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Protective Effects of *Astragalus* Saponin I on Early Stage of Diabetic Nephropathy in Rats

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Abstract. Diabetic nephropathy (DN) has become the leading cause of end stage failure, but no renoprotective treatment has been very available for use in DN. *Astragalus* saponin I (AS I), a component extracted from *Astragalus membranaceus* BUNGE, was studied in experimental DN induced by administration of streptozotocin in male rats. The early DN rats were treated with 3 doses of AS I for 8 weeks to analyze its efficacy with different parameters. By comparison with vehicle-treated DN rats, the renal hypertrophy, the oxidative stress intensity, and the blood glucose level of DN rats were ameliorated by AS I. Also, the microalbuminuria level, advanced glycated end-products either in serum or in kidney cortex, and the aldose reductase activity were significantly reduced. Furthermore, the expression of transforming growth factor β1 mRNA in kidney cortex by RT-PCR analysis was markedly declined. Both the relative grade of mesangium hyperplasia by microscopical observation and the thickness of glomerular base membrane by electron microscope measurement were decreased significantly. Therefore, the results suggest that AS I has therapeutic effects on several pharmacological targets in the progress of DN and is a potential drug for prevention of early stage DN.

Keywords: *Astragalus membranaceus*, *Astragalus* saponin I, diabetic nephropathy

Introduction

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 interaction of metabolic and hemodynamic factors (1). Glucose dependent pathways are activated within the diabetic kidney. These include increased oxidative stress, renal polyol formation, advanced glycated end-products (AGEs) accumulation, and pro-sclerotic cytokines, such as transforming growth factor-β1 (TGF-β1) (2 – 6). These pathways ultimately lead to increased renal albumin permeablility and extracellular matrix accumulation which result in increasing proteinuria, glomerulosclerosis, and tubulointerstitial fibrosis. Therefore, besides the traditional therapeutic methods, the focus has been on some novel strategies very recently. Inhibitors of advanced glycation and aldose reductase are at present under experimental and clinical investigation (7).

Radix Astragali, the root of *Astragalus membranaceus* (Fisch.) BUNGE, is a crude drug widely used in traditional Chinese medicine. Known biologically active constituents of *Astragalus membranaceus* represent two major classes of chemical compounds, polysaccharides, and saponins. It was demonstrated that *Astragalus membranaceus* has an inhibitive effect on oxidative stress induced by metal (8). One of the most famous components among them is *Astragalus* saponin I (AS I) since it was demonstrated to have various bioactivities such as lymphocyte proliferation (9), immunuregulation (10), and anti-liver injury (11) in animal models. Up to now, more data has been reported about its positive inotropic action (12), fibrinolytic potential (13), anti-tumor activity (14), and scavenging effects on O_2^-, and $^{\bullet}OH$ (15). More recently, extract of *Astragalus* showed a definite protective effect on the ischemic reperfusion injured kidney (16) and could inhibit

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proliferation and matrix over-synthesis of human renal mesangial cells and downregulate expression of β1 integrin on its surface (17). Furthermore, the secretion of insulin and C-peptide increased gradually with the prolonging action of AS I on rats (18). All of the above data suggest that extract of *Astragalus* or AS I might have beneficial effects on DN, which was consistent with the fact that *Astragalus membranaceus* was frequently used for the treatment of DM in traditional Chinese medicine.

In our present work, we studied the possible influence of AS I on the parameters that indicate protective effects against the progress of DN, and evaluated its potential therapeutic effects on DN.

Materials and Methods

Drugs

AS I (No. 20020818) was extracted in our laboratory from *Astragalus membranaceus* (Fisch.) BUNGE. (purchased from Shandong, China) and was authenticated in aspects of melt point, mass spectrogram, and infrared spectrogram by Professor Zhao Fuzhong (Department of Chemistry, Nanjing Medical University). The yield of AS I was 0.2 – 0.3% and its purity was higher than 95% by HPLC method. AS I was suspended in 1% carboxymethyl cellulose (CMC) solution at different concentration for the purpose of p.o. administration. Epalrestat (Lot No. 990921), an aldose reductase inhibitor, serving as a positive control drug, was kindly presented by Shanghai Institute of Pharmaceutical Industry (Shanghai, China) and also suspended with 1% CMC solution.

Animals and protocol

Male Sprague-Dawley rats (Certificate No. SYXK 2001-0010, weighing 160 – 180 g) were obtained from Laboratory Animal Center of Nanjing Medical University. Laboratory animal care followed the Guiding Principles for Care and Use of Laboratory Animals of Nanjing Medicine University. Early DN was induced by i.p. administration of 60 mg/kg of streptozotocin (STZ) (Lot No. P5639; Biomol. Research Lab. Inc., Plymouth Meeting, PA, USA), dissolved in pH 4.5 citrate buffer immediately before injection, while rats that received the same volume of citrate buffer (2.5 mL/kg) served as a control normal standard group (NS group). The diabetic state was confirmed by assaying blood glucose at the third day after the injection of STZ. The rats with blood glucose concentrations higher than 13.88 mmol/L were randomly allotted into 5 groups: DN rats treated with CMC solution (DN group); DN rats treated with 3, 6, and 12 mg/kg of AS I for ASIL, ASIM, and ASIH group, respectively; and DN rats treated with 100 mg/kg of epalrestat (EPS group). The same volume of CMC solution was orally administrated to the NS group and DN group. The animals were housed at a controlled environmental temperature (24 ± 1°C, a 12-h light alternating with 12-h darkness cycle, lights on at 7:00 AM) and were allowed food and water ad libitium. After 8 weeks, the urine and blood samples were collected. After animals were sacrificed, fresh kidney cortices were stored in formaldehyde solution for light microscopic observation. Meanwhile, 1 mm × 1 mm × 1 mm cubes of kidney cortices were fixed in 2.5% glutaraldehyde for electron microscopic measurement. The remaining portions of the kidneys were stored at −75°C until analyzed.

