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Chrysophanol ameliorates oxidative stress and pyroptosis in mice with diabetic nephropathy through the Kelch-like ECH-associated protein 1/nuclear factor erythroid 2-related factor 2 signaling pathway

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Diabetic nephropathy (DN), a microvascular complication of diabetes, increases the risk of all-cause diabetes and cardiovascular mortalities. Moreover, oxidative stress and pyroptosis play important roles in the pathogenesis of DN. Rhubarb is widely used in traditional medicine, and chrysophanol (Chr), a free anthraquinone compound abundant in rhubarb, exhibits potent antioxidant properties and ameliorates renal fibrosis. Therefore, this study aimed to investigate the effects of Chr on renal injury, oxidative stress, and pyroptosis in mice with DN. A DN model was established by feeding the mice a high-sugar and fat diet and injecting them with 50 mg/kg streptozotocin as a positive control. The DN mice had significantly impaired renal function, thickened glomerular thylakoids and basement membranes, increased fibrous tissue, and inflammatory cell infiltration. Superoxide dismutase (SOD) levels were reduced, malondialdehyde (MDA) levels were increased, interleukin (IL)-1β and IL-18 increased, and cleaved caspase-1, caspase-1, and gasdermin D (GSDMD) involved in the process of pyroptosis were upregulated in DN. Kelch-like ECH-associated protein 1 (Keap1) expression was upregulated, and nuclear factor erythroid 2-related factor 2 (Nrf2) expression was downregulated. Compared to those in the DN group, the Chr-treated mice with DN had improved renal dysfunction, weakened glomerular thylakoid and basement membrane thickening, and reduced fibrous tissue proliferation and inflammatory cell infiltration. Additionally, Chr increased SOD levels, decreased MDA, IL-1β, and IL-18, down-regulated caspase-1, cleaved caspase-1, GSDMD, and Keap1 expression, and upregulated Nrf2 expression, which reversed the DN. Therefore, Chr reduced oxidative stress and pyroptosis in DNmice by activating the Keap1/Nrf2 pathway.

Keywords: Diabetic nephropathy, Chrysophanol, Keap1/Nrf2 pathway, Oxidative stress, Pyroptosis

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#Equal contribution
Abbreviations: Chr, chrysophanol; DN, Diabetic nephropathy; GSDMD, gasdermin D; Keap1, Kelch-like ECH-associated protein 1; MDA, malondialdehyde; Nrf2, nuclear factor erythroid 2-related factor 2; SOD, Superoxide dismutase

INTRODUCTION

Diabetic nephropathy (DN) is the most common and serious microvascular complication of diabetes. More than 15–40% of patients with diabetes mellitus develop DN during their lifetime. Moreover, DN affects patients' quality of life and increases the risk of all-cause diabetes and cardiovascular mortality (Afkarian *et al.*, 2013). Furthermore, DN is characterized by progressive renal impairment with the onset of glomerular basement membrane thickening and widening of the thylakoid matrix (Tervaert *et al.*, 2010). This is followed by trace urine protein, hypertension, edema, renal insufficiency, and even renal failure, leading to end-stage renal disease (ESRD) (Hirawa *et al.*, 2001; Sagoo & Gnudi, 2020).

The glomerular filtration barrier comprises podocytes, which are highly differentiated glomerular epithelial cells, and kidney diseases linked to diabetes may result from damage to podocytes (Lin *et al.*, 2020). Cell death, including apoptosis, necrosis, autophagy, pyroptosis, and ferroptosis, has been observed in the process of DN (Sifuentes-Franco *et al.*, 2018; Al Mamun *et al.*, 2021; Chen *et al.*, 2021). Pyroptosis is a newly discovered form of programmed cell death that has recently attracted increasing attention. In contrast to apoptosis, pyroptosis is a lysis and inflammatory death dependent on inflammatory vesicles and cystatinases and is characterized by plasma membrane rupture, cell swelling, and dissolution, which is mediated by the gasdermin D (GSDMD) protein family (Shi *et al.*, 2017). Evidence suggests that oxidative stress and inflammation play key roles in the development of DN. Chronic hyperglycemia in patients with diabetes is thought to trigger a downstream cascade response. Excessive production of reactive oxygen species (ROS) in podocytes under high-glucose conditions can activate nucleotide-binding domain, leucine-rich–containing family, pyrin domain-containing-3 (NLRP3) to induce podocyte scorching, whereas mitochondrial autophagy can regulate cellular homeostasis and modulate ROS production (Lin *et al.*, 2019; Qiu *et al.*, 2019; Xiaodong & Xuejun, 2022). Podocytes have a huge energy requirement to maintain adequate mitochondrial numbers and normal function; thus, they are more vulnerable to mitochondrial damage (Gujarati *et al.*, 2020; Su *et al.*, 2020a).

