or eight-hour urine collections after voluntary voiding be used instead of twenty-four-hour collections for the estimation of creatinine clearance in healthy subjects? Pharm World Sci, 1998; 20(6), 258-63.

9. Oktem F, Ozguner F, Yilmaz HR, et al. Melatonin reduces urinary excretion of N-acetyl-beta–D-glucosaminidase, albumin and renal oxidative markers in diabetic rats. Clin Exp Pharmacol Physiol, 2006; 33(1-2), 95-101.

10. Tojo A, Onozato ML, Fukuda S, et al. Nitric oxide generated by nNOS in the macula densa regulates the afferent arteriolar diameter in rat kidney. Med Electron Microsc, 2004; 37(4), 236-41.

11. Nakagawa T, Sato W, Glushakova O, et al. Diabetic Endothelial Nitric Oxide Synthase Knockout Mice Develop Advanced Diabetic Nephropathy. J Am Soc Nephrol, 2007; 18(2), 539-50.

12. Ota T, Takamura T, Ando H, et al. Preventive effect of cerivastatin on diabetic nephropathy through suppression of glomerular macrophage recruitment in a rat model. Diabetologia, 2003; 46(6), 843-51.

13. Ogawa Y, Goto T, Tamasawa N, et al. Serum cystatin C in diabetic patients. Not only an indicator for renal dysfunction in patients with overt nephropathy but also a predictor for cardiovascular events in patients without nephropathy. Diabetes Res Clin Pract, 2008; 79(2), 357-61.

14. Algenstaedt P, Schaefer C, Biermann T, et al. Microvascular Alterations in Diabetic Mice Correlate With Level of Hyperglycemia. Diabetes, 2003; 52(2), 542-9.

15. White NH, Sun W, Cleary PA, et al. Prolonged Effect of Intensive Therapy on the Risk of Retinopathy Complications in Patients With Type 1 Diabetes Mellitus: 10 Years After the Diabetes Control and Complications Trial. Arch Ophthalmol, 2008; 126 (12), 1707-15.

16. Deb DK, Chen Y, Zhang Z, et al. 1,25-Dihydroxyvitamin D3 suppresses high glucose-induced angiotensinogen expression in kidney cells by blocking the NF-κB pathway. Am J Physiol Renal Physiol, 2009; 296(5), F1212-8.

17. Schena FP, Gesualdo L. Pathogenetic mechanisms of diabetic nephropathy. J Am Soc Nephrol, 2005; 16 suppl 1, S30-3.

18. Mahmud N, O'Connell MA, Stinson J. Tumour necrosis factor-alpha and microalbuminuria in patients with inflammatory bowel disease. Eur J Gastroenterol Hepatol, 1995; 7(3), 215-9.

19. Suqimoto K, Tsuruoka S, Fujimura A. Effect of enalapril on diabetic nephropathy in OLETF rats: The role of an anti-oxidative action in its protective properties. Clin Exp Pharmacol Physiol, 2001; 28(10), 826-30.

20. Donahue RP, Stranqes S, Rejman K, et al. Elevated cystatin C concentration and progression to pre-diabetes: the Western New York study. Diabetes Care, 2007; 30(7), 1724-9.

21. Yashiro M, Kamata T, Seqawa H, et al. Comparisons of cystatin C with creatinine for evaluation of renal function in chronic kidney disease. Clin Exp Nephrol, 2009; 13(6), 598-604.

22. Singh D, Whooley MA, Ix JH, et al. Association of cystatin C and estimated GFR with inflammatory biomarkers: the Heart and Soul Study. Nephrol Dial Transpl ant, 2007; 22(4), 1087-92.

23. Satoh M, Fujimoto S, Haruna Y, et al. NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. Am J Physiol Renal Physiol, 2005; 288(6), F1144-52.

24. Soliman GZ. Blood lipid peroxidation (superoxide dismutase, malondialdehyde, glutathione) levels in Egyptian type 2 diabetic patients. Singapore Med J, 2008; 49 (2), 129-36.

25. Fulianq HU, Hepburn HR, Xuan H, et al. Effects of propolis on blood glucose, blood lipid and free radicals in rats with diabetes mellitus. Pharmacol Res, 2005; 51(2), 147-52.

26. Tikoo K, Tripathi DN, Kabra1 DG, et al. Intermittent fasting prevents the progression of type I diabetic nephropathy in rats and changes the expression of Sir2 and p53. FEBS Lett, 2007; 581(5), 1071-8.

27. Seqhrouchni I, Drai J, Bannier E et al. Oxidative stress parameters in type I, type II and insulin-treated type 2 diabetes mellitus; insulin treatment efficiency. Clin Chim Acta, 2002; 321(1-2), 89-96.

28. Sekeroğlu MR, Sahin H, Dülger H, et al. The effect of dietary treatment on erythrocyte lipid peroxidation, superoxide dismutase, glutathione peroxidase, and serum lipid peroxidation in patients with type 2 diabetes mellitus. Clin Biochem, 2000; 33 (8), 669-74.

29. Lal MA, Brismar H, EklÖf AC, et al. Role of oxidative stress in advanced glycation end product-induced mesangial cell activation. Kidney Int, 2002; 61(6), 2006-14.

30. Wang Z, Jiang T, Li J, et al. Regulation of Renal Lipid Metabolism, Lipid Accumulation, and Glomerulosclerosis in FVBdb/db Mice With Type 2 Diabetes. Diabetes, 2005; 54(8), 2328-35.

31. Mezzano S, Aros C, Droguett A, et al. NF-kappaB activation and overexpression of regulated genes in human diabetic nephropathy. Nephrol Dial Transplant, 2004; 19(10), 2505-12.

32. Galkina E, Ley K. Leukocyte recruitment and vascular injury in diabetic nephropathy. J Am Soc Nephrol, 2006; 17(2), 368-77.

33. Sanz AB, Sanchez-Nino MD, Ramos AM, et al. NF-κ B in Renal Inflammation. J Am Soc Nephrol, 2010; 21(8), 1254-62.

34. Park MH, Song HS, Kim KH, et al. Cobrotoxin Inhibits NF-κB Activation and Target Gene Expression through Reaction with NF-κB Signal Molecules. Biochemistry, 2005; 44(23), 8326-36.

35. Gilbert RE, Cooper ME. The tubulointerstitium in progressive diabetic kidney disease: More than an aftermath of glomerular injury? Kidney Int, 1999; 56(5), 1627-37.

36. Kelly DJ, Gilbert RE, Cox AJ, et al. Aminoguanidine ameliorates overexpression of prosclerotic growth factors and collagen deposition in experimental diabetic nephropathy. J Am Soc Nephrol, 2001; 12(10), 2098-107.