Measurement of renal function and biochemical indexes

Blood glucose was measured by the glucose oxidase method with the kits purchased from Dong-Ou Bioengineering Co., Ltd. (No. 2002110002; Wenzhou, China). Urinary albumin was determined by radioimmunoassay using kits purchased from Department of Isotope, China Institute of Atomic Energy (No. 20021203; Beijing, China). Creatinine was assayed by the picric acid method, while the catalase activity was assayed by chemical colorimetry using kits from Jiancheng Bioengineering Institute (No. 20021122; Nanjing, China). The final value of microalbuminuria was represented by the ratio of albumin measured in urine versus creatinine in urine. Kidney index was 1000 × kidney weight / body weight.

Total antioxidative capability in body fluids, including ascorbate, protein thiols, bilirubin, urate, and α-tocopherol, is an important prognostic or diagnostic index for diabetes and atherosclerosis. When 2,2'-azino-bis-3-ethylzenzothiazoline-6-sulphonic acid (ABTS) is incubated with a peroxidase (such as metmyoglobin) and hydrogen peroxide, the relatively long-lived radical cation $ABTS^{\bullet+}$ is formed. In the presence of antioxidant reductants and hydrogen donors in body fluids, the absorbance of this radical cation is quenched to an extent related to the antioxidant capacity of the added fluid (19). In our experiment, 2.0 mL of ABTS/myoglobin reagent was mixed with 20 μL of sample and a further 180 μL of diluent that flushed the sample probe. The initiator of the reaction, hydrogen peroxide (0.675 mmol/L, 250 μL), was added last to obtain a final concentration in the cuvette of 75 μmol/L. After 6-min incubation, the absorbance of the $ABTS^{\bullet+}$ was read at 734 nm by a spectrophotometer (UV-1600; Ruili Instrument Co., Beijing, China), and the value of total antioxidative capability was calculated.

Collagen IV, a main component of the extracellular

matrix, was determined by radioimmunoassay (20), using kits from Shanghai High Biotech Center (No. 20021201; Shanghai, China). AGEs either in serum or renal cortex were represented by fluorescence spectrophotometry (fluorospectrophotometer RF-5300; Shimadzu, Kyoto), and the final value of AGEs was described as the ratio of fluorescence absorbency (AUF) versus the total protein which was measured by biuret colorimetry (21). The aldose reductase activity was measured with the fluorospectrophotometer with the reagents of β-NADPH (Lot No. 81k7059; Sigma Co., St. Louis, MO, USA) and DL-glyceraldehyde (Lot No. 120k2618, Sigma) (22), and its activity was defined as the micromoles of β-NADPH oxidized per min at 37°C, which was represented by the decrease in the fluorescence absorbance.

RT-PCR for the relative quantities of transforming growth factor (TGF)-β1 mRNA in kidney cortex

A reverse transcription PCR procedure was performed to determine the relative quantities of TGF-β1 mRNA in kidney cortex, while β-actin mRNA, the house- keeping gene, was used as an internal control (23). Total RNA was extracted from kidney cortex with TRIzol (Lot No. 1134369; Invitrogen Co., Carlsbad, CA, USA). First-strand cDNA synthesis was reverse-transcribed from 2 μg of mRNA in transcription buffer and 200 U MMLV reverse transcriptase (System 160419; Promega, Madison, WI, USA) at 42°C for 1 h, followed by immediately cooling on ice.

PCR amplification was performed using Taq polymerase (Lot No. 101405; Promega, Shanghai, China) in a total volume of 50 μL. The upstream and downstream primers for rat TGF-β1 mRNA were 5'-CCCGCATC CCAGGACCTCTCT-3' and 5'-CGGGGGACTGGC GAGCCTTAG-3', yielding a 519-bp product, whereas those for β-actin were 5'-GCTGCGTGTGGCCCCT GAG-3' and 5'-ACGCAGGATGGCATGAGGGA-3', yielding a 252-bp product. PCR conditions were as follows: 35 cycles, denaturating at 94°C for 30 s, annealing at 54°C for 60 s, and extending at 72°C for 60 s with initial heating at 94°C for 5 min and final extending at 72°C for 7 min. The PCR products were separated by 1% agrose (Lot No. 051363; Biowest, Miami, FL, USA) electrophoresis, and the band densities were analyzed using laser densitometry (Gel Doc 1000; Bio-Rad, Richmond, CA, USA). The relative quantities of TGF-β1 mRNA in kidney cortices were represented by the ratio of band density of TGF-β1 versus that of β-actin.

Morphological observation and measurement of the relative grade of mesangium hyperplasia and the thickness of glomerular base membrane (GBM)

Kidney cortex samples stored in formaldehyde solution were embedded with paraffin and stained with HE. Each HE-stained sample in each group was observed under a light microscope, and the relative grades of mesangium hyperplasia were scored from 0 to 6 according to their morphological change (24). Three kidney samples from each of the experimental groups were randomly chosen for electron microscopic observation. Specimens were embedded in epoxy resin and cut into ultrathin sections and then stained with plumbum citrate for ultrastructural observation under transmission electron microscope (JEM 1200EX; Jeol, Tokyo). Five photos were taken at different views for each kidney sample. The images were amplified 10 K and then the photos were scanned into computer to measure the thickness of GBM using an image analysis system (Leica Qwin Standard V2.6; Leica Microsystems Ltd., Welzlar, Germany).

Statistical analyses

Statistical analysis was performed to compare the effects of AS I on early DN rats using one-way analysis of variance (ANOVA) and *t*-test for different groups. Data are expressed as the mean ± S.D.; the value of $P<0.05$ was considered significant.