Rhubarb is a common traditional Chinese medicine widely used in treating many diseases related to the circulatory, digestive, endocrine, respiratory, and skel-

etal systems, as well as infectious diseases (Rokaya *et al.*, 2012). Chrysophanol (Chr) is a natural anthraquinone component isolated from Rhubarb spp and is also known as chrysophanic acid and 1,8-dihydroxy-3-methylanthraquinone. Recent reports have demonstrated that Chr has many beneficial effects, such as anti-inflammatory, anticancer, antidepressant, and neuroprotective (Su *et al.*, 2020b). Renal fibrosis is effectively improved by Chr as the NKD2/NF-κB pathway is inhibited (Gu *et al.*, 2022). Additionally, Chr alleviates cisplatin-induced nephrotoxicity by simultaneously inhibiting oxidative stress, apoptosis, and inflammation (Ma *et al.*, 2021). Ovarian cancer cell death is induced by Chr which also inhibits cancer invasiveness via mitochondrial calcium overload (Lim *et al.*, 2018). Significant evidence suggests that Chr has strong antioxidant and ameliorating effects on renal fibrosis. However, the Chr function in DN progression remains unknown.

Nuclear factor erythroid 2- related factor 2 (Nrf2) and the endogenous inhibitor, Kelch-like ECH-associated protein 1 (Keap1), are ubiquitous and evolutionarily conserved intracellular defense mechanisms that regulate the redox imbalance caused by oxidative stress (Yang *et al.*, 2013; Bellezza *et al.*, 2018). However, Nrf2 can form an inactive complex with Keap1, which controls the subcellular localization and steady-state level under normal physiological conditions. Activation of the Nrf2 signaling pathway downregulates high glucose-induced ROS levels and podocyte apoptosis. When Nrf2 expression is downregulated, podocytes exposed to high glucose levels exhibit severe mitochondrial dysfunction (Zhang *et al.*, 2018).

Benazepril is considered an effective drug for treating DN because the medication reduces kidney volume and alleviates DN-associated pathological changes (Xue *et al.*, 2017). In the present study, we evaluated the renal pathology of mice with DN, and blood samples were tested for blood glucose, oxidative stress, and pyrogenic levels to assess the effect of Chr, which provides a further understanding of the protective mechanisms of Chr in DN.

MATERIALS AND METHODS

Animal protocols

C57BL/6 mice (6 weeks old, males) were obtained from the Chengdu Dossy Experimental Animals Company, China. The mice were randomized into six groups (seven mice per group): control, DN, DN + 2.5 mg Chr, DN + 5 mg Chr, DN + 10 mg Chr, and DN + benazepril. The control and DN groups were administered 0.9% saline, and the DN + 2.5 mg Chr, DN + 5 mg Chr, and DN + 10 mg Chr groups were administered with Chr at a dose of 2.5, 5, and 10 mg/kg/d, respectively. The benazepril group was administered benazepril at 1 mg/kg/d (Zhao *et al.*, 2016; Dou *et al.*, 2020; Ma *et al.*, 2021). All the mice were placed in an animal room without specific pathogens and under controlled temperature (24°C±1°C) and humidity (50–70%). The mice were adapted to a 12-h light and 12-h dark cycle. The control group was fed standard pellet feed, and the remaining mice were fed high-sugar and high-fat diets for six weeks. They were then injected with 50 mg/kg streptozotocin (STZ) to establish a DN model. Fasting blood glucose levels >7.0 mmol/L were considered to indicate an incidence of diabetes. When the model was successfully established, Chr gavage was administered for eight weeks. Benazepril was used as the positive control. All experiments were performed by the guidelines for the care and use of laboratory animals. The kidneys of the mice were weighed, and kidney-to-body weight ratios (KBWR) were calculated based on the weight of the mice. All experimental procedures and protocols were approved by the Medical Ethical Committee of West China Hospital of Sichuan University (20230829001).

