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Cornuside Ameliorates Diabetic Nephropathy Possibly by Regulating Angiogenesis and MAPK Signaling

Fang Xiang,[1] Xin Li[2] and Wei Hu[3]

[1]Department of Nephrology, Songshan General Hospital, Chongqing, China
[2]Department of Urology, Songshan General Hospital, Chongqing, China
[3]Department of Nephrology, University-Town Hospital of Chongqing Medical University, Chongqing, China

Diabetic nephropathy (DN) is a prevalent diabetic complication seriously threatening patients' health and lives. Cornuside is an iridoid glycoside compound with various pharmacological properties. Nonetheless, whether cornuside has a protective effect against DN remains unillustrated. In this study, a rat model for DN was established by streptozotocin (STZ) injection. Rat podocytes were stimulated with high glucose (HG) to mimic a DN microenvironment *in vitro*. Several indicators linked to kidney function were evaluated. Periodic Acid-Schiff (PAS) and hematoxylin-eosin staining were implemented for renal histologic analysis. Immunofluorescence staining of CD31 was used for the detection of neovascularization. Western blotting was employed to assess levels of angiogenic factors and MAPK signaling-related proteins in the kidney or podocytes. The results showed that cornuside administration alleviated STZ-elicited renal dysfunction, as evidenced by the reduction in fasting blood glucose, proteinuria, serum creatinine, and blood urea nitrogen. Cornuside attenuated renal pathological lesions in DN rats. Cornuside repressed angiogenesis in DN rat kidney tissues and podocytes. Cornuside blocked MAPK signaling in HG-stimulated podocytes. In conclusion, cornuside alleviates renal injury in DN rats possibly by hindering angiogenesis and MAPK signaling.

Keywords: angiogenesis; cornuside; diabetic nephropathy; hyperglycemia; renal dysfunction
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Introduction

Diabetic nephropathy (DN) is a prevalent and severe complication of diabetes, typically manifested by proteinuria, edema, and renal dysfunction (Dai et al. 2022). It is considered a primary inducer of end-stage renal disease (ESRD), contributing to high morbidity and mortality in patients with diabetes (Samsu 2021). Multiple risk factors are associated with DN pathogenesis, including hyperglycemia, hypertension, genetic predisposition, aging, and inflammation (Tziomalos and Athyros 2015). Current treatments for DN mainly focus on adjusting the modifiable risk factors, such as glycemic control, lipid lowering with statins, and blood pressure control with renin-angiotensin system blockade (Xie et al. 2022). Nonetheless, these treatment methods exert limited renoprotective effects in preventing DN from progressing to ESRD (Esmat et al. 2022). Hence, there is a pressing need to explore new strategies to prevent DN progression.

Angiogenesis is a complicated physiological process referring to the formation of new blood vessels from pre-existing vasculature (Corvera et al. 2022). Glomerular angiogenesis has been indicated to contribute to the pathogenesis of DN (Guo et al. 2005). Angiogenesis is mediated by angiogenic factors, such as vascular endothelial growth factor (VEGF), its receptor FLK1, angiopoietins, and the receptor Tie-2 (Zacharek et al. 2007). VEGF is the most potent pro-angiogenic factor upregulated in DN patients and animal models and is predominantly expressed by podocytes in the glomerulus (Kim et al. 2005). Angiopoietin (Ang)-2 competitively binds to Tie-2 with Ang-1, and negatively regulates Ang-1/Tie-2 signaling during angiogenesis (Felcht et al. 2012). Elevation of Ang-2 level has been observed in animals with DN (Ichinose et al. 2005). Evidence suggests that multiple signaling pathways participate in mediating angiogenesis, including the mito-

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Correspondence: Wei Hu, University-Town Hospital of Chongqing Medical University, No. 55, University City Middle Road, Shapingba District, Chongqing 401331, China.
e-mail: huwei@hospital.cqmu.edu.cn

gen-activated protein kinase (MAPK) signaling pathway (Claesson-Welsh and Welsh 2013). Additionally, many studies have illustrated that inhibition of MAPK signaling ameliorates renal injury, oxidative stress, and inflammation in DN animal models (Malik et al. 2017; Ma et al. 2021).

