

Anhuienoside C Attenuates Podocyte Injury in Diabetic Nephropathy Rats

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Abstract

Objective: The present study evaluated the nephroprotective effects of anhuienoside C (AC) against diabetic nephropathy (DN) in rats.

Material and Methods: Diabetic nephropathy was induced by administration of a high-fat diet (HFD) for 8 weeks and intraperitoneal administration of streptozotocin (STZ; 30 mg/kg) at the end of the fourth week of this protocol. Effects of AC on blood glucose levels, renal function markers, and mediators of inflammation in the serum of DN rats were assessed.

Results: Anhuienoside C treatment reduced the blood glucose levels and attenuated the increased levels of renal injury markers in DN rats. Anhuienoside C also increased podocyte counts; alleviated the changes in podocin, desmin, and nephrin protein levels; and ameliorated the altered pathophysiology in the kidney tissues induced by DN. Compared with the DN group, the levels of inflammatory markers and mediators of oxidative stress were reduced in the serum and kidney tissues of the AC-treated groups. Moreover, treatment with AC ameliorates the altered expression of podocin, nephrin, and desmin proteins in the renal tissue of HFD/STZ-induced kidney-injured rats.

Conclusion: In conclusion, AC protected against podocyte injury by regulating nuclear factor kappa-light-chain-enhancer of activated B cells/protein kinase B pathway in a rat model of DN.

Keywords

anhuienoside C, diabetic nephropathy, high-fat diet, streptozotocin, podocyte

Introduction

Diabetic kidney disease is a chronic complication associated with diabetes, and its prevalence is rising worldwide.¹ A variety of changes occur during the histopathology of diabetic nephropathy (DN) including mesangial expansion, glomerular hypertrophy, and increased thickness of the glomerular basement.² Podocytes, the glomerular basement membrane, and the fenestrated endothelium play important roles in filtration at the kidney barrier.³ For example, podocytes are responsible for the filtration of proteins, and thus, injury to these cells and the glomeruli can lead to proteinuria.⁴ In cases of DN, the podocyte count decreases, which in turn enhances proteinuria,⁵ and the clinical loss of podocytes leads to glomerulosclerosis as well as the progression of kidney injury. It has been shown that podocyte integrity is maintained by regulation of desmin, podocin, and nephrin protein levels⁶ and that apoptosis in podocytes and nephrons is regulated by reduced interactions between nephrin and nephrin-p85 due to decreases in protein kinase B (Akt)

activity.⁷ Several molecules can reduce apoptosis in podocytes by regulating Akt activity⁸ and have been used as conventional therapies to manage DN via the control of dyslipidemia, blood glucose levels, and hypertension. However, these therapies cannot control the progression of DN, and thus, alternative treatment options for the management of DN are needed.

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There are several herbs, and the molecule source from natural origin shows promising effect in the treatment of several chronic disorders including nephropathy. Literature suggest that herbs that have strong antioxidant and anti-inflammatory properties produce beneficial effect in the management of diseases.⁹⁻¹⁴ Cellular apoptosis process starts due to increase in the oxidative stress and mediators of inflammation which activates the death receptors.¹⁵ Report suggests that, due to alteration in the metabolism, diabetes leads to development of ischemia and increases the level of inflammation and oxidative stress, which causes renal injury.¹⁶ Oxidative stress and inflammation are majorly involved in the pathogenesis of diseases, and molecules sourced from natural origin have potential to ameliorate both of these factors.¹⁷ Anhuinoside C (AC) is chemically a triterpenoid saponin isolated from *Anemone flaccida* Fr. Schmidt (Ranunculaceae).¹⁸ *Anemone flaccida* (Di Wu) is traditionally used as medicine for the treatment of rheumatoid arthritis and inflammation in China and it majorly contains saponin is AC. Anhuinoside C reported to show beneficial effect for the treatment of fracture and rheumatoid arthritis.¹⁹ Moreover, in lipopolysaccharide-stimulated macrophages, AC reduces the production of nitric oxide.^{18,20} Thus, the present study determines the protective effect of AC against renal failure.

Materials and Methods

Animals

Male Sprague Dawley rats (180-200 g) were maintained under a 12-hour light/dark cycle and standard conditions at a temperature of $24\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$ and relative humidity of $60\% \pm 5\%$, and guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International were used for the experimentation and animal use.²¹ All study protocols were approved by the Institutional Animal Ethical Committee of Chongqing General Hospital, University of Chinese Academy of Science (IACE/CGH/UC-AS/2018/02).

