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Protein kinase C inhibitor chelerythrine attenuates partial unilateral ureteral obstruction induced kidney injury in neonatal rats

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ABSTRACT

The present study aimed to evaluate the renoprotective effects of chelerythrine (CHE), a protein kinase C inhibitor, on neonatal rats after partial unilateral ureteral obstruction (UUO) surgery. New born Sprague Dawley rats were subjected to partial UUO 48 h after birth and received a daily intraperitoneal injection of 5 mg/kg CHE. At 21-day age, the rats were scarified and the kidneys were collected for analysis. Results showed that CHE treatment significantly increased kidney weight and restored renal function in the obstructed kidney. Histological examination demonstrated that CHE attenuated renal injury by reducing renal parenchymal loss and preventing glomerular and tubular degeneration. In addition, CHE inhibited partial UUO-induced upregulated kidney injury molecule-1 expression and apoptosis and renal fibrosis. Moreover, as a PKC inhibitor, CHE significantly inhibited PKCα and PKCβ membrane translocation. This action may be associated with its effects of anti-apoptosis and anti-fibrosis and contribute to the renoprotection. This short-term study suggests that CHE is beneficial for obstructive nephropathy in neonatal rats and provides foundation for further studies to reveal the long-term effects of CHE on obstructive nephropathy in children and infants.

1. Introduction

In children and infants, congenital obstructive nephropathy (CON) constitutes the most common identifiable cause of chronic kidney disease (CKD), with a prevalence of one in 1500 [1–4]. CON initiates during the development of urinary tract in the fetus and is characterized by renal tubular dilatation and parenchymal damage [5]. The injuries including glomerular sclerosis, tubular atrophy, and interstitial fibrosis will be continued after birth [6,7]. Partial unilateral ureteral obstruction (UUO) has been developed in the fetal or neonatal experimental animals to investigate the pathological mechanisms of CON [8]. Animal studies have revealed that UUO leads to nephron loss and late formation of atubular glomeruli and delays maturation of kidney [9,10]. Importantly, relief of the moderate UUO minimized nephron loss and attenuated tubule atrophy and interstitial fibrosis, but not in the severe UUO [11]. Severe CON will ultimately lead to serious renal dysfunction which may need renal transplantation [12]. Thus, it is urgent to develop new therapies which can effectively improve long term renal functions and attenuate renal injury.

Protein kinase C (PKC) is a superfamily of serine-threonine kinases which regulate diverse cellular functions. Since the first report in the late 1980s [13,14], PKC has been discovered to participate in various biological functions including cell proliferation, differentiation, apoptosis and tissue development [15]. In the kidney, PKC has been found to be involved in numerous physiological and pathological processes. Of the 13 isoenzymes in PKC family, PKCα and PKCβ are well described in renal diseases. PKCβ contributes to the development of cyclosporine A-induced tubulointerstitial fibrosis [16]. PKCα and PKCβ participate in the pathogenesis of diabetic nephropathy and high-glucose-induced renal fibrosis [17]. Knock-out of PKCβ attenuates renal hypertrophy in streptozotocin-induced diabetic mice [17]. shRNA against PKCα improves glomerular filtration rate in rats with fulminant hepatic failure [18]. In addition, PKC inhibitors also exhibit effective renal protective effects on ischemia-reperfusion damage in renal transplants and partial unilateral ureteric obstruction (UUO) induced renal injury [19,20]. Thus, inhibition of PKC isoforms may be a potent therapeutic strategy in renal diseases.

Chelerythrine (CHE), a natural benzophenanthridine alkaloid, has been found to have a wide range of biological activities and exert antitumor [21], antidiabetic [22] and anti-inflammatory effects [23]. CHE