Renal function evaluation

After eight weeks of drug treatment, the mice were fasted overnight. All the mice were intraperitoneally injected with 1% sodium pentobarbital. Whole blood was centrifuged (12 000 rpm, 4°C, 15 min) to obtain serum. A fully automatic biochemical analyzer, Hitachi 7600-110, was used to measure serum creatinine (Scr), blood urea nitrogen (BUN), and 24-hour urine protein levels.

Hematoxylin and eosin (H&E) and MASSON trichrome staining

Masson trichrome and H&E staining were performed as described previously (Wang *et al.*, 2022). After execution of the mice, kidney samples were isolated and fixed in paraformaldehyde for 24 h, subsequently embedded in paraffin, and sectioned (4–5 μm). Tissue sections were stained with H&E and Masson trichrome stains.

Enzyme-linked immunosorbent assay (ELISA)

After eight weeks of drug treatment, the samples of kidney cortex from each group of mice were collected, homogenized in 0.05% phosphate-buffered saline (PBS), and centrifuged at 2500 rpm for 20 min. Anti-interleukin (IL)-1β and anti-IL-18 were purchased from R&D (Minneapolis, MN, USA). The supernatant was collected, and the levels of IL-1β and IL-18 were detected using corresponding ELISA kits according to the manufacturer's instructions.

Determination of superoxide dismutase (SOD) and malondialdehyde (MDA) activity

After treatment, the kidney cortex was collected from each group of mice, homogenized in 0.05% PBS, and centrifuged at 2500 rpm for 20 min. SOD and MDA kits (NJJCBIO) were used. Optical density was measured using a miniature multifunction panel reader (Biotek Instruments, Inc.). SOD and MDA activities in each group were calculated, according to the manufacturer's instructions.

Western blot (WB) analyses

The cortex tissue samples of the kidneys from each group were homogenized with protease inhibitors, lysis buffer, and a 1 mM mixture of phenylmethylsulfonyl fluoride. Total protein concentration was assayed using a bicinchoninic acid protein assay kit. Equal amounts of total protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 20 μg per well and transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was immersed in tris-buffered saline Tween-20 with 3% bovine serum albumin for 1.5 h. The membrane was then incubated with the following specific primary antibodies (1:1000) overnight at 4°C: anti-Keap1, anti-Nrf2, anti-Pro-Caspase-1, anti-cleaved Caspase-1, and anti-GSDMD. The membranes were then incubated with