Cornuside (7-O-Galloylsecologanol, Fig. 1A) is an iridoid glycoside isolated from the fruit of *Cornus officinalis* Sieb. et Zucc which has long been used as a traditional herbal medicine (Ryu et al. 2022). Evidence indicates that cornuside has various pharmacological properties, such as anti-inflammatory, neuroprotective, and anti-septic (Kim et al. 2022; Shi et al. 2022). Song et al. (2011) proposed that cornuside protects against the acute hepatic injury induced by carbon tetrachloride. Moreover, Kim et al. (2023) demonstrated that cornuside could ameliorate particulate matter-elicited lung damage. Importantly, it was indicated that cornuside exerts an ameliorative effect on diabetes-induced testicular injury (Liu et al. 2021). Nevertheless, it is unclarified whether cornuside also has a therapeutic effect on DN caused by diabetes. Additionally, a previous report suggested that cornuside plays an anti-allergic role in rats in part by blocking MAPK signaling (Li et al. 2016).

Herein, we intended to probe the functions of cornuside and its potential mechanisms in DN using a DN rat model and an *in vitro* podocyte model. It was speculated that cornuside might alleviate renal injury by regulating angiogenesis and MAPK signaling.

Materials and Methods

Rats

Male Sprague-Dawley (SD) rats (6-7 weeks, 180-220 g; Cavens, Changzhou, China) were housed in an SPF environment (12-h light/dark cycle, temperature 22 ± 1°C, humidity 55-60%) with free access to food and water. All animal experiments were performed following the NIH Guide for the Care and Use of Laboratory Animals. Approval for this study was obtained from the Ethics Committee of the University-Town Hospital of Chongqing Medical University.

Induction of diabetes

To induce DN, rats were injected intraperitoneally with 65 mg/kg streptozotocin (STZ; Solarbio, Beijing, China) in 0.1 M citrate buffer (pH 4.5). Two days later, rats with fasting blood glucose > 300 mg/dl were considered diabetic and were used subsequently as DN models.

Animal grouping

Twenty-four rats were randomly grouped as 1) control group, 2) control + cornuside group, 3) DN group, and 4) DN + cornuside group (n = 6 /group). Two weeks after modeling, rats in groups 2 and 4 were administrated with cornuside (100 mg/kg/day; purity > 99.9%, MedChemExpress, Shanghai, China) via oral gavage for 8 weeks, while those in groups 1 and 3 were administrated with an equal amount of 1% carboxymethyl cellulose. The dose of cornuside was determined based on previous reports (Liu et al. 2021). Blood glucose level was assessed every week from week 3. A schematic diagram of the experimental procedure is shown in Fig. 1B.

Sample collection

After the last drug administration, rats were placed in metabolic cages to collect 24 h urine samples. Then, rats were anesthetized by injection of pentobarbital sodium (60 mg/kg). Blood samples were collected from the abdominal aorta and centrifuged at 3,000 rpm to obtain the serum. The rats were then euthanized, and the kidneys were collected. The left kidney was fixed in 10% neutral formalin for histological analysis and the right kidney was stored at 80°C for

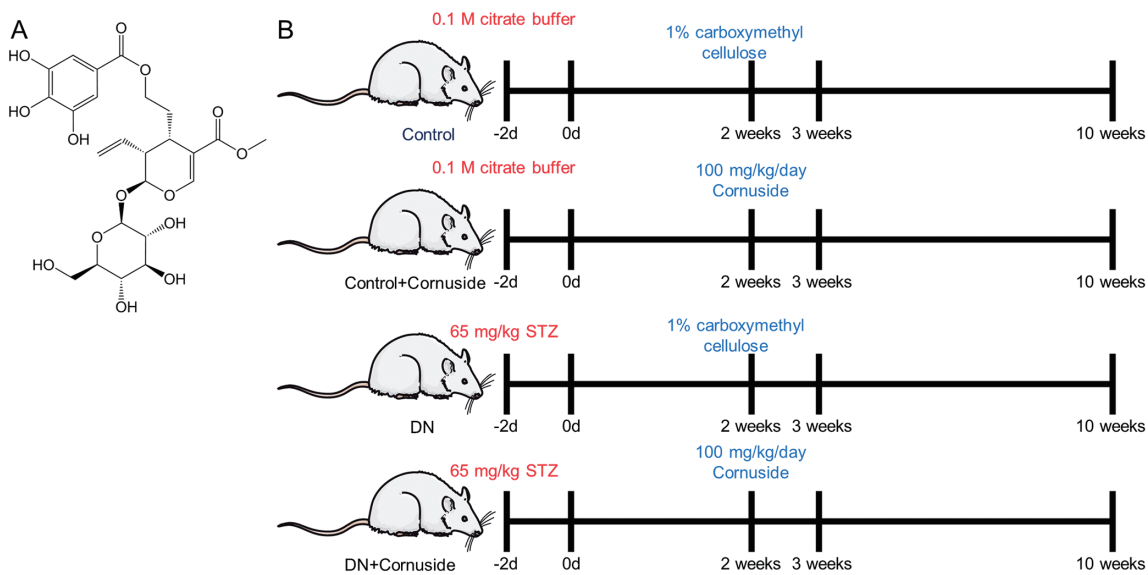

Fig. 1. Experimental design of this study.
A. Chemical structure of cornuside. B. The schematic diagram showing the experimental procedure of the study.

further use.