Chemicals

Anhuinoside C was provided by Guangdong Province Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, China. The kits used to estimate biochemical parameters and the enzyme-linked immunosorbent assay (ELISA) kits used to assess the mediators of inflammation and oxidative stress were purchased from Runyu Biotechnology Co. The antibodies used in the Western blot analyses were obtained from Santa Cruz Biotechnology.

Experimental Procedures

All the 40 healthy experimental animals received a high-fat diet (HFD) with a total calorie count of 40 kJ/kg (20% fat, 22% protein, and 45% carbohydrate) for 8 weeks, while the control animals received a diet with a total calorie count of 20 kJ/kg (5% fat, 20% protein, and 52% carbohydrate).²² At the end of week 4 of the 8-week period, a single dose of streptozotocin (STZ;

30 mg/kg)²³ was administered intraperitoneally to the rats. Blood glucose levels were measured in all animals 72 hours after the administration of STZ, and animals with a glucose level $>200\text{ mg/dL}$ were considered diabetic. Subsequently, the animals were divided into 4 groups, each group containing 10 animals: the control group, DN group, AC 20 mg/kg group (oral administration of 20 mg/kg dose of AC for 8-12 weeks), and the AC 40 mg/kg group (oral administration of 40 mg/kg AC for 8-12 weeks).

Determination of Renal Function Parameters

All animals were anesthetized at the end of the protocol, and blood was withdrawn from each subject. Serum was isolated by centrifuging the blood at 2000 rpm for 10 minutes. Kits were used to estimate the levels of triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, creatinine, and blood urea nitrogen (BUN) in the serum as well as microalbuminuria in the urine.

Determination of Inflammatory Mediators

The ELISA kits were used to determine the concentrations of inflammatory cytokines, including interleukin (IL) 6, IL-10, transforming growth factor beta 1 (TGF- β 1), and tumor necrosis factor α , in the serum of DN rats, according to the manufacturer's instructions.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

To determine apoptosis levels, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were conducted as described previously. Briefly, kidney tissues were sectioned at a thickness of 4 μm using a microtome, and the TUNEL assays were performed according to the manufacturer's instructions. Image Pro Plus 6.3 software was used to observe and count TUNEL-positive cells and total cells.

Determination of Histopathological Changes

Isolated kidneys were fixed in 10% formalin for 1 day at room temperature, and a standard protocol was performed to prepare the histological slides. Briefly, the kidney tissues were dehydrated with ethanol and then seeded into liquid paraffin. Next, a wax cube of the kidney sample was prepared, and 4- μm -thick slices of renal tissue were sectioned using a microtome. The tissue sections were then subjected to the Jones' periodic acid-Schiff staining procedures, and alterations of the histopathological changes in the kidney tissues were evaluated using Olympus BX50 bright field microscope (Leica Microsystems).

Determination of Oxidative Stress

Malondialdehyde (MDA) and glutathione (GSH) levels and catalase (CAT) and superoxide dismutase (SOD) activities were estimated in kidney tissues using ELISA kits according to the manufacturer's instructions.

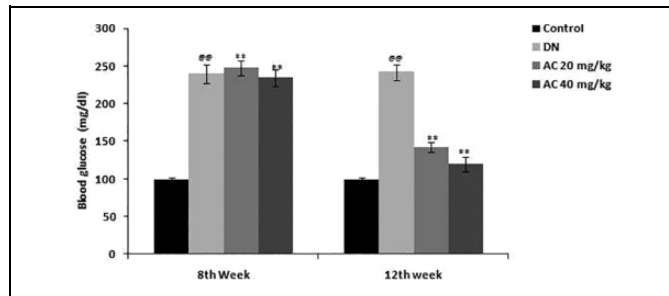


Figure 1. Anhuienoside C attenuates serum glucose concentrations in HFD/STZ-induced DN rats at weeks 8 and 12 of the protocol. Mean \pm SEM (n = 10). $@@P < .01$ compared with the control group. $**P < .01$ compared with the DN group. AC indicates anhuienoside C; DN, diabetic nephropathy; HFD, high-fat diet; SEM, standard error of the mean; STZ, streptozotocin.

Western Blot Assays

Total protein extraction from the kidney tissues was accomplished by lysing the tissues with a solution of 150 mM NaCl, 50 mM Tris-HCl, NP40 protein lysis buffer, and 5 mM EDTA (pH 8.0), supplemented with a protease inhibitor cocktail. The protein lysates were centrifuged for 10 minutes at 13 400 rpm, and the supernatants were collected for further examination. The DC Protein Assay was performed to estimate the total protein concentration. The isolated proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes, and the membranes were blocked in 5% fresh non-fat dry milk.