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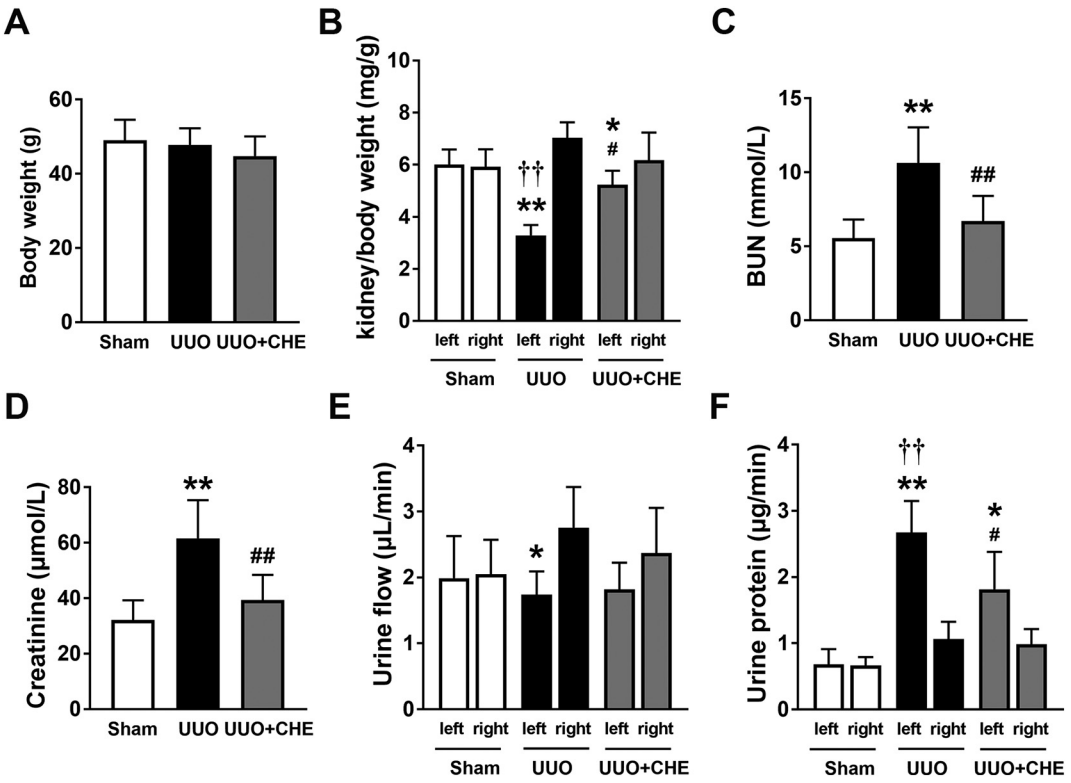

Fig. 1. CHE improves renal function in partial obstructed kidneys. A, body weight, B, kidney weight/body weight ratio, C, BUN, D, serum creatinine, E, urine flow, F, urine protein. Data are expressed as the mean ± SD (n = 5). *P < 0.05 vs. the sham-operated kidneys, **P < 0.01 vs. the sham-operated kidneys, #P < 0.05 vs. the obstructed kidneys, ††P < 0.01 vs. the contralateral kidneys.

is a selective PKCα and PKCβ antagonist [24]. Although some studies indicate that PKC-independent mechanisms are involved in the pharmacological mechanisms of CHE [25,26], it is still extensively used and studied as a specific PKC inhibitor. Juan et al. has demonstrated that chelerythrine can protect renal function after UUO in adult rats [19]. In the present study, we evaluated the effect of CHE on a UUO neonatal rat model and investigated the possible renoprotective action of CHE on neonates.

2. Material and methods

2.1. Animals

New born Sprague Dawley (SD) rats obtained from Liaoning Changsheng biotechnology Co. Ltd. (Benxi, China) were randomly divided into sham group, UUO group, and UUO + CHE group (n = 10 per group). The rats in the UUO and UUO + CHE groups were subjected to partial UUO operation within 48 h of birth according to the previous description [27]. Briefly, the left ureter was isolated and a 0.20-mm steel wire segment was placed next to the left ureter at the ureteropelvic junction. The wire was ligated with the ureter using a single 8–0 nylon suture then removed. The rats in sham group underwent the same operation without ligation. After the operation, the rats in the UUO + CHE group received a daily intraperitoneal injection of 5 mg/kg CHE (purity > 98%, dissolved in 10% DMSO, Aladdin regents Co. Ltd., Shanghai, China) and the rats in the sham and UUO groups received equal amounts of vehicle [19]. At day 21 after birth, the rats were weighed and the urine flow and urine protein were analyzed as described previously [28]. Then, the rats were sacrificed by intraperitoneal injection of 150 mg/kg pentobarbital sodium. All the animal experiments were approved by the Institutional Animal Ethics Committee of China Medical University.

2.2. Tissue collection and preparation

The kidneys were removed and weighed immediately. Five rats were randomly selected for histological examination. The kidneys were fixed by immersion in 10% formalin for 24 h, embedded in paraffin, and sagittally sectioned at 5 μm. The other five rats were used for molecular biological analyses. The kidneys were stored at −80 °C until use. Homogenates were prepared on ice with RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and the protein concentration was determined using the BCA protein assay kit (Beyotime).