Results

Effects of AS I on physical behaviors, blood glucose, microalbuminuria, and kidney index

Physical behaviors of early DN rats in our experiment were described as follows: hypopraxia, cachexia, yellowish and damp fur, kyphosis, body shake, ptosis, polyuria polydipsia, and tardy weight gaining, while rats in NS and ASIH groups were vibrant, vigorous, white and tidy fur, and weight gaining. The mean body weights of NS, DS, ASIL, ASIM, ASIH, and EPS groups on the day of sacrifice were 333 ± 99, 235 ± 49, 224 ± 58, 282 ± 61, 252 ± 73, and 258 ± 56 g, respectively.

Figure 1 shows the effects of AS I on blood glucose, microalbuminuria, and kidney index of rats. The blood glucose level and microalbuminuria of the DN group were significantly higher than those of the NS group ($P<0.01$), indicating that our early DN model was successful. Low dose of AS I slightly reduced blood glucose, microalbuminuria, and kidney index for early DN rats, but differences were not significant. Moderate and high dose of AS I treatment significantly reduced the above three parameters in early DN rats ($P<0.05$ or

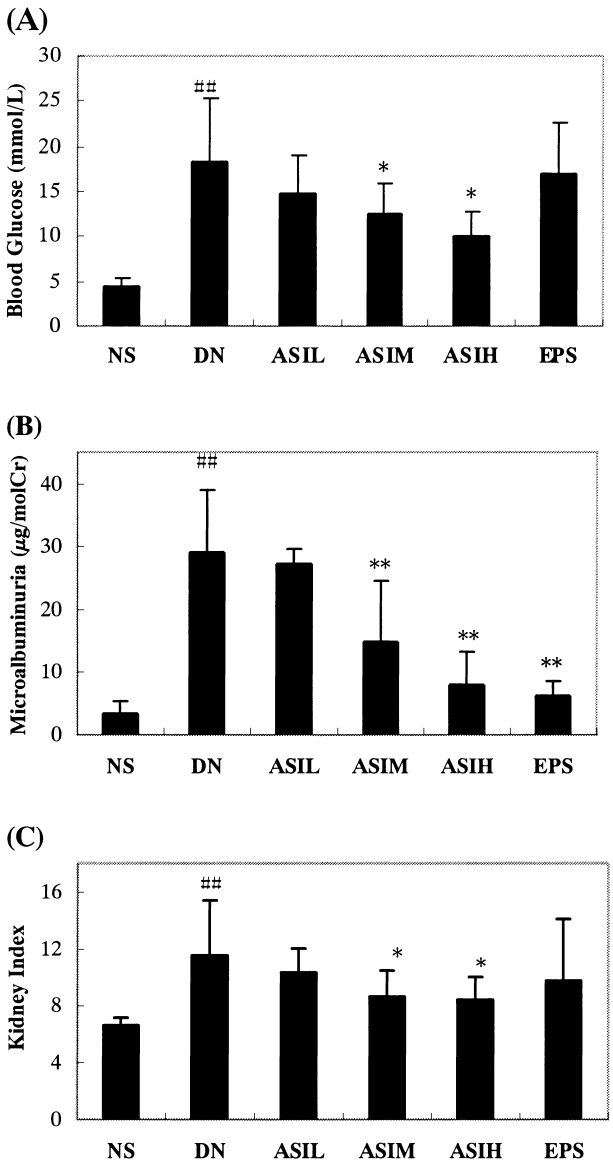

Fig. 1. Effects of AS I on blood glucose (A), microalbuminuria (B), and kidney index (C) of rats. NS, DN, ASIL, ASIM, ASIH, and EPS represent normal standard rats treated by CMC, DN rats treated by CMC, DN rats treated by 3 mg/kg of AS I, DN rats treated by 6 mg/kg of AS I, DN rats treated by 12 mg/kg of AS I, and DN rats treated by 100 mg/kg of epalrestat, respectively. $^{\#\#}P<0.01$, compared with normal standard rats using ANOVA by *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, compared with DN rats by *t*-test. Data are presented as mean ± S.D.

$P<0.01$).

Effects of AS I on extracellular matrix

Collagen IV levels in kidney cortex of NS, DN, ASIL, ASIM, ASIH, and EPS groups were 0.490 ± 0.124, 0.753 ± 0.259, 0.713 ± 0.293, 0.652 ± 0.125, 0.598 ± 0.171, and 0.715 ± 0.185 μg/g, respectively (Fig. 2). Compared with that of normal rats, the collagen IV

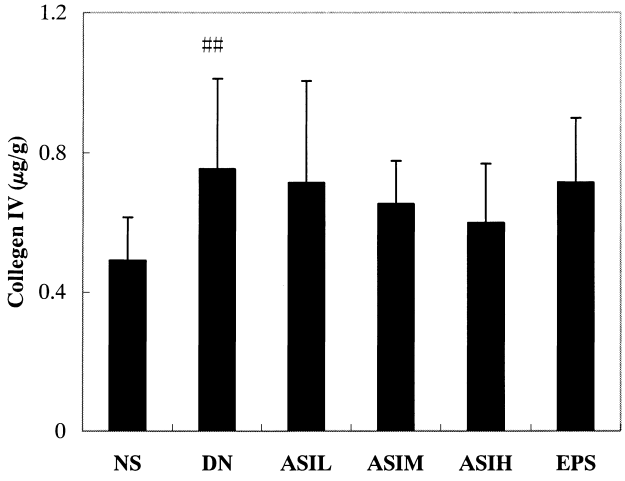

Fig. 2. Effect of AS I on extracellular matrix (collagen IV level in kidney cortex) of rats. NS, DN, ASIL, ASIM, ASIH, and EPS represent normal standard rats treated by CMC, DN rats treated by CMC, DN rats treated by 3 mg/kg of AS I, DN rats treated by 6 mg/kg of AS I, DN rats treated by 12 mg/kg of AS I, and DN rats treated by 100 mg/kg of epalrestat, respectively. $^{\#\#}P<0.01$, compared with normal standard rats using ANOVA by *t*-test. Data are presented as mean ± S.D.

level in kidney cortex of early DN rats had been significantly increased ($P<0.01$). Three doses of AS I slightly decreased the collagen IV level, but the difference was not significant.