Next, the membranes were incubated overnight at 4 °C with primary antibodies (Santa Cruz Biotechnology) against podocin (1:100), nephrin (1:200), desmin (1:200), p-PI3K (1:100), PI3K (1:100), p-Akt (1:200), Akt (1:200), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B; 1:100), p-NF- κ B (1:100), and β -actin (1:100). Subsequently, the membranes were incubated in secondary antibodies at room temperature for 60 minutes. The blots were visualized by chemiluminescence, and densitometric analysis of the protein bands was performed using ImageLab software.

Statistical Analysis

All data are expressed as the mean \pm standard error of the mean (n = 10), and all statistical analyses were performed using GraphPad Prism 5.0 software. The groups were compared using Dunnett post hoc test, and a *P* value $< .05$ was considered to indicate statistical significance.

Results

Anhuienoside C Ameliorates the Blood Glucose of HFD/STZ-Induced Kidney-Injured Rats

Figure 1 shows the effects of AC on the blood glucose concentration in HFD/STZ-induced DN rats at weeks 8 and 12 of the protocol. Compared with the control group, the administration

of HFD/STZ enhanced blood glucose levels by 2.5-fold in all experimental groups after week 8 of the protocol. Furthermore, the blood glucose levels were higher in the DN group than the control group at week 12 of the protocol but lower in the AC 20 mg/kg-treated groups than the DN group.

Anhuienoside C Ameliorates the Alterations in Renal Function Markers of HFD/STZ-Induced Kidney-Injured Rats

Anhuienoside C treatment ameliorated the DN-induced changes in serum and urine markers of renal function in HFD/STZ-treated rats (Figure 2); these alterations confirmed the occurrence of renal injury. Compared with the control group, the DN group had significantly higher serum levels of creatinine (Scr) and BUN (*P* $< .01$) and urine level of microalbuminuria. However, compared with the DN group, the AC-treated group exhibited significant decreases in the serum levels of Scr and BUN and in the urine level of microalbuminuria.

Effects of AC on Lipid Profiles

Diabetes leads to alterations in serum lipid profiles, which contributes to the development of DN. The effects of AC on the lipid profiles of HFD/STZ-induced DN rats are shown in Figure 3. The levels of total cholesterol, triglycerides, and LDL cholesterol were significantly (*P* $< .01$) higher, whereas the HDL cholesterol level was lower in the DN group than in the control group. However, AC treatment attenuated the DN-induced changes in total, LDL, and HDL cholesterol and triglyceride levels in the serum of HFD/STZ-treated rats.

Effects of AC on Podocyte Counts

Podocyte counts in the kidney tissues of rats treated with AC and HFD/STZ were estimated by TUNEL staining (Figure 4). Compared with the control group, the number of TUNEL-positive cells increased by up to 8-fold in the kidney tissues of the DN group. However, compared with the DN group, there was a significant reduction in the number of TUNEL-positive cells (ie, podocyte injury) in the kidney tissues of the AC-treated groups.

Anhuienoside C Ameliorates the Histopathological Changes in the Renal Tissue of HFD/STZ-Induced Kidney-Injured Rats

Histopathological changes in the kidney tissues were measured in AC- and HFD/STZ-treated rats (Figure 5A and B). The kidney tissues of the control group exhibited a normal mesangial matrix and a thin glomerular basement membrane, whereas the kidney tissues of the DN group exhibited an increased mesangial matrix and thicker glomerular basement membrane. However, AC treatment reversed these histopathological changes in the kidney tissues of HFD/STZ-induced DN rats (Figure 5A). Additionally, the percentage of the glomerular surface area in the kidney tissues was increased in the DN group compared with the control

