Protective Effect of Tanshinone IIA on the Early Stage of Experimental Diabetic Nephropathy

Su Kang KIM,^{a,#} Kyung-Hee JUNG,^{a,#} and Byung-Cheol LEE^{*,b}

^a Kohwang Medical Research Institute, Kyung Hee University; and ^b Department of Internal Medicine, College of Oriental Medicine, Kyung Hee University; 1 Hoegi-dong, Dongdaemun-ku, Seoul 130–702, Korea.

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Diabetic nephropathy (DN) has become the leading cause of end stage renal failure, and prevention or retardation of DN has become a major goal in biomedical research. In this study, Tanshinone IIA, a component extracted from *Salvia miltiorrhiza*, was studied in experimental rats in which DN was induced by streptozotocin (STZ) treatment. The DN rats were treated with 10 mg/kg of Tanshinone IIA for 12 weeks to analyze its renoprotective effect with different parameters. Renal hypertrophy and 24-h urinary protein excretion were ameliorated by Tanshinone IIA. Moreover, advanced glycation end-products (AGEs), angiotensin II (Ang II), transforming growth factor β_1 (TGF- β_1), collagen IV, and monocyte/macrophage (ED-1) either in the serum or kidney were significantly reduced. These results suggest that Tanshinone IIA might have protective effects on several pharmacological targets during the progression of DN, and could be a potential drug for the prevention of DN.

Key words Tanshinone IIA; diabetic nephropathy; advanced glycation end product; angiotensin II; transforming growth factor- β_1 ; collagen IV

More than 30% of diabetes mellitus patients develop clinically evident diabetic nephropathy (DN) 10 to 20 years from the onset of diabetes mellitus. DN seems to occur as a result of the interaction between metabolic and hemodynamic factors.¹⁾ DN is characterized functionally by glomerular hyperfiltration and proteinuria, and histologically by the expansion of the glomerular mesangium which is related to the loss of renal function.²⁾ Chronic hyperglycemia, a necessary prerequisite for the development of DN, eventually leads to the formation of long-lived non-enzymatic glycosylated proteins that are referred to as advanced glycation end-products (AGEs).³⁾ Moreover, AGEs increase extracellular matrix (ECM) production, such as collagen IV, via transforming growth factor- β_1 (TGF- β_1).⁴⁾ In addition, inhibitors of angiotensin-converting enzyme (ACE), a key enzyme for hypertension control, significantly delay the onset and progression of DN.⁵⁾ Furthermore, it has been reported that ACE inhibitor (ACEi) can also inhibit the formation of AGEs, hence these inhibitors can potentially mediate diabetic complications, including nephropathy.⁶⁾

Tanshinone IIA is one of the major diterpenes from *Salvia miltiorrhiza* (*S. miltiorrhiza*) referred to as ‘Danshen’ in traditional Chinese medicine (Fig. 1).⁷⁾ In the past, Danshen has been commonly used to treat inflammatory diseases such as edema, arthritis, hepatitis, and endangitis.⁷⁾ Danshen is also traditionally used in Asia to treat hypertension.⁸⁾ Both aqueous and lipid soluble fractions of Danshen contain active components responsible for its observed clinical effects. The two active hydrophilic components of Danshen are dan-

shensu and magnesium tanshinolate B, and cryptotanshinone and Tanshinone IIA are the two lipophilic components.⁸⁾ Tanshinone IIA has antioxidant properties and protects against lipid peroxidation *in vitro* and *in vivo* making it a potential antidote for free radical-based disorders.^{9,10)} In addition, it has been reported that Tanshinone IIA has neuroprotective effects during cerebral ischemia and attenuates Ang II-induced cardiac hypertrophy.^{11–13)} Moreover, a study by Kwark *et al.* showed that Tanshinone IIA may also be a therapeutic drug in the treatment of bone diseases such as osteoporosis.¹⁴⁾ Based on previous studies, there is a great interest in the therapeutic potential of Tanshinone IIA. To evaluate the effect of Tanshinone IIA on DN, we studied the possible influence of Tanshinone IIA on parameters such as blood glucose level, urine protein, and expression of AGEs, Ang II, TGF- β_1 , collagen IV, and monocyte/macrophage infiltration in experimental DN rats.

MATERIALS AND METHODS

Tanshinone IIA Tanshinone IIA was purchased from the Chinese Institute for Drug and Biological Product Control (Chengdu, People’s Republic of China). Tanshinone IIA was solved in 0.9% saline for oral administration.