Effects of AS I on AGEs in kidney cortex and in serum

Table 1 shows that the AGEs levels in serum and in kidney cortex of DN rats were greatly higher than those of NS rats ($P<0.01$). Serum AGEs level of the ASIL group was reduced very significantly ($P<0.01$). Furthermore, the AGEs levels in kidney cortex and in serum decreased significantly in ASIM, ASIH, and EPS groups, compared with the DN group ($P<0.01$).

Effect of AS I on oxidative stress

Table 2 showed that the catalase activity and total antioxidative capability were markedly increased in ASIM, ASIH, and EPS groups ($P<0.05$ or $P<0.01$), whereas a low dose of AS I had no significant effects on these two parameters.

Effects of AS I on aldose reductase activity in erythrocyte

Aldose reductase activity in erythrocytes of the NS group and DN group were 6.90 and 27.29 U, respectively ($P<0.01$) (Fig. 3). Aldose reductase activities of ASIL, ASIM, and ASIH groups were 24.91, 17.18, and 9.88 U, and significant differences existed when compared with that of the DN group, respectively

Table 1. Effects of AS I on the level of advanced glycated end-products (AGEs) in serum and kidney cortex of rats

		AGEs (AUF/mg protein)	
Group	number of rats	serum	kidney cortex
NS	10	4.70 ± 0.94	0.54 ± 0.18
DN	10	17.25 ± 2.50##	2.30 ± 0.71
ASIL (3 mg/kg)	5	7.64 ± 0.61**	1.75 ± 0.37
ASIM (6 mg/kg)	11	6.68 ± 1.58**	1.26 ± 0.28**
ASIH (12 mg/kg)	10	7.57 ± 2.79**	1.14 ± 0.49**
EPS (100 mg/kg)	10	8.46 ± 2.07**	1.60 ± 0.36*

##P<0.01, compared with the NS group by t-test; *P<0.05, **P<0.01, compared with the DN group by t-test. Statistical analysis was performed using ANOVA. Data are presented as mean ± S.D.

Table 2. Effects of AS I on total antioxidative capability (T-AOC) and catalase activity (CAT) in serum of rats

Group	number of rats	T-AOC (U/mL)	CAT (U/mL)
NS	10	15.85 ± 0.99	5.37 ± 1.13
DN	10	6.94 ± 1.81##	3.74 ± 0.91##
ASIL (3 mg/kg)	5	7.33 ± 0.75	4.61 ± 0.47
ASIM (6 mg/kg)	11	12.86 ± 1.31**	5.46 ± 0.81*
ASIH (12 mg/kg)	10	13.76 ± 4.22**	5.89 ± 1.22*
EPS (100 mg/kg)	10	9.00 ± 0.70**	4.33 ± 0.40

##P<0.01, compared with the NS group by t-test; *P<0.05, **P<0.01, compared with the DN group by t-test. Statistical analysis was performed using ANOVA. Data are presented as mean ± S.D.

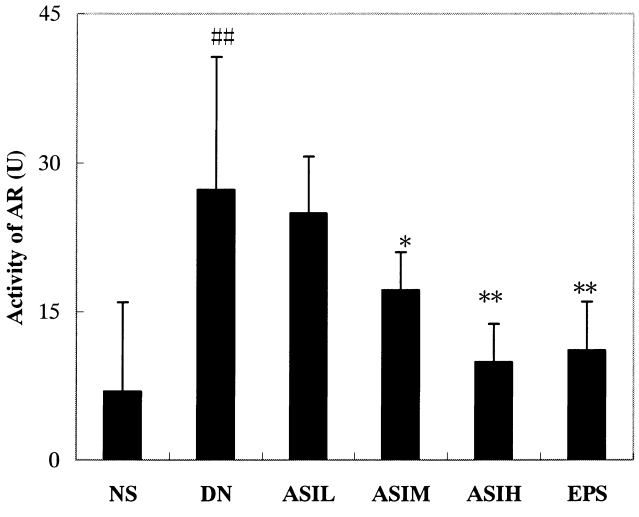

Fig. 3. Effect of AS I on aldose reductase (AR) activity in erythrocyte of rats. NS, DN, ASIL, ASIM, ASIH, and EPS represent normal standard rats treated by CMC, DN rats treated by CMC, DN rats treated by 3 mg/kg of AS I, DN rats treated by 6 mg/kg of AS I, DN rats treated by 12 mg/kg of AS I, and DN rats treated by 100 mg/kg of epalrestat, respectively. ##P<0.01, compared with normal standard rats using ANOVA by t-test; *P<0.05, **P<0.01, compared with DN rats by t-test. Data are presented as mean ± S.D.

(P<0.01). Furthermore, aldose reductase activity showed a good dose-dependence to AS I. Consistently, epalrestat, an aldose reductase inhibitor, also showed significantly reduced aldose reductase activity from 27.29 to 11.07 U (P<0.01).

Effects of AS I on the relative quantity of TGF-β1 mRNA in kidney cortex

In Fig. 4, we found that the relative quantity of TGF-β1 mRNA in kidney cortex of DN rats (1.21 ± 0.25) significantly increased, when compared with that of NS rats (0.55 ± 0.09) (P<0.01). Compared with the DN group, low dose of AS I only slightly reduced TGF-β1 mRNA level to 1.07 ± 0.24, and no statistical difference existed. Moderate and high dose of AS I decreased the level of TGF-β1 mRNA to 0.80 ± 0.12 and 0.67 ± 0.12, respectively (P<0.01). Epalrestat had the same effects as a moderate dose of AS I; statistical difference was found when compared with the DN group (P<0.01).