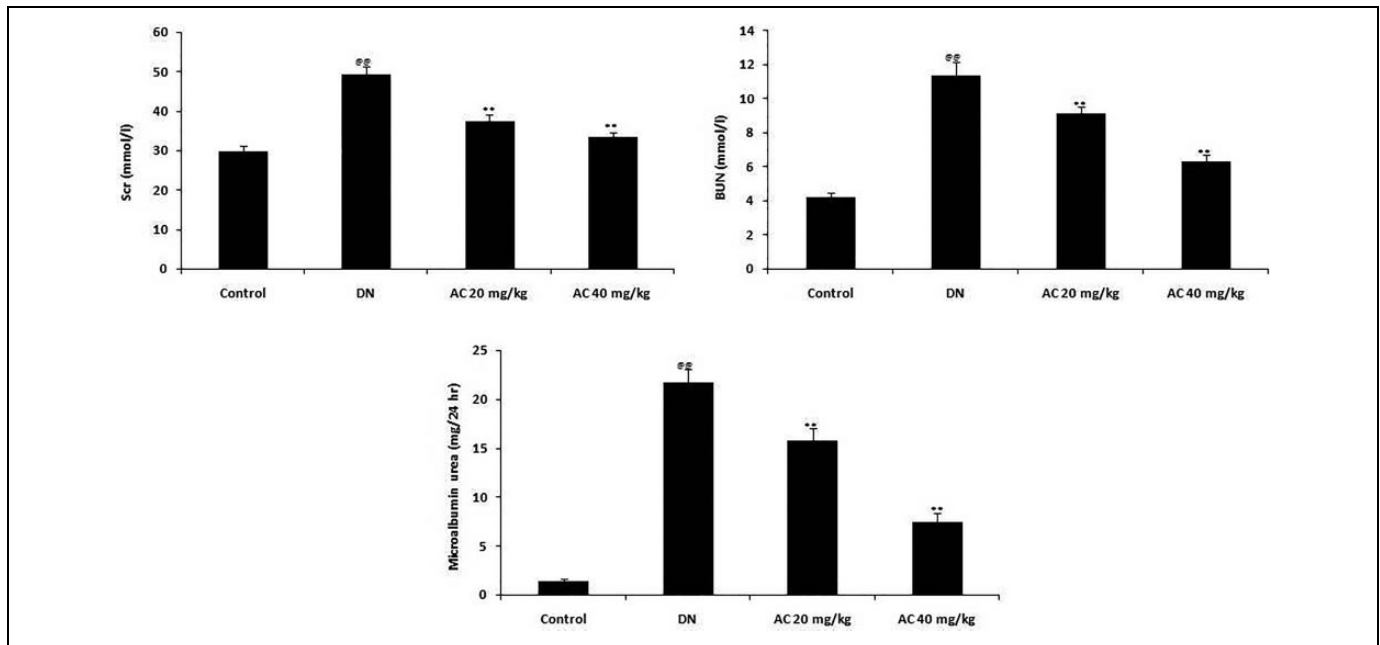


Figure 2. Anhuiesoside C attenuates the levels of renal function markers in the serum and urine of HFD/STZ-induced DN rats. Mean \pm SEM (n = 10). @@P < .01 compared with the control group. **P < .01 compared with the DN group. AC indicates anhuiesoside C; DN, diabetic nephropathy; HFD, high-fat diet; SEM, standard error of the mean; STZ, streptozotocin.

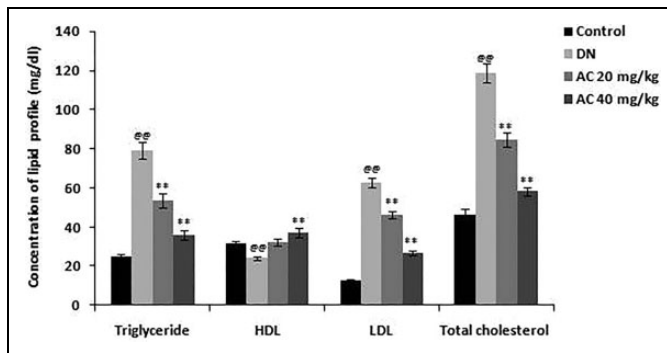


Figure 3. Anhuiesoside C attenuates the serum lipid profiles in HFD/STZ-induced DN rats. Mean \pm SEM (n = 10). @@P < .01 compared with the control group. **P < .01 compared with the DN group. AC indicates anhuiesoside C; DN, diabetic nephropathy; HFD, high-fat diet; SEM, standard error of the mean; STZ, streptozotocin.

group but was significantly reduced ($P < .01$) by AC treatment compared with the DN group (Figure 5B).

Anhuiesoside C Ameliorates the Oxidative Stress Markers in the Renal Tissue of HFD/STZ-Induced Kidney-Injured Rats

Markers of oxidative stress were assessed in the kidney tissues of rats treated with AC and HFD/STZ using ELISA kits. Compared with the control group, the MDA level was increased and the GSH level decreased in the kidney tissues of the DN group. Moreover, compared with the control group, there were significant reductions in SOD and CAT activities in the kidney

tissues of the DN group. However, AC treatment attenuated the DN-induced altered levels of the oxidative stress markers in the kidney tissues of the rats (Table 1).

Anhuiesoside C Ameliorates the Biochemical Markers in the Renal Tissue of HFD/STZ-Induced Kidney-Injured Rats

Markers of inflammation such as IL-6, IL-10, TNF- α , and TGF-1 β were assessed in the kidney tissues of rats treated with AC and HFD/STZ using ELISA kits (Figure 6). Compared with the control group, levels of inflammatory mediators were increased in the kidney tissues of the DN group. However, AC treatment attenuated the DN-induced altered levels of mediators of inflammation in the kidney tissues of the rats.