2.3. Staining

The renal tissue sections were dewaxed for staining. For structural examination, the sections were subjected to hematoxylin and eosin (H&E) and periodic acid Schiff base (PAS) staining using commercial staining solutions (Baso Diagnostic Inc., Zhuhai, China). Parenchymal thickness was determined using the method previously described [29]. The areas of glomeruli were measured in 50 glomeruli from 5 animals in each group [30]. The number of atrophied tubes was determined according to the apoptotic nuclei as previously described [27]. For immunohistochemical staining, the sections were treated with 3% (v/v) H_2O_2 for 15 min and blocked with normal goat serum for another 15 min. Then, the sections were incubated with anti-α-smooth muscle actin (α-SMA, 1:200, bs-0189R, Bioss, Beijing, China) or fibronectin (1:50, 15613-1-AP, Proteintech) at 4 °C overnight and incubated with horseradish peroxidase-conjugated secondary immunoglobulin G (1:5000, Beyotime). The staining was visualized using DAB solution (Solarbio Science & Technology, Co., Ltd., Beijing, China). For cell apoptosis examination, the sections were stained using the in-situ cell death detection kit (Roche Diagnostics, Basel, Switzerland) following the manufacture's protocol. For collagen deposition determination, the sections were stained with Sirius red solution (Solarbio). The stained

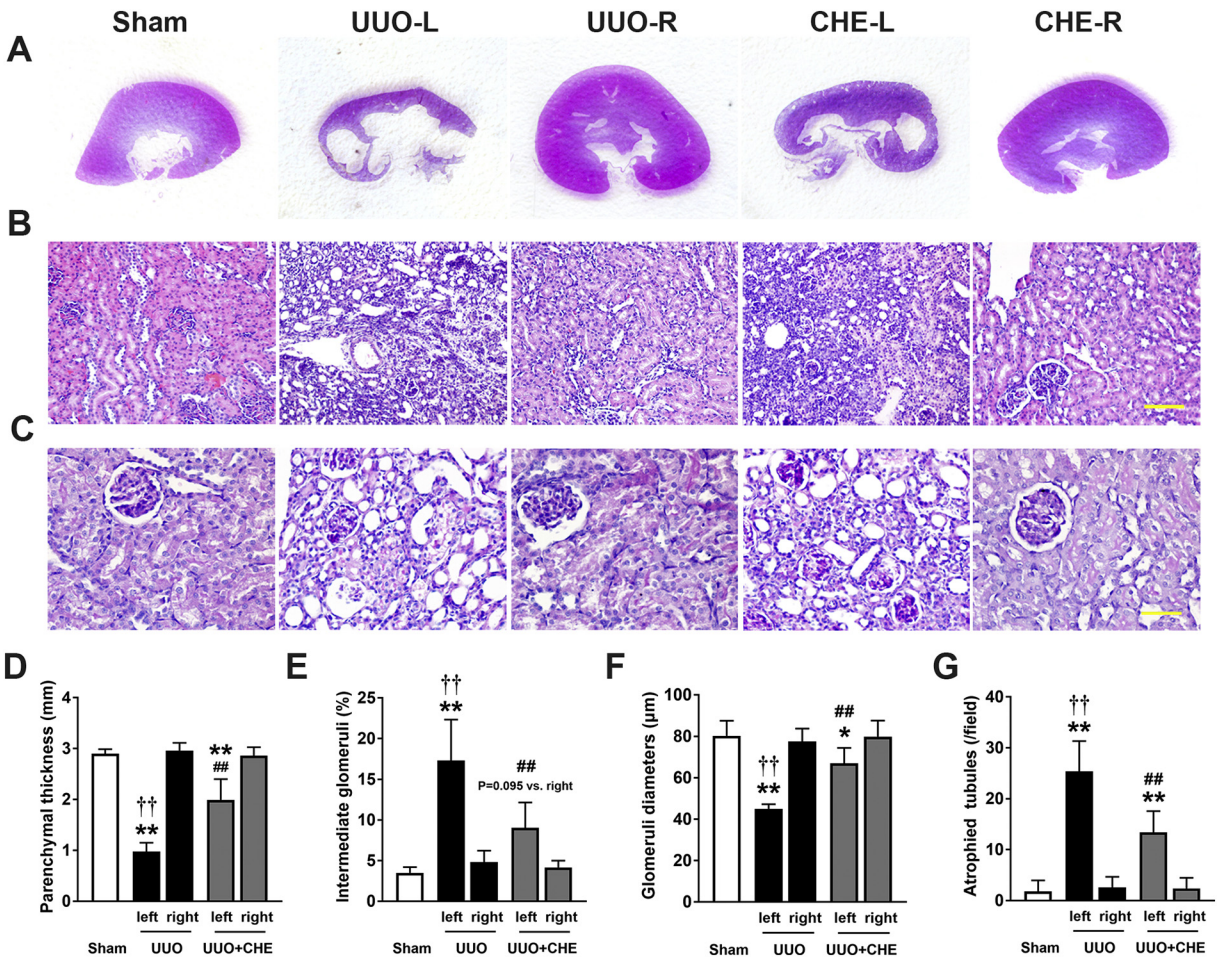

Fig. 2. CHE attenuates partial obstruction-induced renal injury in neonatal rats. A and B, H&E staining, C, PAS staining, D, analysis of parenchymal thickness of the kidneys according to the H&E staining, E, F and G, analyses of the proportion of intermediate glomeruli, glomeruli diameters, and atrophied tubules, respectively, according to the PAS staining. Scale bars: 100 μm. Data are expressed as the mean ± SD (n = 5). *P < 0.05 vs. the sham-operated kidneys, **P < 0.01 vs. the sham-operated kidneys, $^{\#\#}$P < 0.01 vs. the obstructed kidneys, ††P < 0.01 vs. the contralateral kidneys.