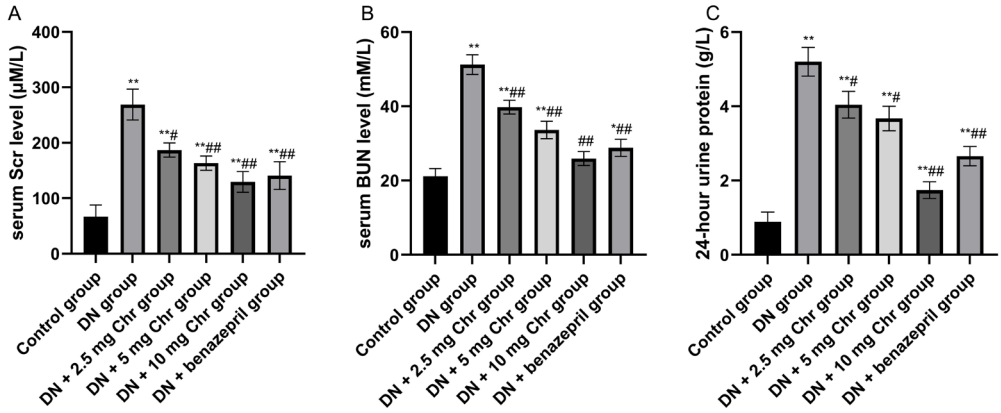

Figure 1. Effects of chrysophanol (Chr) on serum creatinine (Scr, A), blood urea nitrogen (BUN, B) and 24 h urine protein (C) in mice with diabetic nephropathy (DN) analyzed by sarcosine oxidase method, Uricase-GIDH method, and enzyme colorimetry, respectively. Data are presented as mean ± S.D. Data were compared by one-way ANOVA and Tukey's multiple comparison test. $^{*}P<0.05$, $^{**}P<0.01$ as compared with control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ as compared with DN group. n=7.

peroxidase-coupled anti-rabbit or anti-mouse antibodies for 2 h at room temperature. Super-enhanced chemiluminescence reagent (Applygen Technologies Inc, Beijing, China) was added to the membrane to visualize the target band. The band intensity was calculated using ImageJ software, and the relative protein intensities were calculated. Anti-Keap1, Nrf2, and caspase-1 were purchased from Servicebio (Wuhan, China), cleaved caspase-1 antibody was purchased from Santa Cruz (1:200, Dallas, TX, USA), and GSDMD and actin antibodies were obtained from Abcam (Cambridge, MA, USA).

Statistical analyses

Data were analyzed using SPSS software (version 20.0; GraphPad Software, La Jolla, CA, USA), and GraphPad Prism 8.0 software was used for drawings. All data are expressed as mean ± standard deviation. Statistical analyses were performed using a one-way analysis of variance and Tukey's multiple comparison tests for multiple group comparisons. Statistical significance was considered at $P<0.05$.

RESULTS

Chrysophanol improves Renal Dysfunction in C57BL/6 mice

As displayed in Fig. 1, after eight weeks of treatment, the levels of Scr, BUN, and 24-hour urine protein in the DN group were significantly higher than those in the control group. Moreover, Chr demonstrated varying levels of reduced renal dysfunction in the mice, indicating that Chr was effective in inhibiting a decrease in renal

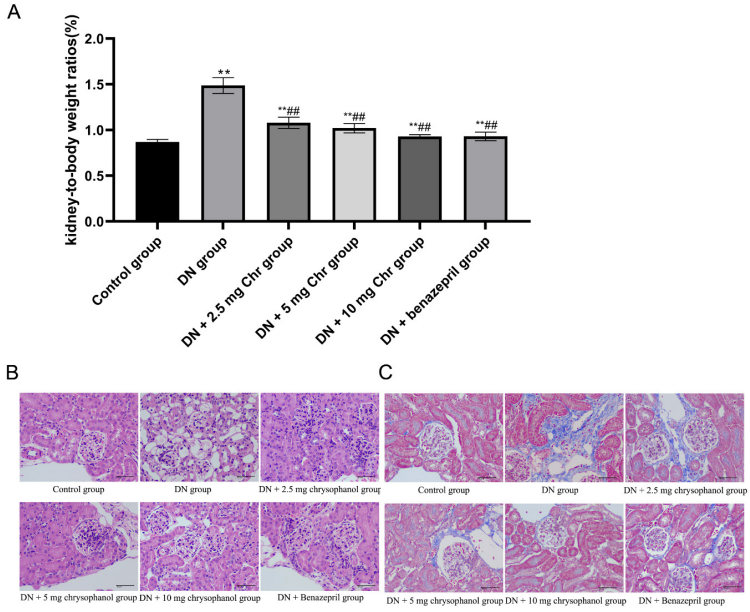

Figure 2. Effects of chrysophanol (Chr) on kidney-to-body weight ratios (A) and pathological features (B and C) in mice with diabetic nephropathy (DN) analyzed by weighing, hematoxylin-eosin staining and Masson staining, respectively. Data are presented as mean ± S.D. Data were compared by one-way ANOVA and Tukey's multiple comparison test. $^{**}P<0.01$ as compared with control group; $^{\#\#}P<0.01$ as compared with DN group. n=7. Magnification: ×40. Scale bar: 50 μm.