Measurement of proteinuria, serum creatinine, and blood urea nitrogen (BUN)

The levels of 24 h proteinuria (C035-2-1), serum creatinine (C011-2-1), and BUN (C013-1-1) were estimated using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as per the manufacturer's protocols.

Histologic analysis

Kidney tissues were embedded in paraffin and cut into sections (4 μm). After deparaffination and rehydration, the renal tissues were stained with hematoxylin-eosin (H&E; G1120, Solarbio) and Periodic Acid Schiff (PAS; G1281, Solarbio) following the instructions from the manufacturer. A microscope (Leica Microsystems, Shanghai, China) was employed for the observation of the pathological changes in the kidneys. Semi-quantitative evaluation of glomerular diameter and mesangial matrix index was conducted using ImageJ software. Four non-overlapping sections were taken from each renal sample, and five high-power visual fields were randomly chosen from each section. The percentage of PAS-positive area in the glomerulus was denoted as the mesangial matrix index.

Immunofluorescence staining

The paraffin-embedded sections were dewaxed and rehydrated, followed by heat-mediated antigen retrieval in Tris/EDTA buffer (pH 9.0). To block endogenous peroxidase activity, the sections were treated with 10% normal goat serum and 1% bovine serum albumin in Tris-buffered saline for 2 h. Then, the sections were incubated with an anti-CD31 primary antibody (ab222783, Abcam) overnight at 4°C, and then incubated with the secondary goat anti-rabbit antibody (ab150077, Abcam) for 2 h at room temperature. The sections were counterstained with DAPI for nuclear labeling. Lastly, the images were captured with a fluorescence microscope (Leica Microsystems). Twenty glomeruli per renal section were viewed and analyzed using ImageJ software to quantify the CD31-positive glomerular area.

Cell culture

The rat podocytes were purchased from BeNa Culture Collection (Beijing, China) and incubated in DMEM (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco) in a humidified environment (5% CO_2, 37°C). To induce a hyperglycemic condition, podocytes were serum-starved for 24 h and treated with 30 mM high glucose (HG) or 5.6 mM normal glucose (NG) with/without 10 μM cornuside for 24 h. In some experiments, podocytes were treated with NG plus recombinant human VEGF (10 ng/ml; ab259412, Abcam) or with HG plus an anti-VEGF antibody (10 μg/ml; ab214424, Abcam).

ELISA

VEGF concentration in kidney tissues and podocytes was determined using a rat VEGF ELISA kit (ab100786, Abcam) following the manufacturer's instructions.

Western blotting

Proteins from kidneys and podocytes were extracted using RIPA buffer (Solarbio) and estimated using a bicinchoninic acid assay kit (Beyotime). Protein samples (20 μg) were separated by 10% SDS-PAGE, blotted onto polyvinylidene fluoride membranes (Beyotime), and blocked with 5% defatted milk. The membranes were then incubated with primary antibodies (listed in Table 1) overnight at 4°C and washed thrice before incubating at room temperature with the secondary antibody (ab7090, Abcam) for 2 h. Lastly, protein bands were visualized using an ECL kit (Solarbio) and detected using an automatic chemiluminescence image analysis system (Bio-Best; Siemon, Los Angeles, CA). The band intensity was analyzed using ImageJ software.

Table 1. Primary antibodies used in western blotting.

Target	Host species/Clonality	Cat. No	Concentration
VEGF	Rabbit monoclonal	ab214424*	1:1000
FLK1	Rabbit monoclonal	ab221679*	1:1000
Ang-2	Rabbit monoclonal	ab155106*	1:1000
Tie-2	Rabbit polyclonal	19157-1-AP#	1:500
p-ERK	Rabbit monoclonal	ab201015*	1:1000
ERK	Rabbit monoclonal	ab184699*	1:10000
p-JNK	Rabbit monoclonal	ab76572*	1:5000
JNK	Rabbit monoclonal	ab179461*	1:1000
p-p38	Rabbit polyclonal	ab4822*	1:1000
p38	Rabbit monoclonal	ab170099*	1:1000
GAPDH	Rabbit monoclonal	ab181602*	1:10000

* All from Abcam, Shanghai, China. # From Proteintech, Wuhan, China.