Anhuiesoside C Ameliorates the Podocin, Nephrin, and Desmin Expression in the Renal Tissue of HFD/STZ-Induced Kidney-Injured Rats

The protein levels of podocin, nephrin, and desmin were determined in the kidney tissues of AC- and HFD/STZ-treated rats (Figure 7) because these proteins regulate the function of podocytes. Compared with the control group, the protein levels of podocin and nephrin were decreased, whereas that of desmin were increased, in the kidney tissues of the DN group. However, AC treatment ameliorated the altered protein levels of podocin, nephrin, and desmin in the kidney tissues of HFD/STZ-induced DN rats.

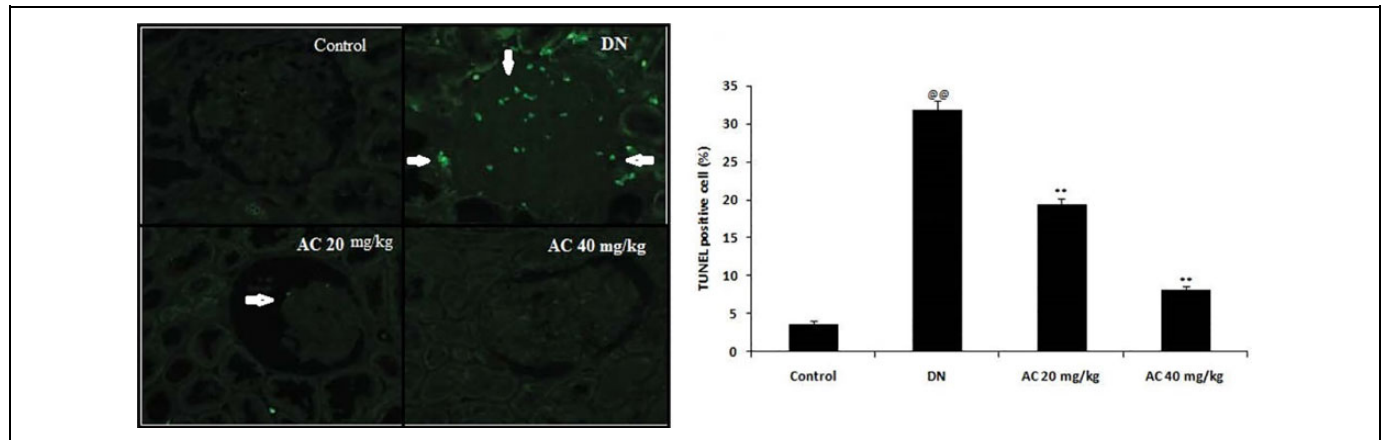


Figure 4. Anhuiesinide C attenuates podocyte counts in the kidney tissues of HFD/STZ-induced DN rats. Mean \pm SEM (n = 10). @@ $P < .01$ compared with the control group. ** $P < .01$ compared with the DN group. AC indicates anhuiesinide C; DN, diabetic nephropathy; HFD, high-fat diet; SEM, standard error of the mean; STZ, streptozotocin.