sections were observed under an optic microscope (DP73, Olympus, Tokyo, Japan). The immunohistochemical and Sirius red staining were quantified using a computerized image-analysis program (Image Pro-plus 6.0).

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from the renal tissues was isolated using a Total RNA Extraction kit (BioTeke Corporation, Beijing, China) following the manufacturer's protocol. The reverse transcription was performed using oligonucleotide primer and super M-MLV (BioTeke, Beijing, China). The quantitative PCR reaction mixture of 20 μL volume contained 1 μL cDNA template, 10 μL 2 × Power Taq PCR Master Mix (BioTeke), 0.3 μL SYBR Green (Solarbio), and 0.5 μL of each primers of kidney injury molecule-1 (KIM-1) gene (*KIM-1*: forward: 5′-ATTCCCACAAGTCCTCC AAC-3′, reverse: 5′-TGTCACAGTGCCATTCCAGT-3′). The reactions were performed on an Exicycler 96 (Bioneer, Daejeon, Korea). The *KIM-1* mRNA levels were normalized against β-actin and presented as $2^{-\Delta\Delta Ct}$.

2.5. Membrane and cytosol protein extraction and Western blot analysis

The membranous and cytosolic protein of the renal tissues was extracted using the Membrane and Cytosol Protein Extraction Kit (Beyotime) following the manufacture's protocol. The concentration of the protein was determined using the BCA Protein Assay Kit (Beyotime). Proteins were separated on 10% acrylamide gels by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis, transferred to polyvinylidene fluoride membranes, and incubated with anti-kidney injury molecule-1 (KIM-1) antibody (1:1000, ab47634, Abcam, Cambridge, UK), anti-PKCα antibody (1:500, #2056, Cell Signaling Technology, Danvers, MA, USA), anti-PKCβ antibody (1: 500, D261349, Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China), anti-Na^+/K^+-ATPase antibody (1:200, sc-58628, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-β-actin (1: 500, bsm-33139M, Bioss, Beijing, China) overnight at 4 °C. The membranes were then washed with PBST and incubated with bovine anti-goat or anti-mouse secondary antibody (1:5000, Beyotime) at 37 °C for 45 min. The protein blots were visualized by using ECL solution (Beyotime) and the grey values were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). All the expressions of proteins in the membrane were normalized to Na^+/K^+-ATPase and that in the cytosol were normalized to β-actin.

2.6. Statistical analysis

All data were presented as mean ± standard deviation (SD). In the examination of renal function, the difference between the data from unilateral kidney was analyzed using two-way analysis of variance (ANOVA). In the other examinations, the difference between the data from left kidney of the sham, UUO, and UUO + CHE groups were analyzed using one-way ANOVA. The difference between the data from