Statistical analysis

Data were presented as the mean ± standard deviation. Differences in different groups were evaluated by one-way ANOVA followed by Tukey's *post hoc* analysis using GraphPad Prism 8.0.2 (GraphPad, San Diego, CA). For blood glucose level evaluation, two-way ANOVA was used. $p < 0.05$ indicated statistical significance.

Results

Cornuside improves renal function in DN rats

To evaluate the protection role of cornuside in DN, we detected the indexes related to renal function. As displayed by the results, relative to those in the control rats, fasting blood glucose levels in DN rats were prominently higher (Fig. 2A). A significant reduction in body weight was observed in DN rats relative to the control ones (Fig. 2B). Moreover, STZ injection caused a marked increase in kidney weight (Fig. 2C) and proteinuria excretion (Fig. 2D) in rats, confirming that STZ triggered renal injury. Additionally, DN rats exhibited significantly higher levels of serum creatinine and BUN than the control rats (Fig. 2E,F). Nonetheless, the above effects in DN rats caused by STZ were prominently attenuated after cornuside administration (Fig. 2A-F), indicating that cornuside could improve renal function in rats with DN.

Cornuside alleviates renal lesions in DN rats

H&E and PAS staining were performed for histological analysis of the kidney tissues in each group. As depicted in Fig. 3A,B, there were no evident pathological changes in the kidney samples of the control group. In comparison to the control group, the DN group exhibited marked glomerular hypertrophy, tubular injury, mesangial matrix expansion, and arteriolar hyalinosis. These pathological changes in the kidney tissues were prominently improved in cornuside-administrated DN rats. In addition, semi-quantitation of glomerular diameter and matrix index further confirmed that cornuside alleviated STZ-evoked renal lesions in the DN rat model (Fig. 3C,D).

Cornuside downregulates angiogenesis factors in DN rats

To assess the effect of cornuside on angiogenesis, we detected the expression of several angiogenic factors. Both ELISA and western blotting showed that STZ injection induced upregulation of VEGF, a proangiogenic factor in the kidney tissues of rats, while cornuside administration decreased VEGF expression in STZ-induced DN rats (Fig. 4A-C). In parallel, the results depicted that cornuside abated STZ-evoked upregulation of FLK1, Ang-2, and downregulation of Tie-2 protein expression in rat kidneys (Fig. 4B,D-F). The endothelial cell marker CD31 is widely used as an indicator of neovascularization (Niinimäk et al.