Effects of AS I on morphological change in kidney and the relative grade of mesangium hyperplasia and the thickness of GBM

The light microphotograph showed that glomerular

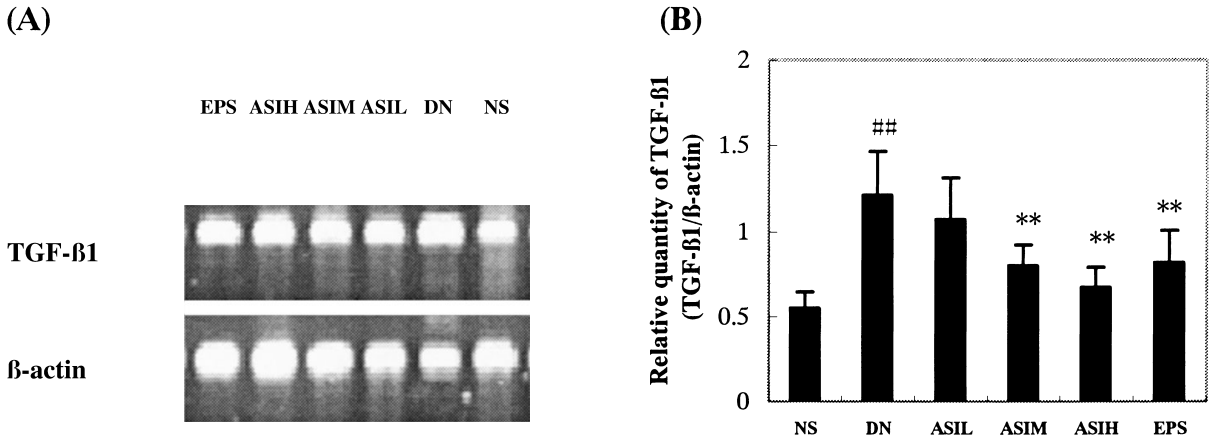

Fig. 4. Effect of AS I on the relative quantity of TGF-β1 mRNA in kidney cortex of rat. NS, DN, ASIL, ASIM, ASIH, and EPS represent normal standard rats treated by CMC, DN rats treated by CMC, DN rats treated by 3 mg/kg of AS I, DN rats treated by 6 mg/kg of AS I, DN rats treated by 12 mg/kg of AS I, and DN rats treated by 100 mg/kg of epalrestat, respectively. A: cDNA samples obtained from the kidney cortex of each rat were amplified for the detection of TGF-β1 mRNA. β-Actin was used as the internal standard in each sample. B: RT-PCR data for relatively quantify of TGF-β1 mRNA performed by densitometric analysis. Statistical analysis was performed using ANOVA. $^{\#\#}P<0.01$, compared with the NS group; $^{**}P<0.05$, compared with the DN group with *t*-test. Data are presented as mean ± S.D.

mesangial hyperplasia existed (Fig. 5). By transmission electron micrographs, the ultrastructure glomerulus of CMC-treated DN rat was changed. The GBM was wrinkled and thickened partly, with effacement of some visceral epithelial cell foot processes and microvillous transformation. After 8-week treatment by 12 mg/kg of AS I, the glomerular capillary loops, GBM, pedicelsa, and mesangial matrix of rats became nearly normal (Fig. 6).

The relative grade of mesangium hyperplasia of the NS group and DN group were 0.6 ± 1.0 and 4.4 ± 1.8, respectively ($P<0.01$). Those of ASIM and ASIH groups were 2.8 ± 1.0 and 2.4 ± 0.8; significant differences existed when compared with that of the DN group, respectively ($P<0.01$). The relative grade of mesangium hyperplasia in ASIL and EPS groups were 3.6 ± 0.9 and 3.6 ± 0.8, respectively; no statistic differences were founded compared with the DN group (Fig. 7).

There was a significant difference in the thickness of GBM between the DN group (219.4 ± 8.5 nm) and NS group (141.6 ± 9.7 nm) ($P<0.01$). The thickness of GBM of ASIL, ASIM, and ASIH groups were 189.3 ± 46.4, 178.9 ± 18.5, and 152.9 ± 6.0 nm, respectively, while that of the EPS group was 160.2 ± 15.9 nm. Compared with the DN group, there were significant differences for the thicknesses of GBM of ASIH, ASIM, and EPS groups ($P<0.05$). Furthermore, the thickness of GBM was dose-dependently decreased with AS I (Fig. 7).

Discussion

DN is characterized by increased urinary albumin (proteinuria) excretion and loss of renal function. Increased proteinuria is a key component of this disease. A number of observational studies have shown correlations between glycemic control and the development of various levels of albuminuria and also decline in glomerular filtration rate (25). However, large long-term prospective, randomized, interventional studies have now definitely proven that improved metabolic control that achieves near-normoglycemia can significantly decrease the development and progression of DN (25). *Astragalus membranaceus* has used for treatment of diabetics for hundreds years in Chinese traditional medicine. Recently, several reports showed good results in glycemic and proteinuria control in the early stage of DN treated with a preparation of *Astragalus membranaceus* or its extract (26, 27). In our study, AS I can ameliorate symptoms in STZ-induced DN rats, decrease the level of blood glucose, microalbuminuria, and renal hypertrophy (kidney index). Therefore, it is very important to investigate the effects of AS I on some intricate factors relative with glucose metabolism, such as oxidative stress, AGEs, polyol pathway, and TGF-β1.