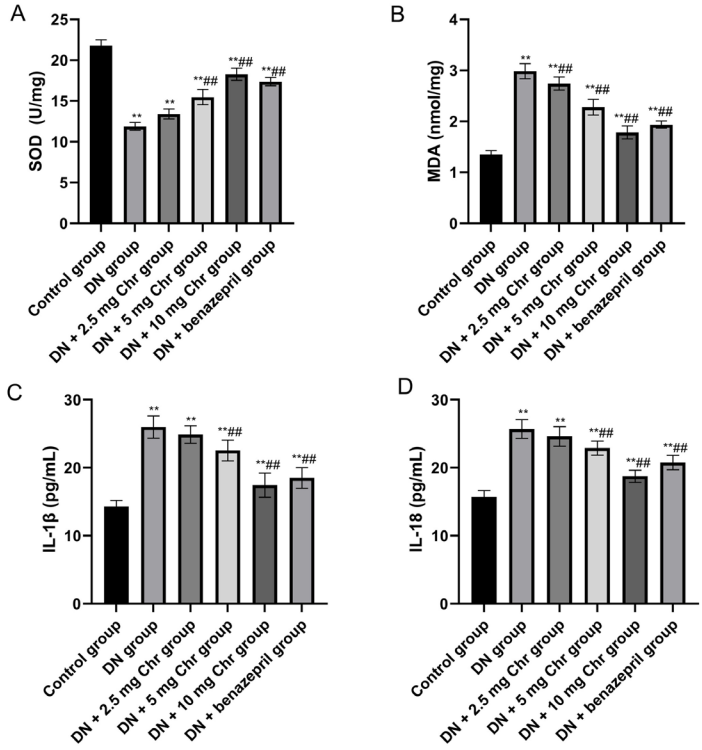

Figure 3. Effects of chrysophanol (Chr) on superoxide dismutase (SOD, A), malondialdehyde (MDA, B), interleukin-1β (IL-1β, C), and interleukin-18 (IL-18, D) in kidneys of mice with diabetic nephropathy (DN) analyzed by Enzyme-Linked Immunosorbent Assay (ELISA).
Data are presented as mean ± S.D. Data were compared by one-way ANOVA and Tukey's multiple comparison test. $^{**}P<0.01$ as compared with control group; $^{\#\#}P<0.01$ as compared with DN group. n=7.

function in the DN mice. Additionally, 10 mg of Chr was more effective than benazepril.

Chrysophanol ameliorates morphological and pathological changes in C57BL/6 mice

The kidneys of each mouse group were evaluated. First, we evaluated the KBWR. The KBWR increased significantly in DN mice, but decreased significantly in Chr-treated mice (Fig. 2A). Figures 2B and 2C illustrate kidney slices stained with H&E and Masson's trichrome stains. Thicker glomerular thylakoid and basement membranes, capillary occlusion, epithelial cell swelling, small amounts of interstitial fibrous tissue hyperplasia, and lymphocytic infiltration were observed in the DN group. Compared to those in the DN group, the Chr-treated DN mice exhibited varying degrees of glomerular thylakoid and basement membrane thickening. Additionally, slight epithelial cell degeneration, fibrous tissue hyperplasia, and lymphocyte infiltration were observed in the 2.5 mg Chr group. In the 5 mg Chr group, swelling of epithelial cells with minimal fibrous tissue hyperplasia was observed. No significant fibrous tissue proliferation or inflammatory cell infiltration was observed in the 10 mg Chr group.