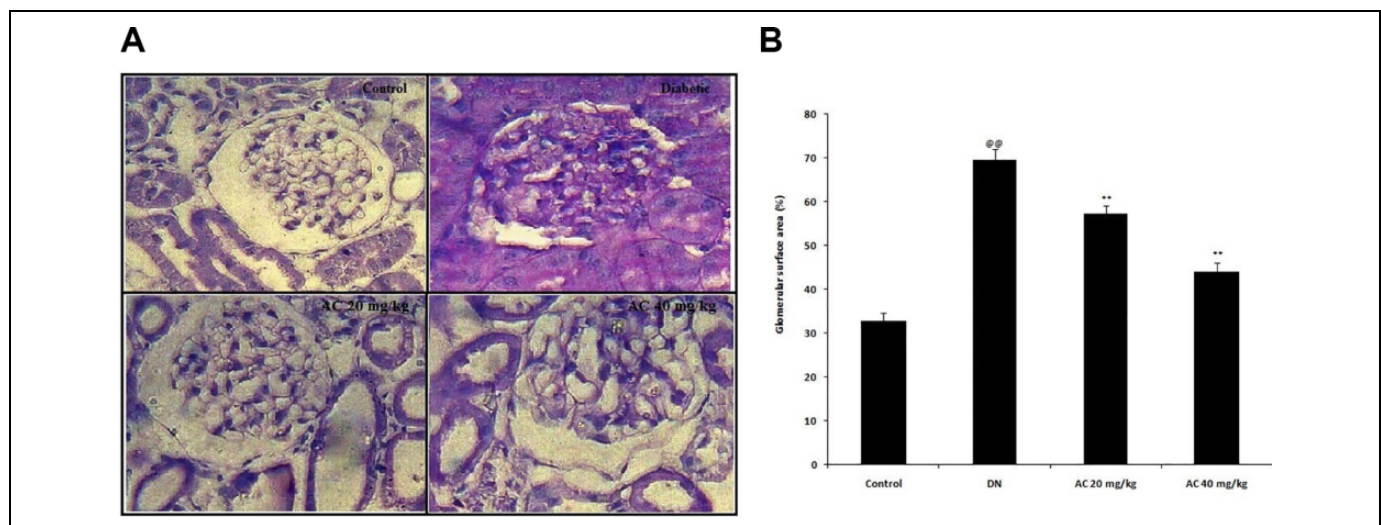


Figure 5. Anhuiesinide C attenuates the histopathological changes in the kidney tissues of HFD/STZ-induced DN rats. A, TS of kidney tissues by PAS staining. B, Percentage of glomerular surface area. Mean \pm SEM (n = 10). @@ $P < .01$ compared with the control group. ** $P < .01$ compared with the DN group. AC indicates anhuiesinide C; DN, diabetic nephropathy; HFD, high-fat diet; PAS, periodic acid–Schiff; SEM, standard error of the mean; STZ, streptozotocin; TS, transverse section.

Table 1. Effects of AC on Markers of Oxidative Stress in the Kidney Tissues of HFD/STZ-Induced DN Rats.^a

S. no.	Group	MDA (nmol/mg protein)	GSH (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
1	Control	1.26 \pm 0.09	10.32 \pm 0.59	3.26 \pm 0.21	0.94 \pm 0.08
2	DN	8.61 \pm 0.26	2.92 \pm 0.14 ^b	1.09 \pm 0.04 ^b	0.21 \pm 0.01 ^b
3	AC 20 mg/kg	5.16 \pm 0.17 ^c	7.69 \pm 0.36 ^c	1.69 \pm 0.13 ^c	0.52 \pm 0.04 ^c
4	AC 40 mg/kg	3.42 \pm 0.12 ^c	4.16 \pm 0.24 ^c	2.54 \pm 0.16 ^c	0.73 \pm 0.06 ^c

^aMean \pm standard error of the mean (n = 10).

^b $P < .01$ compared with the control group.

^c $P < .01$ compared with the DN group.

Anhuienoside C Ameliorates the PI3K/Akt/NF- κ B Signaling Pathway in the Renal Tissue of HFD/STZ-Induced Kidney-Injured Rats

The effects of AC on the protein levels of PI3K, Akt, and NF- κ B in the kidney tissues of HFD/STZ-induced DN rats are shown in Figure 8. Compared with the control group, the PI3K and Akt levels were decreased and the NF- κ B level increased in the kidney tissues of the DN group. Compared with the DN group, the AC-treated group exhibited dose-dependent increases in PI3K and Akt levels and a decrease in the NF- κ B level in kidney tissue homogenates.

Discussion

Diabetic nephropathy is a chronic complication associated with diabetes, and its management remains a challenge. Over the

past few decades, several alternative medicines have shown promise for the treatment of chronic disorders, including diabetes-associated renal failure. Thus, we evaluated the nephroprotective effects of AC in HFD/STZ-induced DN rats. Diabetes-associated renal failure, a chronic complication of diabetes, alters the levels of renal injury markers.^{24,25} Studies have shown that drugs used for the management of renal failure ameliorate renal function, and similarly, the present study demonstrated that AC treatment reduced blood glucose levels and attenuated the alterations in renal injury markers induced by DN. Podocytes play an important role in filtration because the interpodocyte slit membrane acts as a filtration barrier,²⁶ and the development of DN is caused by a decreased podocyte count.²⁷ Additionally, reduced podocyte counts in the kidney tissue increase the albumin level in urine, which causes further injury to nephrons.²⁸ The present study found that AC treatment enhanced the podocyte count and attenuated the altered pathophysiology of kidney tissues in DN rats.

Several factors are responsible for the normal functioning of podocytes, including the structure of the interpodocyte membrane. This membrane is maintained by nephrin, which also regulates normal glomerular function by controlling the release of proteins into the urine.²⁹ Additionally, podocin is an integral membrane protein from the stomatin family that regulates filtration by maintaining the integrity of the slit diaphragm.³⁰ The mechanical stability of these cells is maintained by upregulated expression of desmin, which results in podocyte injury.³¹ Several molecules regulate normal podocyte function by attenuating the protein levels of podocin, desmin, and nephrin.³² The present study demonstrated that AC treatment ameliorated the altered protein levels of podocin, desmin, and nephrin in kidney tissues induced by DN.