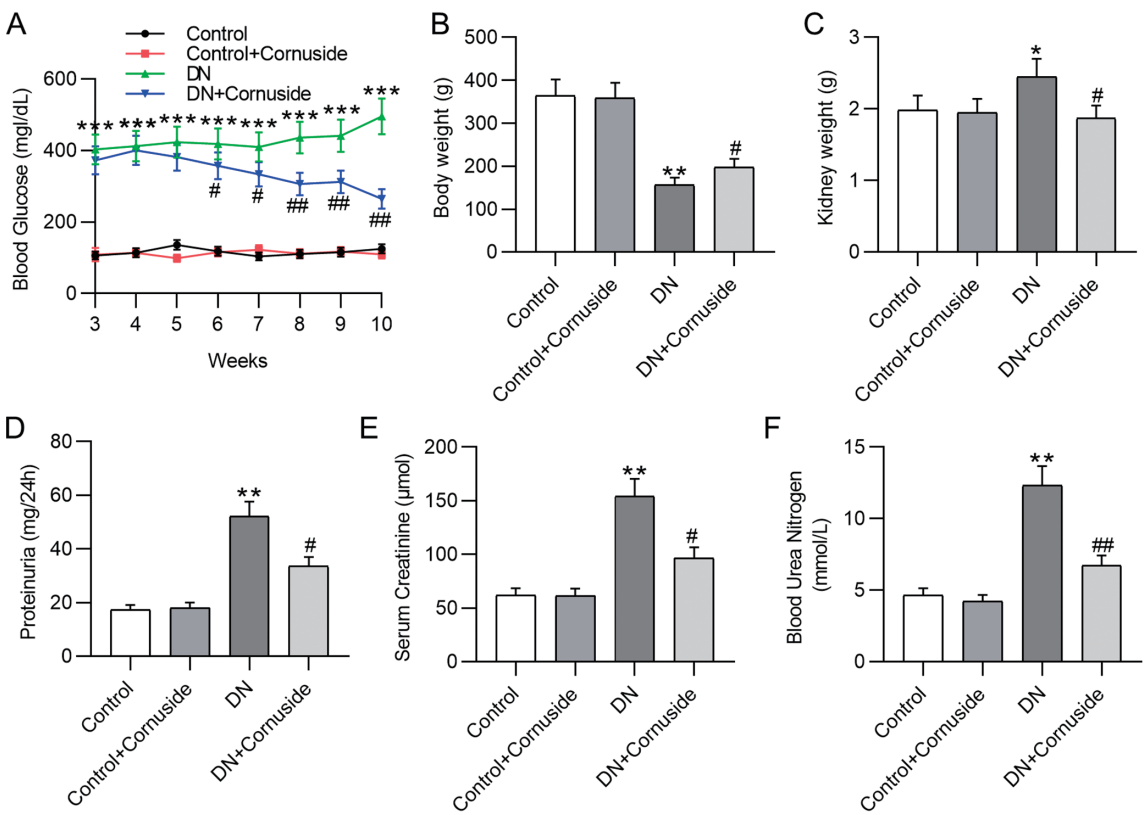

Fig. 2. Cornuside improves renal function in DN rats.
(A) Fasting blood glucose levels from weeks 3 to 10. B-C. Rat body weight (B) and kidney weight (C) measured in the tenth week. D. Proteinuria excretion of 24 h. E-F. Serum creatinine (E) and blood urea nitrogen (F) in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. DN group.