Chrysophanol reduces oxidative damage and pyroptosis of the renal cortex in C57BL/6 mice by regulating the Keap1/Nrf2 pathway

To evaluate kidney oxidative stress injury in the C57BL/6 mice, SOD, MDA, IL-1β, and IL-18 were measured and analyzed in the kidney cortex tissue. In the DN group, SOD significantly decreased, and MDA significantly increased (Fig. 3A and Fig. 3B). However, Chr upregulated SOD and decreased MDA levels in the kidney cortex tissue. Additionally, Chr significantly decreased the levels of IL-1β and IL-18 (Fig. 3C and Fig. 3D), which indicated that Chr could alleviate oxidative stress inflammatory damage in the C57BL/6 mice.

The WB results (Fig. 4A–4G) revealed that the expression of cleaved caspase-1, caspase-1, GSDMD, and Keap1 was upregulated, whereas, the expression of Nrf2 was downregulated in the DN group. Additionally, Chr treatment downregulated the expression of cleaved caspase-1, caspase-1, and GSDMD, which are involved in pyroptosis, downregulated Keap1 protein, and upregulated the expression of Nrf2. These results suggest that Chr inhibits oxidative stress and pyroptosis by regulating the Keap1/Nrf2 signaling pathway.

DISCUSSION

DN is a chronic kidney disease that is a major cause of ESRD and is associated with significant healthcare costs. Although treatment options for DN have progressed, including the use of angiotensin II receptor blockers, angiotensin-converting enzyme inhibitors sodium-glucose co-transporter protein 2 inhibitors, and salt corticosteroid receptor antagonists, the outcomes remain very limited (Fried *et al.*, 2013; Barrera-Chimal *et al.*, 2019; Tesař, 2022). Thus, an urgent need to develop more valid DN regimens is present. Chr a unique anthraquinone, is a primary component of many plant extensions of traditional Chinese medicine. Moreover, Chr has a broad-spectrum curative potential and eco-relevance (Yusuf *et al.*, 2019), and the drug has been validated to