Inflammatory cytokines disrupt glomerular membrane function by altering the dynamics of the extracellular matrix.³³ Several factors contribute to the development of DN, including accumulation of TGF- β 1, which stimulates cellular apoptosis and alters glomerulosclerosis as a result of the decreased

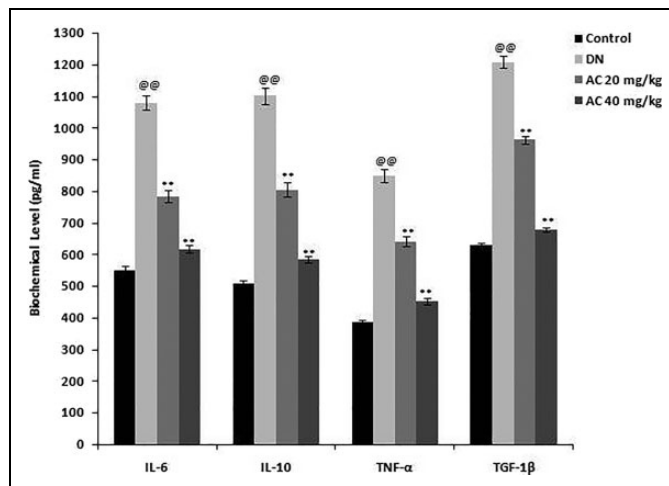


Figure 6. Anhuienoside C attenuates the altered level of mediators of inflammation in the serum of HFD/STZ-induced DN rats. Mean \pm SEM (n = 10). @@P < .01 compared with the control group. **P < .01 compared with the DN group. AC indicates anhuienoside C; DN, diabetic nephropathy; HFD, high-fat diet; SEM, standard error of the mean; STZ, streptozotocin.

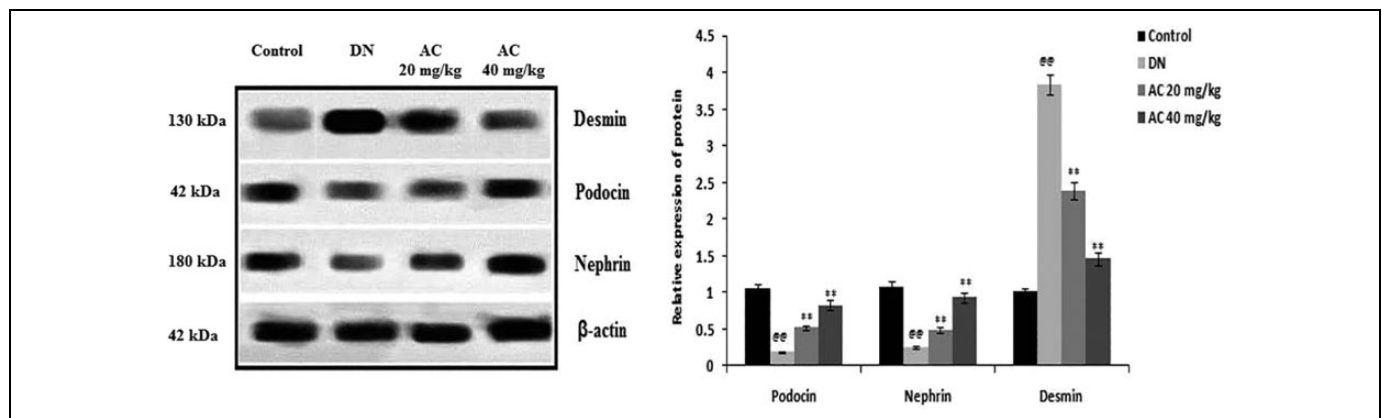


Figure 7. Anhuienoside C attenuates the protein levels of podocin, nephrin, and desmin in the kidney tissues of HFD/STZ-induced DN rats. Mean \pm SEM (n = 10). @@P < .01 compared with the control group. **P < .01 compared with the DN group. AC indicates anhuienoside C; DN, diabetic nephropathy; HFD, high-fat diet; SEM, standard error of the mean; STZ, streptozotocin.