Oxidative stress has been known to play an important role in the development and progression of DN. Moreover, the intensity and durability of oxidative stress facilitate the formation of AGEs, which is the product of the reaction between carbohydrates and free

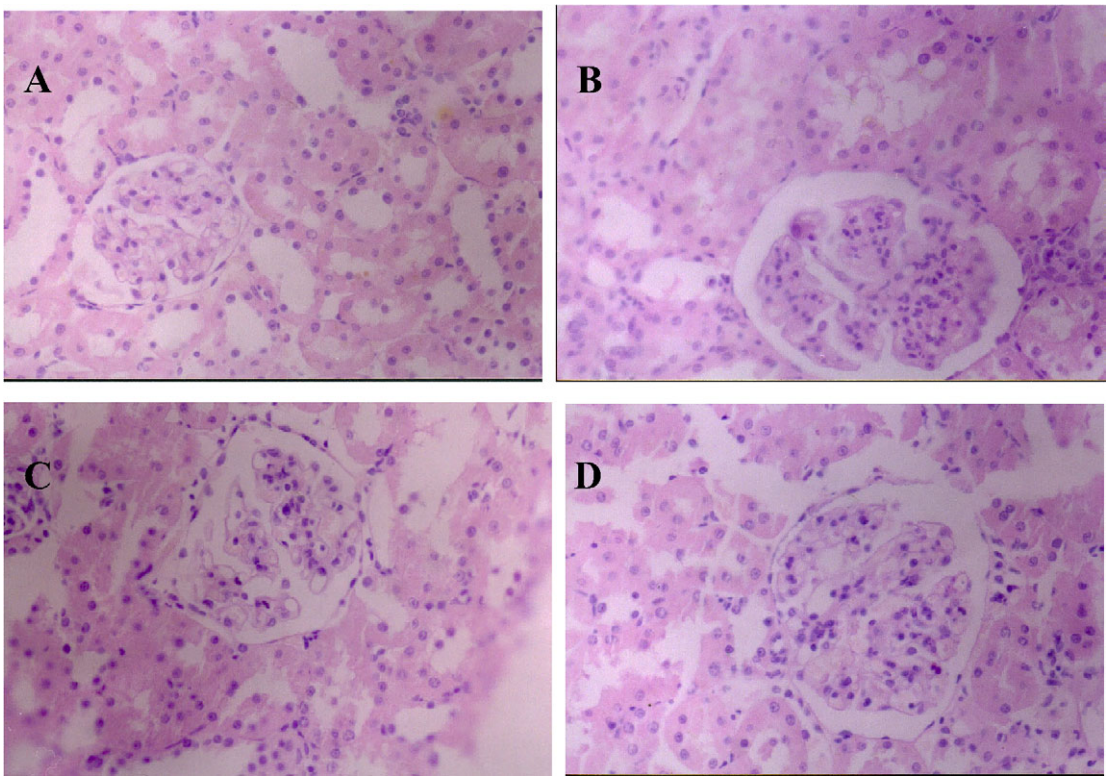

Fig. 5. Representative light microphotographs showing the glomerular mesangial hyperplasia (HE stain, ×40). A: The glomerulus of normal standard rat: the capillary loops are opened and portion of the glomerular is relatively small; B: The glomerulus of CMC-treated DN rat: the capillary loops are opened but narrowed and portion of the glomerular is relatively large. The mesangial hyperplasia is moderate-grade; C: The glomerulus of 6 mg/kg of AS I-treated DN rats: the mesangial hyperplasia is low-grade; D: The glomerulus of 12 mg/kg of AS I-treated DN rats: the mesangial hyperplasia is low-grade.

amino group of proteins. AGEs accumulation happens earlier and with an accelerated rate in diabetes mellitus patients than in non-diabetic individuals (28). AGEs also enhances susceptibility of LDL to oxidation (29), inducing apoptosis in cultured human umbilical vein endothelial cells (30). On the other hand, interactions between AGEs and its specific receptor RAGE induces activation of oxidative stress and stimulates the production and release of cytokines, which amplified the tissue damage (28). Thus, oxidative stress and AGEs interact mutually and upregulate each other.

There is emerging evidence that the formation of reactive oxygen species is a direct consequence of hyperglycemia. Biomarkers for oxidative damage to DNA, lipids, and proteins are also supporting the concept of increased oxidative stress in diabetes and DN (31). In diabetic Akita mice, the levels of N^{ε}-(hexanonyl)-lysine (HEL) and dityrosine, which is related to lipid peroxide-derived protein covalent modification and protein cross-linking, were significantly increased (32). It has been report that the glucose induced increase in fibronectin and collagen IV gene expression can be partially reversed by the addition of two structurally unrelated antioxidants, trolox and α-lipoic acid, in porcine mesangial cells (33). Therefore, glucose-induced oxidative stress by diabetes could play a crucial role in the development and progression of DN, and antioxidant treatment could be a potential therapeutic procedure. Studies showed that antioxidative agents, including taurine, vitamin C, and vitamin E, ameliorate the symptoms of STZ-induced DN in rats, such as preventing glomerular dysfunction; decreasing albuminuria, glomerular TGF-β1 level, and glomerular volume; preventing glucose-induced lipid-peroxidation and collagen production. Also, studies demonstrated that *Astragalus membranaceus* and its extract have an antioxidative effect (34 – 36). In our study, we measured the catalase activity and total antioxidative capability, which are common indicators for changes in the anti-oxidation system. Both of them were significantly increased by AS I in DN rats, strongly suggesting that AS I has effects on antioxidative capability in vivo. On the other hand, we also found that AS I in moderate and high dose very significantly decreased the AGEs