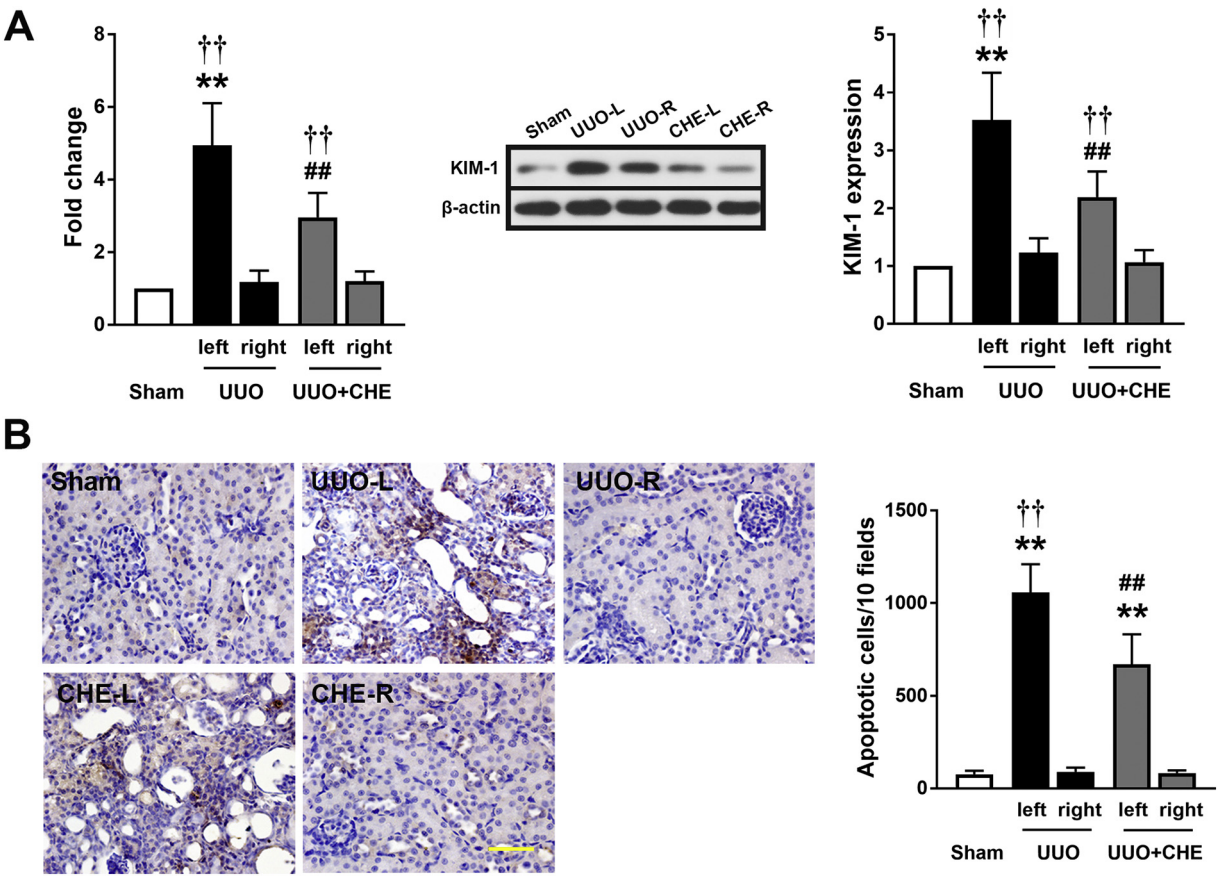

Fig. 3. Effects of CHE on proliferation and apoptosis of tubular cells in partial UUO neonatal rats. A, Quantitative real-time PCR and Western blot analysis s of KIM-1 expression. B, TUNEL staining and quantitative analysis of TUNEL-positive cells. Data are expressed as the mean ± SD (n = 5). **P < 0.01 vs. the sham-operated kidneys, ##P < 0.01 vs. the obstructed kidneys, ††P < 0.01 vs. the contralateral kidneys.

unilateral kidney of the UUO, and UUO + CHE groups were analyzed using two-way ANOVA. The post hoc analysis was using Tukey's test. P value < 0.05 was considered significant difference.

3. Results

3.1. CHE improves renal function in partial UUO neonatal rats

In our study, neither the partial UUO operation nor the CHE administration affected the body weight of the neonatal rats (Fig. 1A). In the sham operated rats, the kidney weight/body weight ratio, urine flow rates and urine protein in left and right kidney were almost the same (Fig. 1). In the UUO rats, the partially obstructed kidney weight was significantly lighter than the intact contralateral kidney and the ipsilateral kidney in the sham operated rats (Fig. 1B). In addition, BUN and serum creatine were significantly increased compared with the sham operated rats (Fig. 1C and D). Furthermore, the urine flow was markedly reduced and the urine protein content was significantly enhanced in the obstructed kidney compared with the intact contralateral kidney and the ipsilateral kidney in the sham operated rats (Fig. 1E and F). These indexes in the intact contralateral kidney were not different from that of sham-operated rats. After CHE administration for 20 days, kidney weight and urine protein of the partially obstructed kidney were partially but significantly restored. However, the urine flow was not changed.

3.2. CHE attenuates partial obstruction-induced renal injury in neonatal rats

The left and right kidney in the sham rats showed the same renal functions. Thus, we selected the left kidney, the ipsilateral kidney of the partially obstructed kidney in the UUO rats, as the sham-operated control for the following examinations.

As illustrated in the results of H&E (Fig. 2A and B) and PAS (Fig. 2C) staining, the kidney of sham-operated rats showed well organized structure and normal morphology of tubules glomeruli. In the partial obstructed kidneys, parenchyma was significantly thinned (Fig. 2A and D) and the histological structure was disordered (Fig. 2B). In addition, unmatured glomeruli and atrophied tubules were found in the partial UUO kidney (Fig. 2C, E–G). According to our results, the partial UUO did not affect the structure of the contralateral kidney. After CHE treatment, these pathological injuries were partially but significantly attenuated.