Animals Diabetes was induced by injecting 45 mg/kg of streptozotocin (STZ) intravenously (dissolved in pH 4.5 citrate buffer immediately before injection) into the tail vein, while controlled normal standard rats (Con group, $n=9$) received 2.5 ml/kg of citrate buffer. Induction of the diabetic state was confirmed by measuring blood glucose levels 72 h after injection of STZ. The rats whose blood glucose concentrations were ≥ 300 mg/dl were randomly divided into two groups: DN rats who received no treatment (DN group, $n=9$) and DN rats that were orally administered a dose of 10 mg/kg of Tanshinone IIA daily (TS group, $n=9$). The animals were housed in a controlled environment ($24 \pm 1^\circ\text{C}$, 12-h light : 12-h dark cycle) and were allowed food and water *ad libitum*. One day before sacrifice at 12 weeks, the animals

Fig. 1. Chemical Structure of Tanshinone IIA

* To whom correspondence should be addressed. e-mail: hydrolee@korea.com

These authors contributed equally to this work.

were housed in metabolic cages for 24 h to obtain urine for the measurement of urine protein. Blood, obtained at decapitation, was collected in lithium heparin tubes, placed on ice, and serum was immediately separated and stored at -20°C . After animals were sacrificed, fresh kidney samples were stored in formaldehyde solution for light microscopic observation. The remaining kidneys were stored at -75°C for later analysis.

Blood and Urine Analyses Plasma glucose, blood urea nitrogen (BUN), creatinine, total cholesterol, low density lipoprotein (LDL) cholesterol, triglyceride, and 24-h urinary protein excretion were analyzed by a commercial laboratory company (SMLab, Seoul, Korea).

Morphologic Analysis Kidney tissue sections ($4\mu\text{m}$ thickness) were stained with periodic acid-Schiff (PAS) and examined by light microscopy in a blinded fashion. The mesangial expansion index (MEI) was scored in four levels from 0 to 3, with the index scores defined as follows.¹⁵⁾ 0, normal glomeruli; 1, matrix expansion in up to 50% of glomeruli; 2, matrix expansion in 50–70% of glomeruli; 3, matrix expansion in 75–100% of glomeruli. Scores were assigned for at least 30 glomeruli from kidney slices from each animal, and the means were calculated. To estimate MEI, the stained PAS slides were measured by two pathologists. The investigator scoring the sections was blinded to the animal group.

Immunohistochemistry Immunostaining was performed on $4\mu\text{m}$ -thick sections after deparaffinization. Microwave antigen retrieval was performed in citrate buffer at pH 6.0 for 10 min prior to peroxide quenching with 3% H_2O_2 in phosphate buffered saline (PBS) for 10 min. Sections were then washed in water and pre-blocked with normal goat or rabbit serum for 10 min. For primary antibody labeling, slides were incubated with anti-AGE (Cosmo Bio, Tokyo, Japan) at a 1:500 dilution, anti-Ang II (Peninsula Laboratories, Belmont, Calif., U.S.A.) at a 1:200 dilution, anti-TGF- β_1 (Santa Cruz, CA, U.S.A.) at a 1:200 dilution, anti-collagen IV antibodies (SouthernBiotech, Birmingham, AL), and anti-macrophage antigen (ED-1; Chemicon International Inc., Temecula, CA, U.S.A.) at a 1:200 dilution for 1 h at room temperature. The sections were then incubated with biotinylated secondary antibodies (1:1000) for 1 h. Following a washing step with PBS, streptavidin-HRP was applied. Finally, the sections were rinsed in PBS and then developed with diaminobenzidine tetrahydrochloride substrate for 10 min. At least three random fields of each section were examined at $\times 400$, which were analyzed by a computer image analysis system, Metavue (Molecular Devices, Sunnyvale, CA, U.S.A.). The density values represent the mean $\pm$ S.E. of the threshold area.

Enzyme-Linked Immunosorbent Assay (ELISA) Serum samples were tested for Ang II and TGF- β_1 by ELISA. The plates were coated overnight with $2\mu\text{g}/\text{ml}$ anti-Ang II and TGF- β_1 capture antibodies (R&D systems, Minneapolis, MN, U.S.A.) in 0.1M Na_2HPO_4 pH 9 buffer and blocked with PBS-Tween 20. Biotin-labeled $1\mu\text{g}/\text{ml}$ anti-Ang II and TGF- β_1 detecting antibodies were used. The plates were developed using streptavidin-horseradish peroxidase (Vector, Burlingame, CA, U.S.A.) and 2,2-azino-bis substrate (Sigma, St. Louis, MO, U.S.A.).