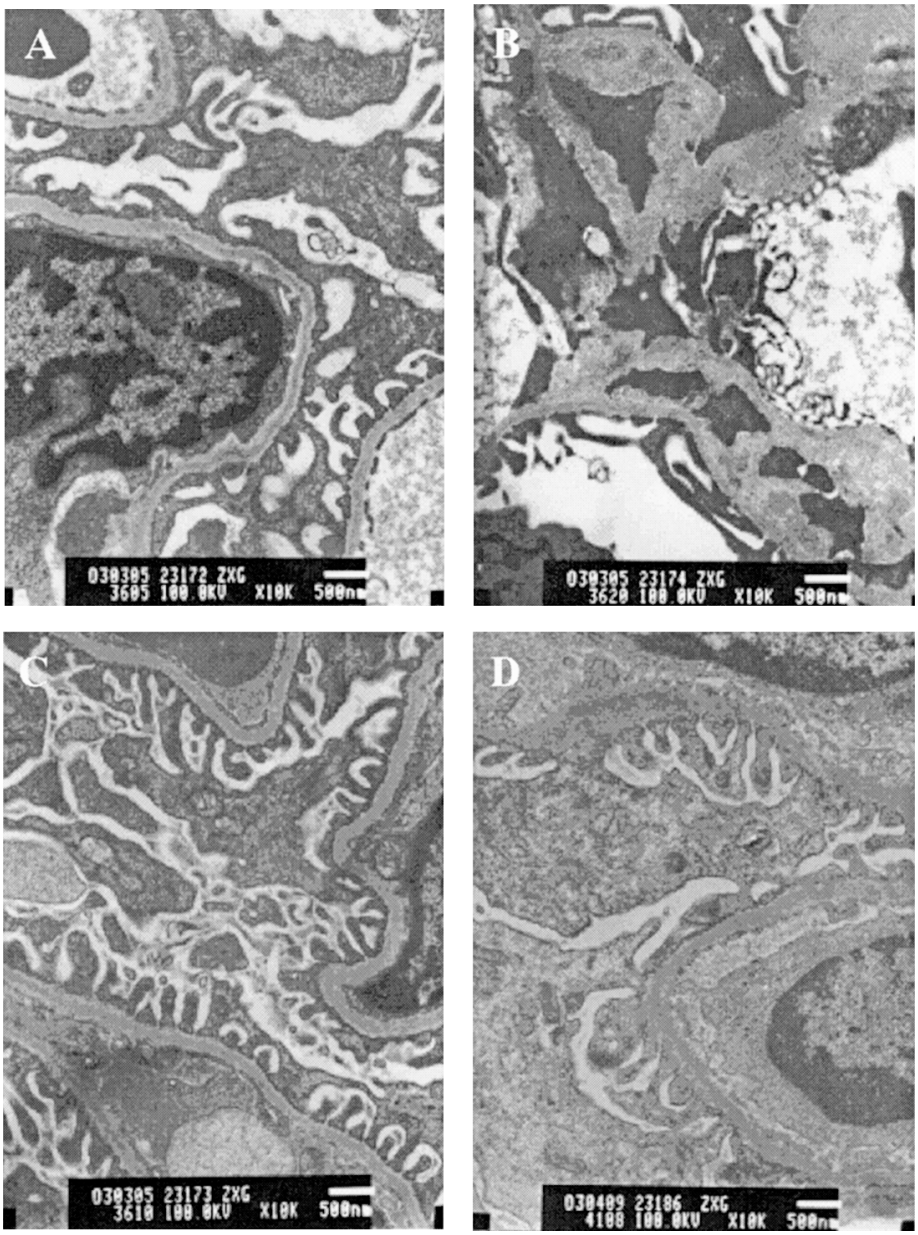

Fig. 6. Transmission electron micrographs showing the ultrastructure of rat glomerulus (×10 k). A: Normal control group of rats: illustrating a portion of the glomerular tuft, the thickness of glomerular basement membrane was normal, and pedicels embedded in the lamina rara externa. B: CMC-treated DN rats: the glomerular basement membrane was wrinkled, and thickened partly, with effacement of some visceral epithelial cell foot processes and microvillous transformation. C: 6 mg/kg of AS I-treated DN rats: a few of visceral epithelial cell foot processes showed microvillous transformation. The glomerular basement membrane, pedicelsa and glomerular capillary loops were almost normal. D: 12 mg/kg of AS I-treated DN rats: the glomerular capillary loops, glomerular basement membrane, pedicelsa and mesangial matrix were normal. Bar = 500 nm.

levels either in kidney cortex or in serum, while a low dose of AS I can still decrease it in serum. This anti-oxidative effect of AS I in our experiment is consistent with other reports (34 – 36). Thus, pharmacological intervention of hyperglycemia-induced diabetic complications would be of great clinical significance and the anti-oxidation and anti-AGEs effects of AS I are very important and may make a beneficial synergism for the prevention of DN.

It is very interesting that AS I has definite effect of normalizing hyperglycemia in DN rats in our experiment. Although we did not measure the insulin or C-peptide in plasma, there are still other reports showing that *Astragalus* saponin and AS I increase the insulin