3.3. CHE modulates and apoptosis and KIM-1 expression in partial UUO neonatal rats

To investigate the tubular injury in partial UUO kidney, Expression of KIM-1 and apoptosis in the kidney were examined. The results of qRT-PCR and Western blot analyses demonstrated that the mRNA and protein expression levels of KIM-1, a hallmark of tubular injury, were markedly upregulated in the obstructed kidneys, and CHE treatment downregulated its expression in both mRNA and protein levels (Fig. 3A). In addition, compared with the sham-operated kidneys, partial UUO induced significantly increased number of TUNEL-positive cells in the kidney, and CHE administration partially but significantly reduce the number of apoptotic cells (P < 0.01 compared with the partial UUO kidneys) (Fig. 3B). The intact contralateral kidney showed no difference compared with the sham-operated kidneys.

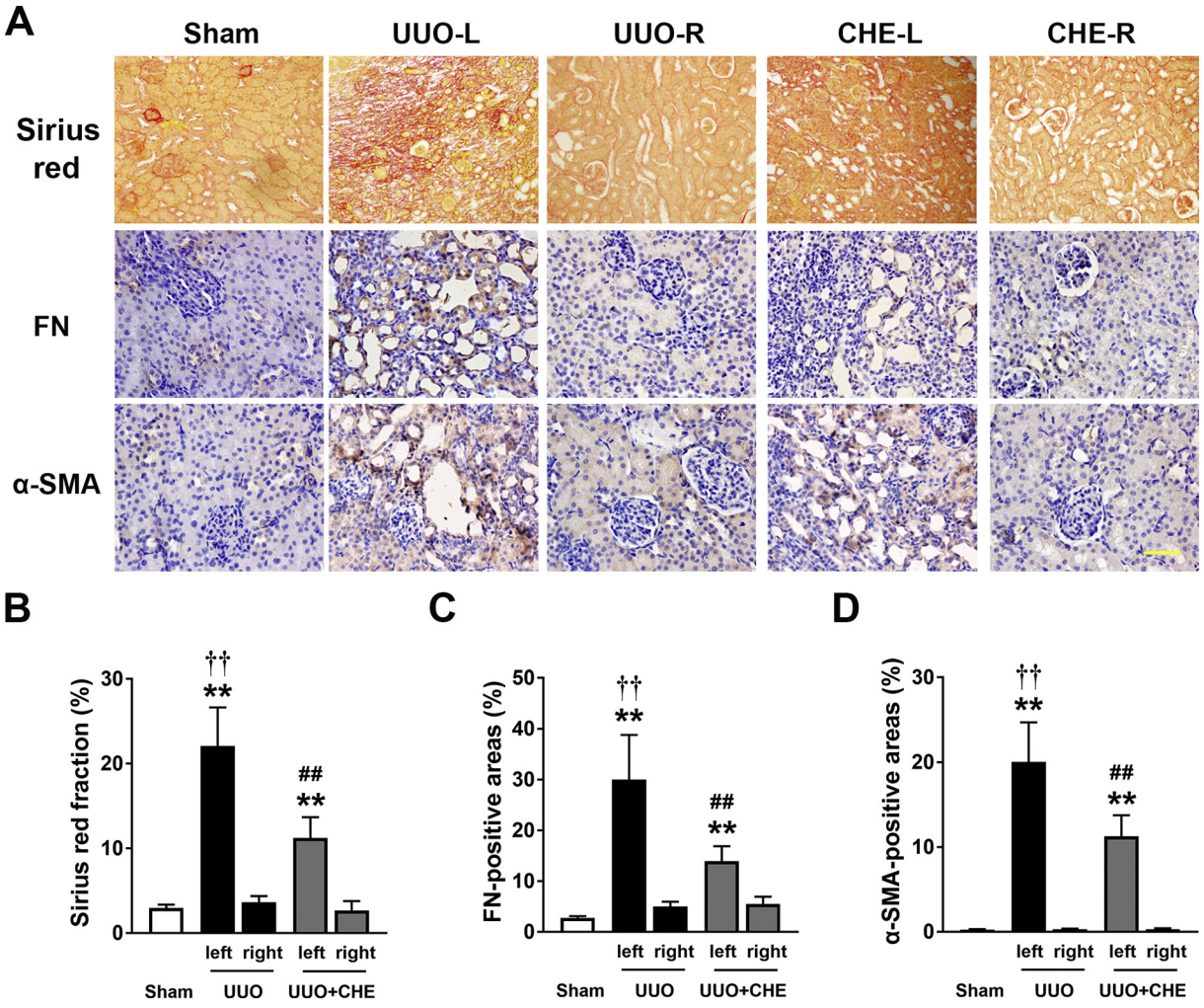

Fig. 4. CHE attenuates partial obstruction-induced renal injury in neonatal rats. A, Sirius red staining and fibronectin and α-SMA immunohistochemical staining. B, analysis of Sirius red staining, C and D, quantitative analysis of fibronectin and α-SMA-positive areas. Scale bar: 100 μm. Data are expressed as the mean ± SD (n = 5). **P < 0.01 vs. the sham-operated kidneys, ##P < 0.01 vs. the obstructed kidneys, ††P < 0.01 vs. the contralateral kidneys. FN: fibronectin.