Statistical Analyses All of the values are expressed as

the mean $\pm$ S.E. Significant differences between the groups were analyzed using a one-way analysis of variance (ANOVA), followed by a non-parametric *post hoc* test (LSD). A *p*-value of 0.05 or less was considered statistically significant.

RESULTS

Functional and Biochemical Parameters The DN group demonstrated significantly higher plasma glucose than the control group ($p < 0.001$) (Table 1). Plasma glucose decreased in Tanshinone IIA treatment group compared to DN group ($p < 0.05$). Furthermore, BUN and 24-h urine protein excretion in DN rats were all markedly elevated compared with controls ($p < 0.01$). As expected, Tanshinone IIA treatment significantly decreased BUN and 24-h urine protein excretion levels ($p < 0.05$).

Kidney Histopathology Structural abnormalities of DN are similar in type 1 and type 2 diabetes,¹⁶⁾ and all renal compartments (glomeruli, tubulointerstitium, and renal vasculature) can be affected. The morphological change of DN is mesangial expansion due to increased mesangial matrix deposition, a mild increase in mesangial cellularity, and hypertrophy of mesangial cells.¹⁷⁾ In this study, kidney sections were stained with PAS for histochemical determination of mucopolysaccharides. PAS reaction was detected in some glomeruli from diabetic animals. Also, mesangial matrix expansion was observed in DN groups (Fig. 2). However, in the Tanshinone IIA treatment group, the PAS reaction was slightly less than in the DN group. In addition, the Tanshinone IIA treatment partially prevented mesangial matrix expansion. The MEI in PAS-stained glomeruli was determined in DN rats. The MEI in the DN group (1.7 ± 0.2) was remarkably increased compared with the control group ($p < 0.01$, 0.3 ± 0.1), whereas the MEI in the Tanshinone IIA treatment group (0.9 ± 0.1) was significantly lower than that of the DN group ($p < 0.05$).

The Protein Expression of AGEs, Ang II TGF- β_1 , Collagen IV and ED-1 We next assessed the effects of Tanshinone IIA on the expression level of AGEs, Ang II, TGF- β_1 , collagen IV, and ED-1 by immunohistochemistry. The expression of AGEs in DN (30.51 ± 1.62) was significantly increased compared with the controls ($p < 0.05$, 10.68 ± 1.74).

Table 1. The Effect of Tanshinone IIA Treatment on Biochemical Parameters of Diabetic Nephropathy (DN) Rats

Parameter	Group		
	Con	DN	TS
Body weight (g)	457.6 $\pm$ 24.84	347.3 $\pm$ 13.18 [#]	348.4 $\pm$ 43.68
Plasma glucose (mg/dl)	140.8 $\pm$ 11.56	608.2 $\pm$ 33.60 ^{###}	530.0 $\pm$ 72.15*
Blood urea nitrogen (mg/dl)	19.8 $\pm$ 0.45	28.1 $\pm$ 1.32 ^{###}	22.0 $\pm$ 2.27*
Creatinine (mg/dl)	0.53 $\pm$ 0.01	0.55 $\pm$ 0.01	0.57 $\pm$ 0.01
Albumin (mg/dl)	2.33 $\pm$ 0.04	2.04 $\pm$ 0.07	2.06 $\pm$ 0.13
Total cholesterol (mg/dl)	76.80 $\pm$ 4.84	96.00 $\pm$ 5.67	76.60 $\pm$ 4.49
LDL cholesterol (mg/dl)	8.00 $\pm$ 0.84	15.71 $\pm$ 2.97	13.20 $\pm$ 1.16
Triglyceride (mg/dl)	128.60 $\pm$ 15.05	160.71 $\pm$ 35.97	178.40 $\pm$ 23.88
Urinary protein excretion (mg/kg/24 h)	22.3 $\pm$ 5.8	174.6 $\pm$ 15.3 ^{###}	77.7 $\pm$ 18.2*

Data are presented as mean $\pm$ S.E. ($n=9$). [#] $p < 0.05$, ^{##} $p < 0.01$, ^{###} $p < 0.001$ vs. Con and * $p < 0.05$ vs. DN, respectively. Con, control; DN, diabetic nephropathy (45 mg/kg of STZ); TS, diabetic rats treated with Tanshinone IIA (10 mg/kg).