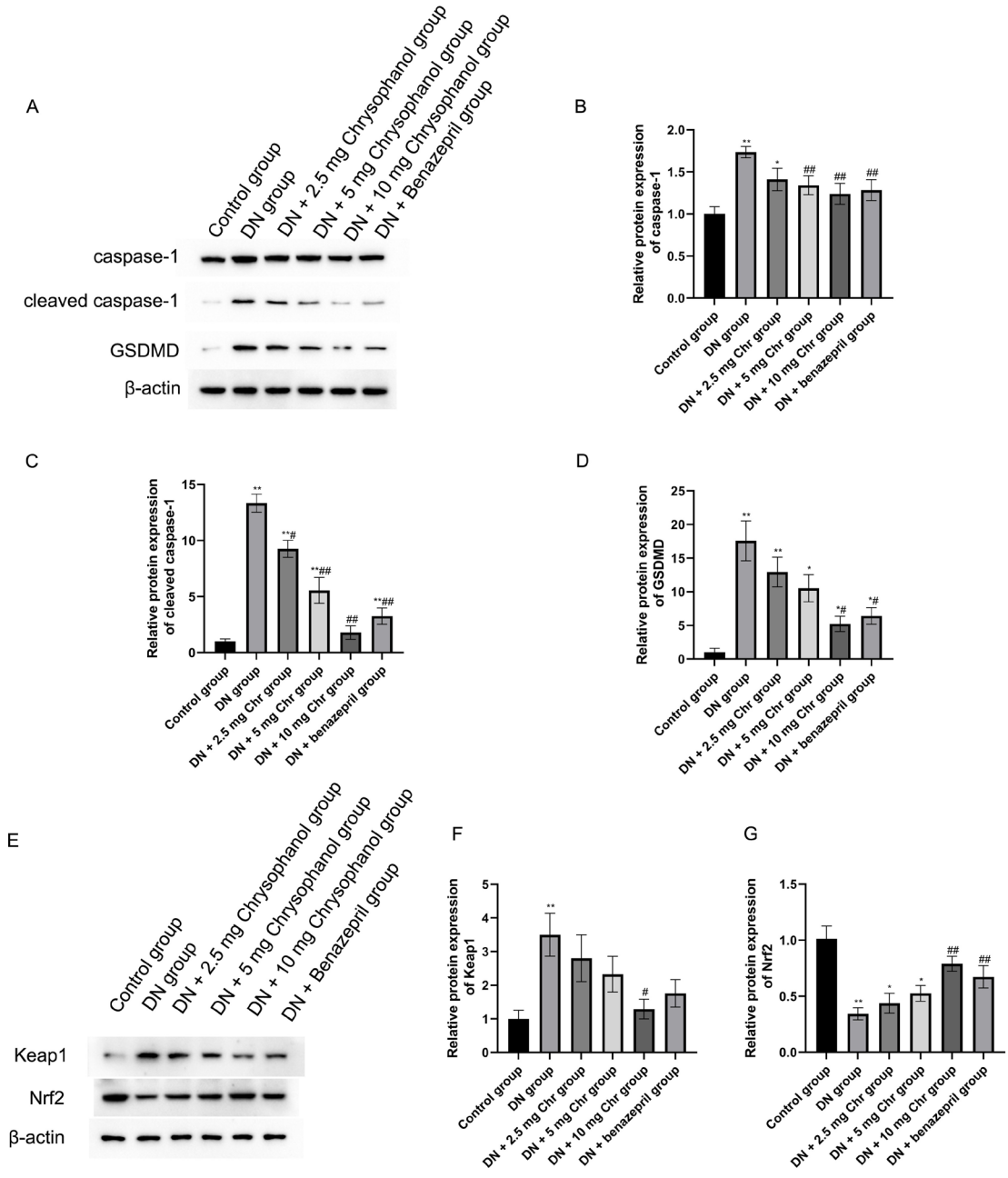

Figure 4. Effects of chrysophanol (Chr) on caspase-1 (A and B), cleaved caspase-1 (A and C), gasdermin D (GSDMD, A and D), kelch-like ECH-associated protein 1 (Keap1, E and F) and nuclear factor erythroid 2-related factor 2 (Nrf2, E and G) in kidneys of mice with diabetic nephropathy (DN) analyzed by western blots (WB).
Data are presented as mean ± S.D. Data were compared by one-way ANOVA and Tukey's multiple comparison test. $^{*}P<0.05$, $^{**}P<0.01$ as compared with control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ as compared with DN group. n=7.

be effective in the prevention and management of diabetes and its complications (Guo *et al.*, 2020). Benazepril is an angiotensin-converting enzyme inhibitor (ACEI) drug commonly used in treating hypertension and heart failure (Yan *et al.*, 2013). Numerous studies have demonstrated that benazepril improves DN and reduces proteinuria in rats (Jin *et al.*, 2014). In this study, we investigated the underlying effects and potential mechanisms of action of Chr on DN using benazepril as a positive control. Our results suggest that Chr can prevent DN by activating the Keap1/Nrf2 pathway to attenuate oxidative stress and cellular scorching and reduce inflammation.

The molecular mechanisms underlying DN include inflammation, fibrosis, metabolism, and hemodynamics. Oxidative stress is considered a critical pathway in diabetic kidney injury in the presence of metabolic and hemodynamic abnormalities. Under normal conditions, ROS levels are too low to maintain cellular homeostasis. However, in high conditions, ROS generation in podocytes increases significantly (Bhatti & Usman, 2015). Exposure of podocytes to ROS leads to α-dystroglycan

deglycosylation, thereby decreasing the number of podocytes. Mitochondrial damage-induced podocyte apoptosis is considered to be the most devastating factor in DN progression (Feng *et al.*, 2019). In this study, we identified that Chr could repair high-fat, high-sugar, and STZ-induced mitochondrial damage, enhance antioxidative capacity, and inhibit the generation of oxidation products, thereby preventing apoptosis of podocytes and alleviating DN.