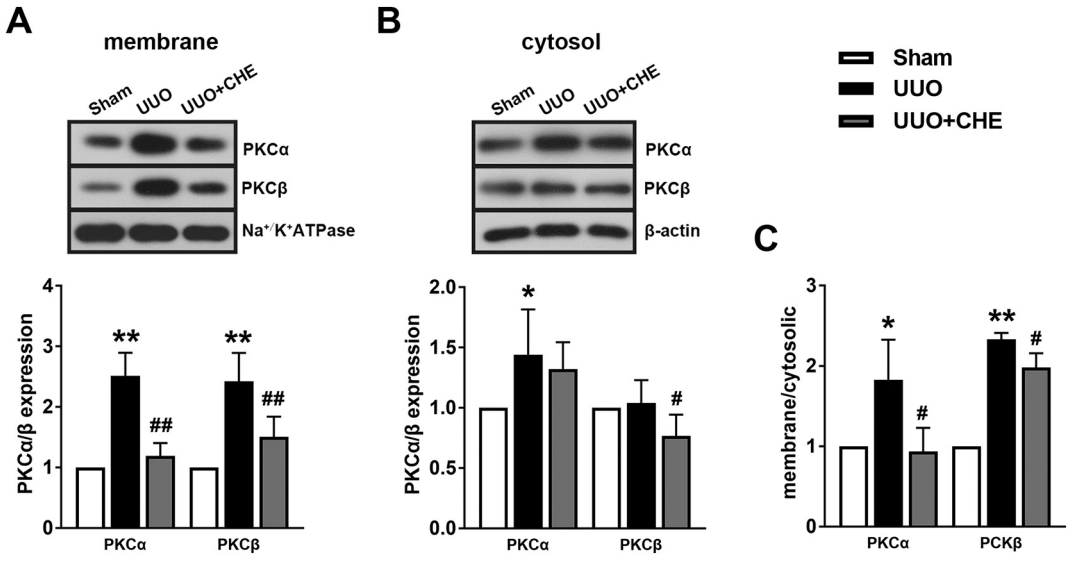

Fig. 5. Effects of CHE on PKC expression in the kidneys of partial UUO neonatal rats. A, Western blot analysis of the expressions of PKCα and β in cell membrane of the kidneys, B, Western blot analysis of the expressions of PKCα and β in cell cytosol of the kidneys, C, ratio of membranous to cytosolic PKC α and PKC β expression. Data are expressed as the mean ± SD (n = 5). **P < 0.01 vs. the sham-operated kidneys, #P < 0.05 vs. the obstructed kidneys, ##P < 0.01 vs. the obstructed kidneys.

3.4. CHE attenuates renal fibrosis in partial UUO neonatal rats

In the results of Sirius red staining, markedly increased collagen deposition was found in the partially obstructed kidneys (Fig. 4A and B). In addition, significantly increased fibronectin and α-SMA-positive staining were found in tubulointerstitium of partially obstructed kidneys compared with the sham-operated kidneys and the intact contralateral kidney (Fig. 4A, C and D). In contrast, CHE treatment significantly reduced collagen deposition and downregulated fibronectin and α-SMA expressions in partially-obstructed kidneys.

3.5. CHE inhibits the expression of PKC proteins in the kidneys

Western blotting analysis was used to examine the expression of PKC proteins in the kidneys after treatment of CHE. As shown in Fig. 5A and B, the protein expression level of PKCα was significantly increased in cellular membrane and cytosol in the partial obstructed kidneys. The expression level of PKCβ was also enhanced in membrane but not in cytosol (Fig. 5B). In addition, the ratio of the expression of PKCα and β was markedly increased after the partial UUO surgery, which indicated a translocation of PKC from cytosol to membrane (Fig. 5C). However, the PKC inhibitor CHE treatment obviously suppressed the expressions of PKCα and β in the partial obstructed kidneys and inhibited their membranous translocation.

4. Discussion

Our study aims to extend the renoprotective effects of CHE in adult UUO to in neonatal UUO and to evaluate its anti-apoptotic and anti-fibrotic effects on neonatal UUO rats. In rats, the renal development at birth is incomplete, which is similar to that of the midtrimester human fetus. Thus, this model can mimic the development of CON in humans [28]. Consistent with the previous study, we found an obvious loss of weight in the partial obstructed kidneys. Meanwhile, BUN, serum creatinine and urine protein were markedly increased in the same kidneys, which reflects the damage of renal functions. CHE treatment led to significantly increase in kidney weight and decrease in urine protein in the partial obstructed kidneys, which indicates that CHE may have protective effects in partial UUO neonatal rats.