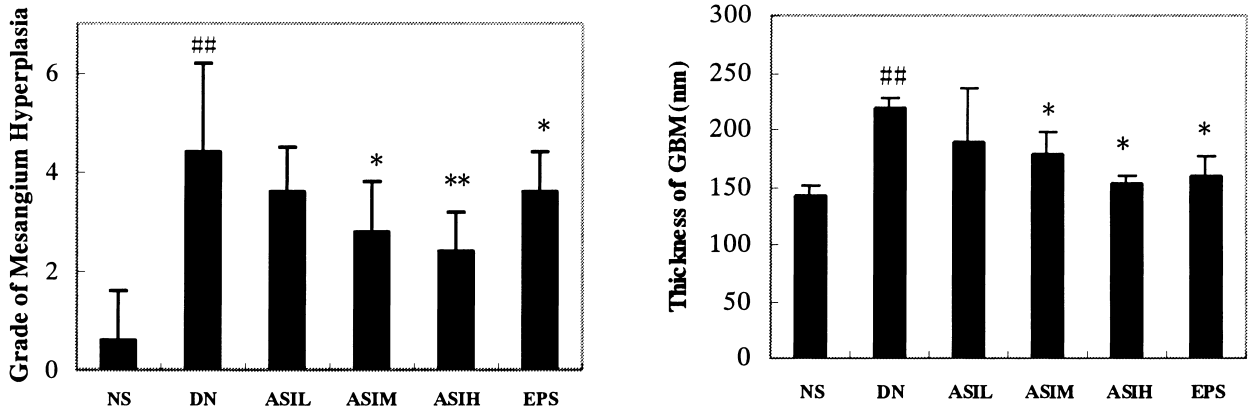

Fig. 7. Effect of AS I on mesangium hyperplasia and the thickness of glomerular base membrane (GBM) of rat. NS, DN, ASIL, ASIM, ASIH, and EPS represent normal standard rats, vehicle-treated DN rats, 3 mg/kg of AS I-treated DN rats, 6 mg/kg of AS I-treated DN rats, 12 mg/kg of AS I-treated DN rats, and 100 mg/kg of epalrestat-treated DN rats, respectively. Statistical analysis was performed using ANOVA. $^{\#\#}P<0.01$, compared with the NS group with *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, compared with the DN group with *t*-test. Data are presented as mean ± S.D.

and C-peptide level and reduced blood glucose level in rat serum (18, 27), indicating that AS I might have a protective effect on pancreatic β-cells. The mechanisms by which AS I decreases the blood glucose level in DN rats with the STZ-destroyed β-cell is still unclear, but this effect may be closely related to its antioxidant effect. Some well-known antioxidants have a β-cell-protective effect in diabetic animals. It is concluded that vitamin E exerts moderate beneficial effects on β-cell functions during short to intermediate length of high glucose exposure (37, 38). According to an intraperitoneal glucose tolerance test, the treatment with *N*-acetyl-L-cysteine (NAC, one kind of antioxidant) retained glucose-stimulated insulin secretion and moderately decreased blood glucose levels (39). Histologic analyses of the pancreases revealed that the β-cell mass was significantly larger in the diabetic mice treated with the antioxidants than in the untreated mice (39). As a possible cause, the antioxidant treatment suppressed apoptosis in β-cells without changing the rate of β-cell proliferation, supporting the hypothesis that in chronic hyperglycemia, apoptosis induced by oxidative stress causes reduction of β-cell mass. The antioxidant treatment also preserved the amounts of insulin content and insulin mRNA, making the extent of insulin degranulation less evident. Furthermore, expression of pancreatic and duodenal homeobox factor-1 (PDX-1), a β-cell-specific transcription factor, was more clearly visible in the nuclei of islet cells after the antioxidant treatment (39). Therefore, antioxidant treatment with AS I can exert beneficial effects in DN, with preservation of in vivo β-cell function. This mechanism of normalizing hyperglycemia of AS I suggests a potential usefulness of antioxidants for treating DN and provides further support for the implication of oxidative stress in β-cell dysfunction in diabetes.

It is well known that the accumulation of polyols in the kidney is involved in the development of DN (40). In diabetic patients, aldose reductase was greatly activated by high glucose and so sorbitol accumulated, which is associated with the depletion of myoinositol (41). The importance of aldose reductase to DN has been emphasized since the increase of aldose reductase activity is associated with enhanced protein kinase C activation and TGF-β1 production in human mesangial cells in response to glucose. The glucose-induced increase in TGF-β1 was prevented by concomitant incubation with epalrestat, an aldose reductase inhibitor, in a dose-dependent manner at a concentration of more than 10^{-6} mol/L in human mesangial cells (4). In our current experiment, we found that AS I significantly reduced aldose reductase activity from 27.29 to 24.91 U (in dose of 3 mg/kg), 17.18 U (in dose of 6 mg/kg), and 9.88 U (in dose of 12 mg/kg); and furthermore, there was a good relationship between this inhibitive effect on aldose redutase activity and AS I dose. This result strongly suggests that AS I is an aldose reductase inhibitor and may justify its clinical application for treatment of DN.

TGF-β1 is considered the pivotal cytokine mediating the progression of DN. In vitro studies have shown that a range of stimuli increase TGF-β1 expression, that is, hyperglycaemia, AGEs, and various products of oxidative stress (42 – 45). Indeed, TGF-β1 seems to be an important factor in the interaction between hemodynamic and metabolic pathways, playing a key role in

the synergy between hypertension and hyperglycaemia in DN. Our data shows that AS I has an inhibitive effect on TGF-β1 mRNA in kidney cortex of DN rats; this is consistent with the previous report about the effect of *Astragalus membranaceus* on TGF-β1 (46). Therefore, from this aspect, AS I can be a prime candidate for prevention and treatment of DN.

Collagen IV and thickness of GBM are usually enlarged during the progression of DN and served as persuasive parameters describing glomerular hypertrophy. Although decrease in the level of extracellular matrix (collagen IV) for 3 doses of AS I has not defined significantly by radioimmunoassay in our experiment, other significantly decreased morphological parameters (the relative grade of mesangium hyperplasia and thickness of GBM) in our study, together with the kidney index, still suggest that AS I may have an effect on preventing renal hypertrophy. These apparent and morphological parameters are strongly consistent with the description on *Astragalus membranaceus* function in traditional Chinese medicine.

In the complicated mechanisms of DN development, the pathway of hyperglcaemia-oxidative stress-AGEs /TGF-β1 is assuredly very important. All of the renal changes in our experiment are probably consequences of changes in blood glucose levels, and the ameliorative effects of AS I on DN are directly or tightly dependent on its effect on the blood glucose level. In conclusion, our data have demonstrated that AS I reduces blood glucose level, intensity of oxidative stress, AGEs level, aldose reductase activity, and level of TGF-β1 mRNA in DN rats, which are important factors relating to the progression of DN. Therefore, taking together these results, AS I has therapeutic effects on several pharmacological targets in the complicated pathological mechanism of DN, and thus, it is worthwhile to be further investigated for its potential pharmacological effects on early DN.

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