The increased expression of AGEs observed in glomeruli was reduced by treatment with Tanshinone IIA ($p<0.05$, 20.26 ± 1.02). Furthermore, the expression of Ang II in the DN group (24.64 ± 0.53) was decreased by Tanshinone IIA ($p<0.05$, 15.06 ± 0.75). Like Ang II, expression of TGF- β_1 in the DN group (29.79 ± 2.11) was significantly higher than in the control group (8.53 ± 1.01), and was located mainly in the glomeruli ($p<0.01$), suggesting that TGF- β_1 expression is correlated with ECM accumulation in glomeruli. However, Tanshinone IIA treatment prevented the enhanced expression of TGF- β_1 (16.25 ± 2.21). Faint collagen IV expression was observed in normal glomeruli, mainly in the glomerular basement membrane (Fig. 3). In the DN group, expression of collagen IV (30.52 ± 1.79) was significantly increased compared with the control group ($p<0.01$, 6.69 ± 0.82), whereas

Tanshinone IIA treatment partially attenuated increased collagen IV expression ($p<0.01$, 14.26 ± 3.17). The expression of ED-1 in DN (11.71 ± 0.05) was significantly increased compared with the controls ($p<0.05$, 0.27 ± 0.20). The increased expression of ED-1 observed in glomeruli was reduced by treatment with Tanshinone IIA ($p<0.05$, 4.45 ± 1.89).

The Production of Ang II and TGF- β_1 As shown in Fig. 4, the level of Ang II in serum of the DN group was significantly higher than that of the control groups ($p<0.01$, 2.84 ± 0.47 ng/ml). Ang II levels in serum in the Tanshinone IIA treatment group was reduced significantly compared to the DN group ($p<0.05$, 1.79 ± 0.03 ng/ml). Furthermore, in the DN group, TGF- β_1 levels in serum (153.4 ± 22.02 pg/ml) was similar to that of Ang II; TGF- β_1 levels in serum, how-

Fig. 2. Histological Analysis of Diabetic Nephropathy (DN) Rats by Periodic Acid Schiff (PAS) Staining

(A) Kidney sections were stained with PAS in streptozotocin (STZ)-induced diabetic nephropathy. (B) Mesangial expansion index (MEI) in PAS-stained glomeruli was scored according to four levels, and the average value for each rat was obtained from the analysis of >30 glomeruli per rat. Data are presented as the mean $\pm$ S.E. ($n=9$). $*p<0.05$ vs. DN. Con, control; TS, diabetic rats treated with Tanshinone IIA (10 mg/kg). Original magnification $\times 400$.

Fig. 3. The Expression of Advanced Glycation End-Products (AGEs), Angiotensin II (Ang II), Transforming Growth Factor- β_1 (TGF- β_1), Collagen Type IV, and ED-1 after Treatment with Tanshinone IIA

(A) Kidney tissues were stained with anti-AGEs, anti-Ang II, anti-TGF- β_1 , collagen IV, and ED-1 antibodies. (B) Quantification of AGEs, Ang II, TGF- β_1 , Collagen IV, and ED-1 expression was performed using Metavue software. Data are presented as the mean $\pm$ S.E. of at least six separate experiments. $*p<0.05$, $**p<0.01$, vs. DN, respectively. Con, control; DN, diabetic nephropathy (45 mg/kg of STZ); TS, diabetic rats treated with Tanshinone IIA (10 mg/kg). Original magnification $\times 400$.

Fig. 4. ELISA Measurements of the Levels of Angiotensin II (Ang II) and Transforming Growth Factor- β_1 (TGF- β_1) in Serum after Treatment with Tanshinone IIA

After treatment with Tanshinone IIA, rats in each group were killed and venous blood was collected. Serum samples were bound with anti-Ang II and anti-TGF- β_1 antibodies. Data are presented as mean $\pm$ S.E. for at least six separate experiments. * $p < 0.05$ vs. DN. Con, control; DN, diabetic nephropathy (45 mg/kg of STZ); TS, diabetic rats treated with Tanshinone IIA (10 mg/kg).

ever, was decreased significantly by Tanshinone IIA treatment ($p < 0.05$, 105.92 ± 10.21 pg/ml).