Partial UUO leads to increased intratubular pressure and decreased glomerular filtration rate, which finally induce tubular atrophy and decreased glomerular volume [31]. In our pathological examinations, an obvious renal parenchymal atrophy was observed in the partial obstructed kidneys, which is corresponding to the loss of kidney weight. In addition, the diameters of glomeruli were decreased and numerous immature glomeruli appeared in the partial obstructed kidneys, which reflect the delay of renal maturation following partial UUO. Tubular injury was also found in the partial obstructed kidneys, as evidenced by the obvious tubular atrophy. Although not completely, CHE treatment of 20 days significantly attenuated partial UUO-induced renal injury, and this effect is associated with the promotion of glomerular maturation and alleviation of tubular atrophy.

Tubular cell injury is an important pathological feature in obstructive nephropathy [32] which occurs at early stage of UUO before renal fibrosis formation. It is induced by the increased intratubular pressure through the caspase pathways. Inhibition of apoptosis can attenuate the following inflammation and fibrosis in obstructed kidneys [33,34]. In the present study, KIM-1, a biomarker for detection of tubular injury in kidney diseases [35] was significantly upregulated in the obstructed kidneys, which indicated the injury of tubules. In addition, TUNEL staining showed that TUNEL-positive cells in kidney was markedly increased after partial UUO in neonatal rats, which indicates that apoptosis are stimulated after partial UUO operation. This result is agreement with the findings in the adult partial UUO [19]. Several PKC isoforms have been reported to be associated with apoptosis including PKCα and PKCβ [36]. According to the existing studies, PKC can regulate both pro- and anti-apoptotic signaling pathways [37]. PKC activates caspase 3 and stimulates cytochrome *c* release [38] to promote cellular apoptosis and phosphorylates Bcl-2 on Ser70 to stabilize Bcl-2 and prevent apoptosis [39]. In this study, partial UUO induced dramatically increased membrane translocation of PKCα and PKCβ, and PKC inhibitor CHE administration completely inhibited PKCα translocation and partially inhibited PKCβ translocation. Meanwhile, the upregulated KIM-1 expression and apoptosis in the obstructed kidneys were diminished. These findings suggest that the anti-apoptotic effect, which may be mediated by PKC inhibition, may contribute to the renal protection of CHE.

Fibrosis is another important pathological character occurred at the late stage in obstructive nephropathy [6]. It is a slow process and is considered to be the final common pathway in various end-stage renal disease [40]. Fibrosis is characterized by aberrant fibroblast proliferation and excess accumulation of extracellular matrix [41]. In the present study, the occurrence of α-SMA expression and increased expression of fibronectin, the markers of myofibroblasts, reflected the abnormal activation of fibroblasts. In line with the changes of these fibrosis-associated proteins, the results of Sirius red staining showed the large fibrotic areas in the obstructed kidneys. CHE treatment inhibited the expressions of α-SMA and fibronectin and attenuated renal fibrosis. Previous studies showed that PKC is involved in the process of fibrosis in different organs including kidney [16]. Juan et al. showed that inhibit PKC by CHE could prevent fibrosis in adult partial UUO [19]. Here we expand this effect to neonatal partial UUO.

The therapeutic effects of PKC inhibitor on renal diseases have been demonstrated in some investigations. Various signaling pathways have been revealed to be involved in the therapeutic mechanisms of PKC inhibitors, including MAPK signaling pathway, TGF-β1 signaling, autophagic process [42,43]. The present study found that a PKC inhibitor CHE prevented apoptosis and fibrosis induced by partial UUO in neonatal rats. The anti-apoptotic and anti-fibrotic actions of CHE may contribute to its renoprotective functions. However, the detailed pharmacological mechanisms of CHE did not include in the present study. The associated protein signals and whether these effects of CHE are PKC inhibition-dependent will be revealed in future studies.

The present study firstly investigates the effects of PKC inhibitor CHE on kidney disease in neonates. This short-term study suggests that CHE is beneficial for obstructive nephropathy in neonatal rats and provides foundation for further studies to reveal the long-term effects of CHE on obstructive nephropathy in children and infants.

CRediT authorship contribution statement

Bo Shi: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing - original draft. **Shixing Li:** Data curation, Formal analysis, Methodology, Software. **Hao Ju:** Methodology. **Xin Liu:** Methodology. **Dan Li:** Methodology. **Ying Li:** Conceptualization, Investigation, Project administration, Resources, Supervision, Validation, Writing - review & editing.

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