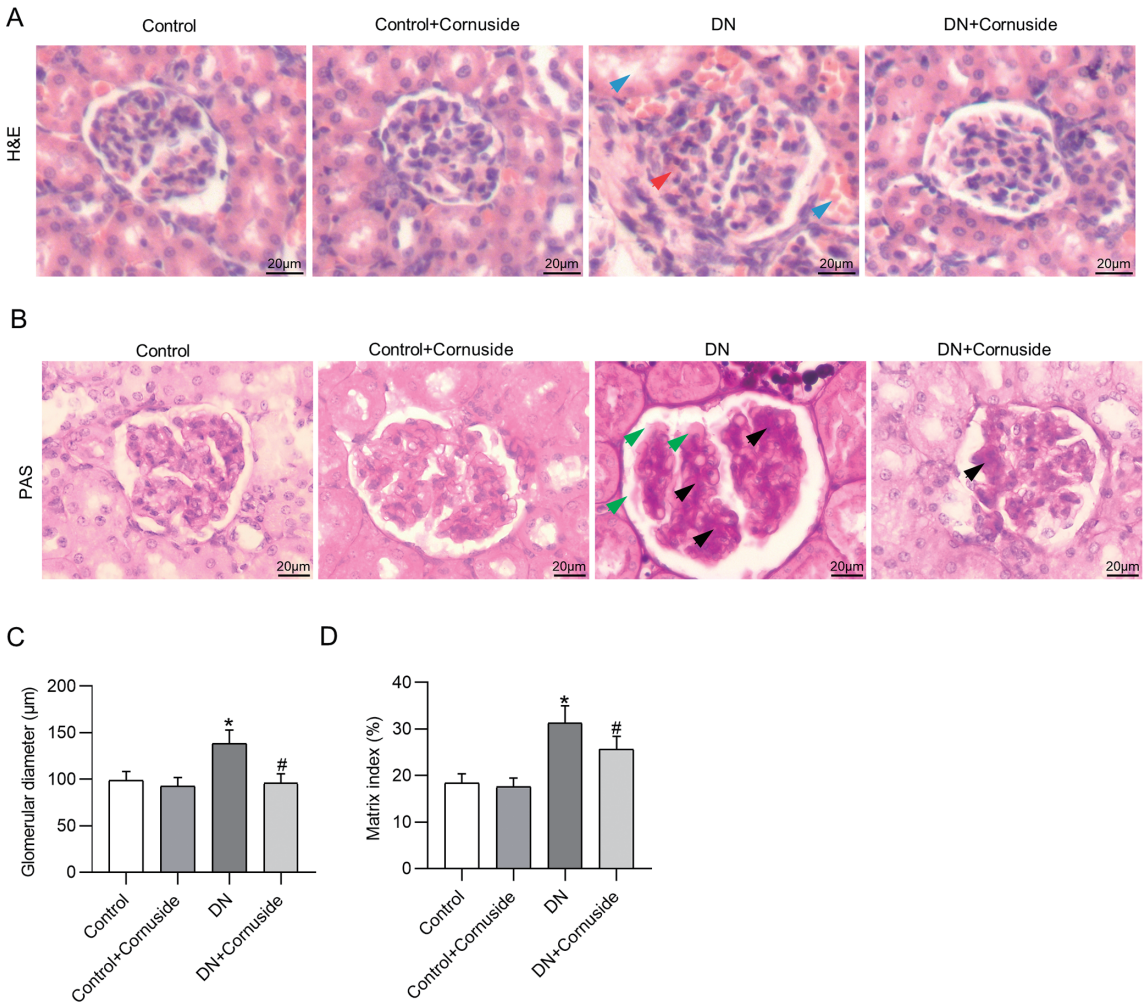

Fig. 3. Cornuside alleviates renal lesions in DN rats.
A-B. Representative images of H&E (A) and PAS staining (B) for histologic analysis of the kidney tissues in each group. Arrows indicate pathological changes: glomerular hypertrophy (red), tubular injury (blue), mesangial matrix expansion (black), and arteriolar hyalinosis (green). C-D. Semi-quantitative results of glomerular diameter (C) and matrix index (D). $^{*}p < 0.05$ vs. Control group; $^{\#}p < 0.05$ vs. DN group.

2018). Immunofluorescence staining showed that the CD31-positive glomerular area was significantly increased in DN rats. However, this effect was abated by cornuside treatment (Fig. 4G,H). These data demonstrated that cornuside hindered angiogenesis in the kidneys of DN rats.

Cornuside reduces VEGF expression and impedes MAPK signaling in HG-treated podocytes

Rat podocytes were treated with HG to mimic a hyperglycemic microenvironment *in vitro*. Notably, VEGF level was markedly elevated in HG-exposed podocytes, while cornuside treatment abated this effect (Fig. 5A). Similar results were shown by western blotting (Fig. 5B,C). Moreover, considering the significance of the MAPK signaling pathway in DN and the regulation of cornuside on this pathway, we evaluated whether cornuside affected MAPK signaling in HG-stimulated podocytes. As expected, HG stimulated the upregulation of p-ERK, p-JNK, and p-p38 in podocytes, whereas cornuside treatment markedly counteracted these effects (Fig. 5D), suggesting that cornuside blocked MAPK signaling in podocytes under HG stimulation. In addition, our results showed that treatment with VEGF induced the phosphorylation of ERK, JNK, and p38 in NG-treated podocytes (Supplementary Fig. S1A,B). Conversely, treatment with the anti-VEGF antibody significantly reduced the high p-ERK, p-JNK, and p-p38 levels in HG-stimulated podocytes (Supplementary Fig. S1C,D).

Discussion

This study examined the renoprotective effect of cornuside and its potential mechanism on DN progression in STZ-induced DN rats and HG-stimulated podocytes. Our study revealed that cornuside administration improved STZ-evoked renal dysfunction and pathological lesions in rats. The mechanism might be associated with inhibition of angiogenesis and MAPK signaling.