DISCUSSION

Multiple mechanisms have been postulated for the development of diabetic nephropathy, including AGEs, type IV collagen, TGF- β , polyol pathway, oxidative stress, inflammation, and hemodynamic changes. Therefore, multi-targeted therapeutic interventions should be required for DN. Recently, it has been reported that several herbs such as *Momordica grosvenori* and *Erigeron breviscapus*, have multi-targeted reno-protective effect in DN.^{18,19} In the present study, we demonstrated the multi-targeted renoprotective effects of Tanshinone IIA on DN. To the best of our knowledge, this is the first report showing that Tanshinone IIA treatment improves DN status according to plasma glucose, BUN, and urine protein excretion. Furthermore, our data indicates that Tanshinone IIA can prevent streptozotocin-induced DN by decreasing the expression of AGEs, Ang II, TGF- β_1 , collagen IV, and ED-1. Also, histological examination revealed that Tanshinone IIA significantly ameliorated diabetic-induced mesangial expansion.

DN is one of the main complications of diabetes, and involves increased urine protein excretion and loss of renal function. Thus, increased urine protein is a key component of DN. Many studies have shown correlations between urine protein level and a decline in glomerular filtration rate.²⁰ Indeed, our study showed that Tanshinone IIA decreased urine protein excretion by 60% in STZ-induced DN, which was similar to anti-proteinuric effects of ACEi or Ang II receptor blocker (ARB) in previous reported studies.^{21,22}

AGEs accumulate in diabetic tissue at an accelerated rate as a consequence of increased glucose concentration in extra-

cellular fluids. The administration of AGEs to non-diabetic animals leads to glomerulosclerosis and albuminuria,²³ suggesting that AGEs alone may be sufficient to induce renal injury in diabetes patients. Recently, it has been reported that AGEs stimulate Ang II production by an interaction with receptor for AGEs.²⁴ Ang II, the primary effector product of the renin-angiotensin system, is thought to be a crucial factor in progressive renal failure.²⁵ In fact, ARBs have shown a blocking effect on the progression of albuminuria, nephropathy development, and decline in kidney function.^{26–28} This study showed that expression of AGEs in the kidney of DN rats was decreased by Tanshinone IIA. Moreover, Tanshinone IIA decreased Ang II production and urine protein excretion in DN.

TGF- β_1 is reported to mediate renal hypertrophy, glomerulosclerosis, and tubulointerstitial fibrosis in DN. Many features of the diabetic state stimulate renal TGF- β_1 activity in both cell culture and *in vivo* systems. Once activated, TGF- β_1 induces the accumulation of matrix proteins²⁹ and stimulates the mRNA expression and production of key ECM molecules such as collagen IV.^{29,30} There are some reports indicating that inhibition of TGF- β_1 significantly decreases the high glucose-induced increase in ECM protein expression.³¹ In the present study, increased TGF- β_1 in DN was markedly inhibited by Tanshinone IIA. The expression of collagen IV was also increased in DN as expected from the increased TGF- β_1 levels, and Tanshinone IIA inhibited the expression of collagen IV consistent with its inhibition of TGF- β_1 expression.

Many studies have reported that Tanshinone IIA has the capacity of anti-inflammation.^{5,32} Fang *et al.* reported that Tanshinone IIA not only inhibits the oxidation but also suppresses the inflammation in atherosclerosis that is a chronic inflammatory disease.³³ We evaluated the effect of Tanshinone IIA on glomerular infiltration of monocyte/macrophages. ED-1 was quantified because they play a major role in early healing and reflect the inflammatory healing process.³⁴ In the present study, increased ED-1 in DN was markedly decreased in Tanshinone IIA treatment.

In summary, Tanshinone IIA treatment in this study decreased glucose, BUN, and 24-h urine protein excretion in a STZ-induced DN rat model. Moreover, Tanshinone IIA significantly inhibited the expressions of AGEs, Ang II, TGF- β_1 , collagen IV, and ED-1 in this model. Tanshinone IIA has protective effects on several pharmacological targets in DN. Therefore, we suggest that Tanshinone IIA might be useful in preventing the development of DN. In the future studies, Tanshinone IIA therapy should be compared to combined ACEi or ARB to establish the benefit of this treatment, as well as to define its optimal dose and clear its primary action mechanism on DN.

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