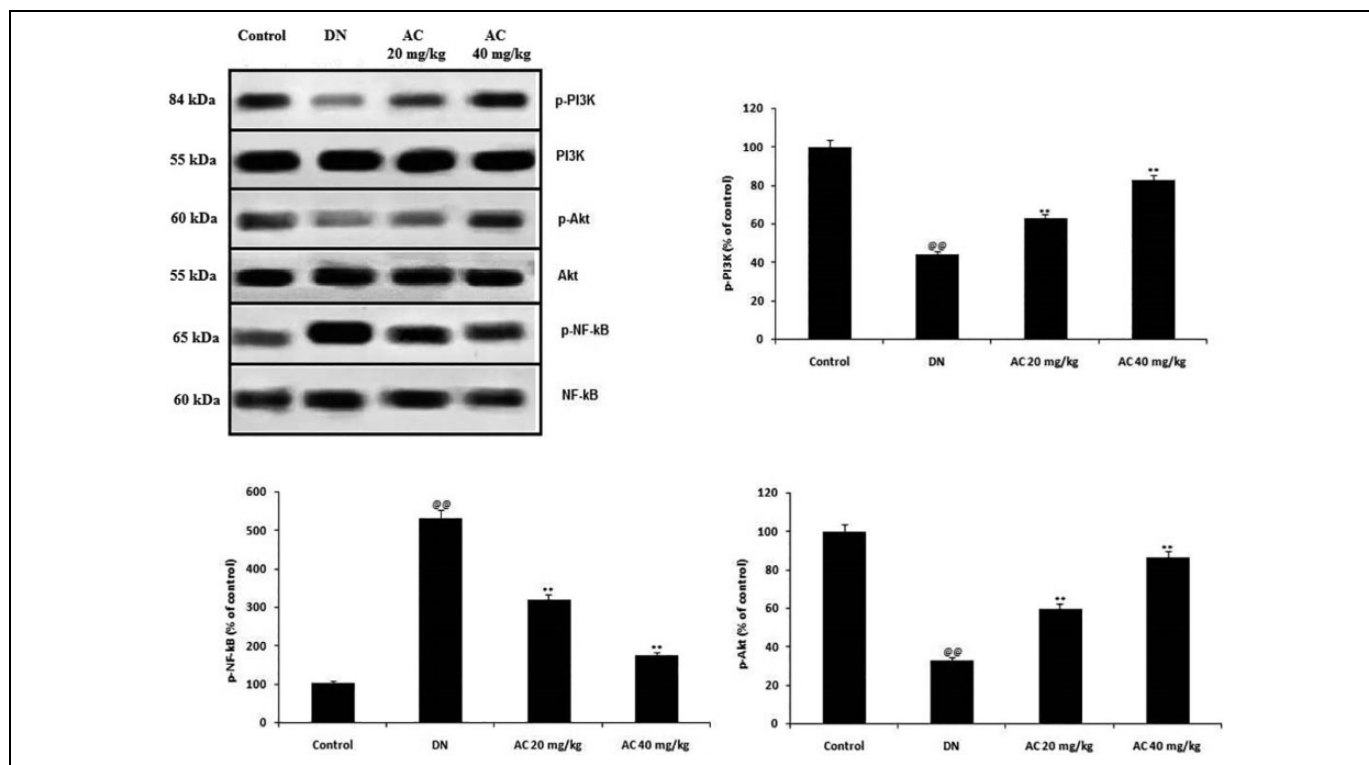


Figure 8. Anhuienoside C attenuates the protein levels of PI3K, Akt, and NF- κ B in the kidney tissues of HFD/STZ-induced DN rats. Mean \pm SEM ($n = 10$). @@ $P < .01$ compared with the control group. ** $P < .01$ compared with the DN group. AC indicates anhuienoside C; Akt, protein kinase B; DN, diabetic nephropathy; HFD, high-fat diet; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; SEM, standard error of the mean; STZ, streptozotocin.

number of podocytes.³⁴ The present study found that the levels of inflammatory mediators were lower in the AC-treated group than in the DN group. Moreover, AC treatment attenuated the DN-induced altered levels of oxidative stress markers in the kidney tissues of DN rats. The PI3K/Akt signaling pathway and NF- κ B also contribute to the regulation of podocyte function. For example, Akt kinase is activated by TGF- β in diabetic kidneys,³⁵ and the morphology and function of podocytes are maintained by nephrin, which involves PI3K. PI3K also controls the excretion of microalbuminuria in DN. The present findings showed that AC treatment ameliorated the DN-induced altered protein levels of PI3K, Akt, and NF- κ B in rat kidney tissues. In conclusion, the present data revealed that AC treatment protected against podocyte injury in HFD/STZ-induced DN rats by regulating the NF- κ B/Akt signaling pathway. Result of the investigation suggests that Ac could be used clinically for the management of DN.

Authors' Note

This study was approved by Chongqing General Hospital, China. C.W. and Y.J. designed the protocol of the study. Y.J. and K.Y. performed the experimental work and collected the data for the present study. K.L. involve in the statistical analysis and histopathology study. C.W. supervised the work and drafted the manuscript, although all the authors contribute to the preparation of manuscript. Final manuscript is approved by all the authors of the presented report.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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