Previous evidence has suggested that several bioactive

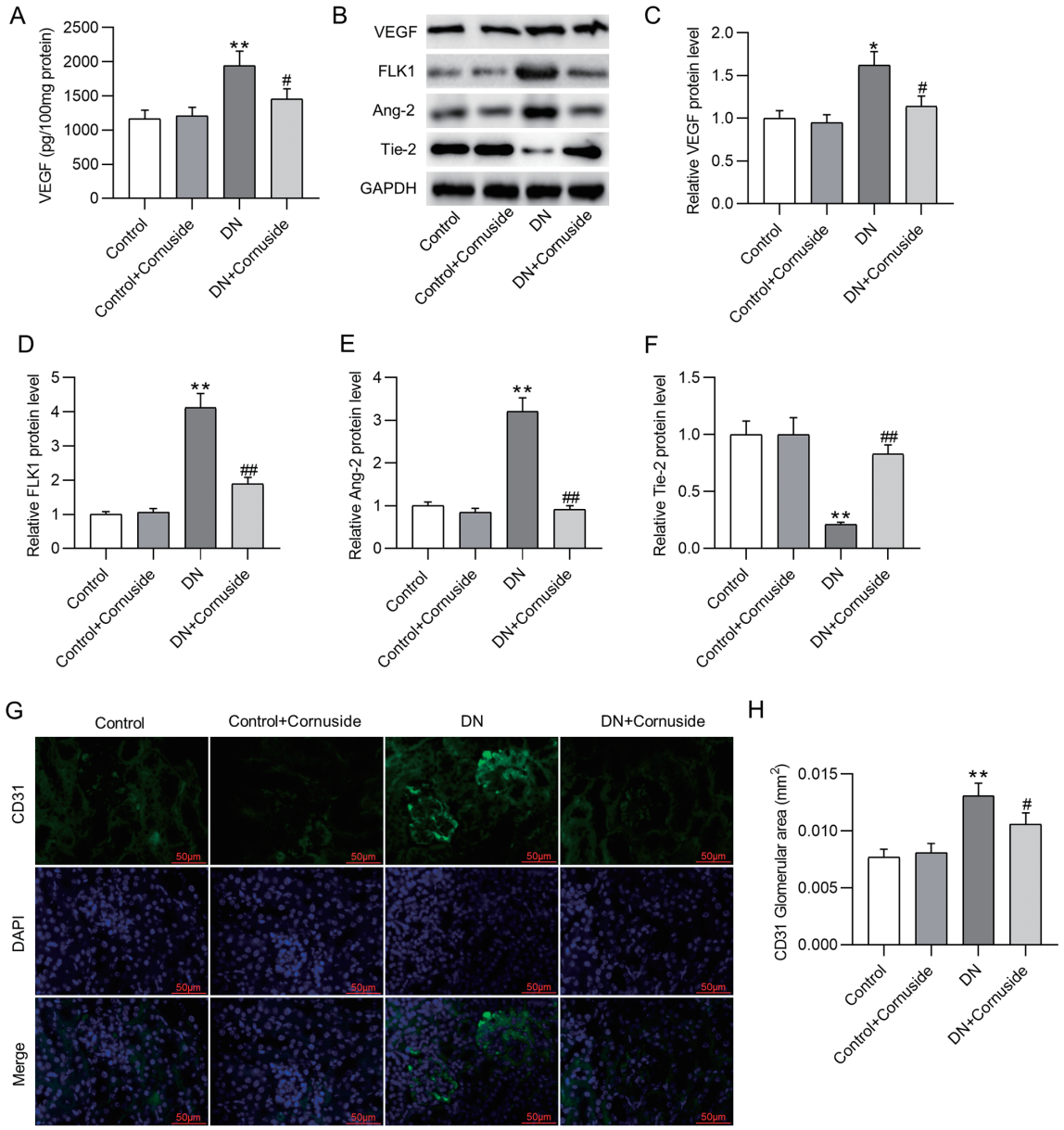

Fig. 4. Cornuside suppresses angiogenesis in DN rats.
A. ELISA for detecting VEGF secretion in the renal tissues. B-F. Western blotting for evaluating protein levels of VEGF, FLK1, Ang-1, and Tie-2 in rat kidneys. G. Representative images of immunofluorescence staining of CD31 in the rat kidneys. H. Quantitative analysis of DC31-positive glomerular area in each group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. DN group.

compounds from *Cornus officinali* have an anti-DN activity (Ma et al. 2014). As a secoiridoid glucoside compound from this plant, cornuside has been indicated to reduce fasting blood glucose levels and alleviate testicular injury in diabetic mice (Liu et al. 2021). Similarly, our results also showed the hypoglycemic effect of cornuside in the DN rat model. Moreover, this study depicted that cornuside decreased 24-h proteinuria, serum creatinine, and BUN in diabetic rats, which serve as critical indicators of renal dysfunction. Additionally, our results showed that cornuside treatment alone did not cause significant changes in the control rats or NG-treated podocytes, indicating that cornuside has low toxicity in normal animals.

Mounting evidence has illuminated that aberrant angiogenesis is tightly linked to DN progression. New blood vessel formation has been observed in glomeruli of diabetic patients, which is considered to be related to enhanced glomerular filtration surface, resulting in glomerular hypertrophy and hyperfiltration in the early stages of DN (Tanabe et al. 2017). Anti-angiogenic therapy has shown promise as a treatment for DN in animal studies (Wen et al. 2013). Previous reports proposed that expression levels of the pro-angiogenic factor VEGF and its receptor FLK1 were upregulated in the kidneys of diabetic