In addition to oxidative stress, inflammation is a key factor in DN development. Several studies have demonstrated that activating inflammatory signaling and infiltrating inflammatory cells are essential for DN progression (Rayego-Mateos *et al.*, 2020). The inflammasome is a multi-protein signaling platform that controls response to inflammation and coordinates antimicrobial host defense (Broz & Dixit, 2016). The NOD-like receptors (NLR), which contain the pyridine structural domain family 3, assemble an intracellular protein complex called the NLRP3 inflammasome when certain pathogen products or sterile danger signals are sensed (Wang *et al.*, 2021). Additionally, NLRP3 is present in natural immune and non-immune cells in the kidney, such as podocytes. NLRP3 recognizes a variety of pathogen- or injury-associated molecular patterns and is involved in intrinsic immune and inflammatory responses (Bai *et al.*, 2017). The NLRP3 inflammasomes mediate pyroptosis and play an important role (Wang *et al.*, 2017; Li *et al.*, 2021). When cells are stimulated externally, they activate the inflammatory complex and Caspase-1, which acts as a direct agonist to cleave IL-1β and IL-18 precursors, thereby recruiting other inflammatory cells and exacerbating inflammatory response by releasing them extracellularly. Simultaneously, they shear the GSDMD, release the active N-terminal (GSDMD-N) to bind to phospholipids in the inner layer of the plasma membrane, and accelerate nuclear pore formation, which releases mature forms of inflammatory factors to induce pyroptosis (Ystgaard *et al.*, 2015; Rathinam & Fitzgerald, 2016; Sborgi *et al.*, 2016; Sun & Scott, 2016). This inflammatory reaction further promotes the activation of NLRP3 inflammatory corpuscles, forming a vicious circle and aggravating the disease. In the present study, we demonstrated that Chr reduced caspase-1 levels and downregulated GSDMD expression in mice with DN to suppress inflammatory responses, thereby preventing pyroptosis and alleviating DN.

Proteinuria is an important indicator of DN progression (KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Diabetes and Chronic Kidney Disease, 2007). However, assessing the severity or prognosis of DN solely based on the degree of proteinuria is accurate (Radcliffe *et al.*, 2017), and pathological changes are a beneficial addition to this assessment. In this study, renal tissue sections from mice in the DN group displayed severe morphological damage and fibrosis. Treatment with Chr restores renal histopathological damage to varying degrees and reduces renal fibrosis in C57BL/6 mice. This implies that Chr may have a protective effect on podocytes in DN. This protective mechanism might be related to the anti-inflammatory and antioxidant properties of Chr.

The present study confirmed that oxidative stress and inflammatory responses are closely associated with DN, and our results suggest that Chr is a potentially effective therapy for DN. Furthermore, because of the common upstream pathogenesis of many diseases, published evidence suggests that Chr may ameliorate various diseases associated with abnormal angiogenesis, including diabetes and cancer, by inhibiting oxidative stress and the Keap1/Nrf2 pathway (Guo & Mo, 2020). We suggest that this profile could also be deduced from other products of natural origin with antioxidant and anti-inflammatory properties for treating diabetic complications. Furthermore, the findings provide conclusive evidence that the Keap1/Nrf2 signaling pathway is a potential target in DN.

Our study has some limitations. Although the current study suggests that Chr can treat DN by attenuating oxidative stress and inflammation, we did not directly elucidate the origin of oxidative stress-induced damage in the mouse models. The pathogenesis of DN is complex and involves various distinct cell types, such as podocytes, glomerular endothelial cells, thylakoid cells, and renal tubular epithelial cells. Numerous studies have established that oxidative stress and inflammation can affect the aforementioned cells, collectively leading to DN progression. Therefore, we hope that more extensive and targeted investigations will be conducted in the future.

CONCLUSION

In summary, our study provides evidence for the primary role and potential mechanisms of action of Chr in DN. The results display that Chr has a considerable impact on upstream events in the pathogenesis of DN rather than just focusing on late inflammatory infiltration and fiber formation. Chr can manage DN by mitigating both oxidative stress and inflammation and the primary mechanism is related to the inhibition of the Keap1/Nrf2 pathway.

Declarations

Conflict of interest. The authors declare no conflict of interest.

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