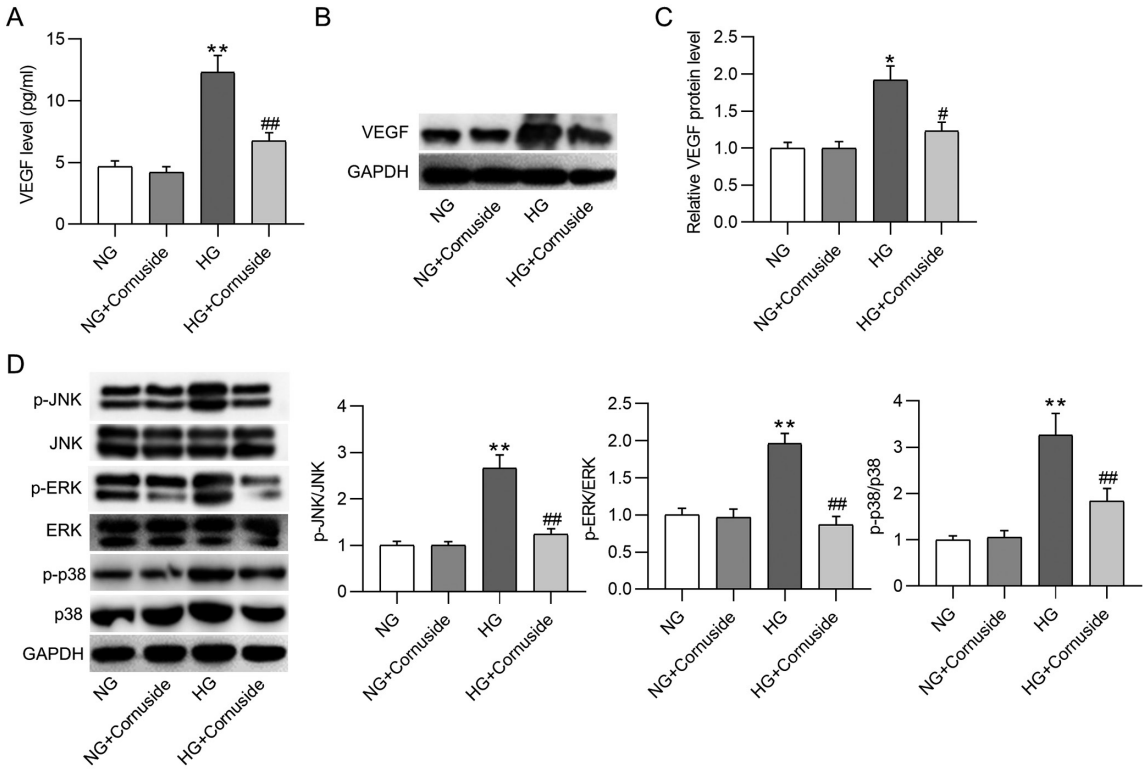

Fig. 5. Cornuside impedes angiogenesis and MAPK signaling in HG-treated podocytes.
A. ELISA for detecting VEGF level in podocytes. B-C. Western blotting of VEGF protein expression in podocytes. D. Western blotting for examining levels of MAPK signaling-related proteins. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. DN group.

rats (Cooper et al. 1999; Chyła-Danił et al. 2023). Furthermore, the Ang/Tie-2 system also plays a pivotal role in angiogenesis. Ang-2, a natural Ang-1 antagonist, represses blood vessel maturation by impeding Tie-2 signaling and also promotes angiogenesis in the presence of VEGF (Wen et al. 2013). Elevated Ang-2 level, along with decreased Tie-2 level, has been observed in DN animal models (Ichinose et al. 2005). Consistent with the aforementioned reports, our results displayed that VEGF, FLK1, and Ang-2 protein levels were elevated, and Tie-2 protein expression was reduced in the kidney tissues of DN rats. These effects were prominently counteracted after cornuside administration, indicating that cornuside could restore STZ-evoked abnormal angiogenesis in rat kidneys.

Podocytes are the crucial function cells in glomeruli. Podocyte injury contributes to glomerular filtration barrier dysfunction and is considered an early hallmark of DN (Ou et al. 2021). Our results further confirmed that cornuside repressed VEGF expression in HG-stimulated podocytes. MAPK signaling transduction is a conserved pathway involved in various pathophysiological processes (Hepworth and Hinton 2021). Importantly, many studies have shown the potential efficacy of several agents in treating DN partially via the inactivation of MAPKs, mainly including ERK, p38, and JNK (Gong et al. 2021; Wang et al. 2022). Activation of ERK or p38 contributes to HG-induced podocyte apoptosis (Wang et al. 2022). Inhibition of MAPK signaling can suppress hyperglycemia-induced podocyte apoptosis and inflammation, thereby attenuating renal injury (Han et al. 2020). Our study depicted that cornuside treatment markedly suppressed the phosphorylation of ERK, p38, and JNK in podocytes under HG stimulation, indicating that cornuside blocked MAPK signaling activation. As mentioned above, Li et al. (2016) proposed that cornuside alleviates allergic response by inactivating MAPK, which supports our findings. However, the precise mechanism of cornuside on MAPK inactivation remains unclear, highlighting the need for further investigation. Additionally, future studies are required to substantiate the involvement of MAPK signaling in cornuside-mediated therapeutic effects on DN.

In conclusion, this study depicts that cornuside ameliorates renal injury and restores abnormal angiogenesis in the DN rat model. Moreover, the renoprotective property of cornuside is possibly associated with the inactivation of MAPK signaling. Our findings may provide new clues for treating DN.

Author Contributions

Fang Xiang was the main designer of this study. Fang Xiang, Xin Li, and Wei Hu performed the experiments and analyzed the data. Fang Xiang and Wei Hu drafted the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Files

Please find supplementary file(s);
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