

Demethylzeylasteral ameliorates inflammation in a rat model of unilateral ureteral obstruction through inhibiting activation of the NF- κ B pathway

QIANG WANG^{1*}, YANMEI XIAO^{2*}, TONGWEI LIU³, HAIBO YUAN³ and CHUNWU LI³

¹Department of Urology, The First Central Hospital of Baoding; Departments of ²Nephrology and ³Urology, The 252nd Hospital of The PLA, Baoding, Hebei 071000, P.R. China

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Abstract. The present study investigated the pharmacodynamic role and therapeutic mechanism of demethylzeylasteral in the suppression of inflammation in a rat model of unilateral ureteral obstruction and reduction in nuclear factor (NF)- κ B pathway activity. The rats in the unilateral ureteral obstruction model were treated with 30-120 mg/kg demethylzeylasteral for 8 weeks. The activities of tumor necrosis factor (TNF)- α , interleukin (IL)-6 and caspase-3/9, and the protein expression levels of cyclooxygenase (COX)-2 and intercellular adhesion molecule-1 (ICAM-1) and NF- κ B p65 were analyzed using ELISA kits and western blot analyses, respectively. Compared with the rats in the unilateral ureteral obstruction model group, demethylzeylasteral treatment markedly inhibited the increased concentrations of serum creatinine and blood urea nitrogen, urinary protein/creatinine ratio, and concentrations of high-density lipoprotein and low-density lipoprotein cholesterol, and prevented kidney damage. In addition, demethylzeylasteral inhibited the levels of TNF- α and IL-6 and suppressed the protein expression levels of COX-2 and ICAM-1 in the kidneys of the rats in the unilateral ureteral obstruction model. Demethylzeylasteral also significantly suppressed the protein expression of NF- κ B p65. The results of the present study suggested that demethylzeylasteral unilateral ureteral obstruction and inhibited inflammation via inhibiting the activation of COX-2, ICAM-1 and NF- κ B p65, and suppressing the activities of caspase-3/9 in rats with unilateral ureteral obstruction.

Introduction

The morbidity rates of chronic kidney disease (CKD) are increasing yearly, however, no effective therapeutic methods have been found (1). Consequently, CKD has become a public health issue, which seriously threatens the health of individuals. Previous studies have predicted that ~23-36% of the worldwide population suffer from CKD (1,2). In central China, ~10.49% of the population suffer from at least one type of disease resulting in renal injury (3). CKD has been generally considered to be closely associated with mortality rates in the young. It is characterized by high rates of mortality, morbidity and cardiovascular disease, whereas rates of control and awareness are low and have various complications. It has been suggested that, regardless of the cause of renal function injury, it is significantly associated with renal fibrotic lesions (2).

At present, as an experimental model can be readily and conveniently established through rat unilateral urethral obstruction, it is used extensively in renal interstitial fibrosis assessments (4). In addition, following model establishment, higher survival rates and a lower incidence of complications occur in the stable model, which can effectively reduce costs and experimental durations (5). A literature review found that unilateral urethral obstruction models have been used in the majority of studies on renal interstitial fibrosis and conclusions have been recognized generally (6). This is sufficient to confirm that this model is stable and has widespread approval from scholars (7).

As an important factor for the control of genetic transcription, nuclear factor (NF)- κ B may be activated by the stimulation of cytokines, protein kinase, viruses and oxidizing agents (8). The activated NF- κ B can induce the expressions of cytokines, growth factors and chemotactic factors, and be involved in pathological processes, including inflammatory responses, fibrosis, tumor development and atherosclerosis (9). Activated NF- κ B can promote the transcription of monocyte chemoattractant protein-1, the excessive expression of which reduces the infiltration of macrophages and interstitial fibrosis (10,11).

Demethylzeylasteral (molecular weight, 480; molecular formula, $C_{29}H_{36}O_6$) is extracted from *Tripterygium wilfordii* (12). It can decrease the receptor activities of

Correspondence to: Dr Qiang Wang, Department of Urology, The First Central Hospital of Baoding, 320 The Great Wall, Baoding, Hebei 071000, P.R. China
E-mail: qiangwangqiang@yeah.net

*Contributed equally

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interleukin (IL)-2 through inhibition of the transformation of T cells, and the formation and release of IL-2 (13). Therefore, demethylzeylasteral may be a drug with high immunosuppressive and low myelosuppressive activities. In addition, according to assessments of the acute and subacute toxicity of demethylzeylasteral, it has not been found to cause renal toxicity (14). In the present study, it was hypothesized that demethylzeylasteral ameliorates inflammation in a rat model of unilateral ureteral obstruction, which was investigated, and it was demonstrated that suppression of the NF- κ B pathway were investigated.

Materials and methods

Animals and establishment of the model of unilateral ureteral obstruction. Specific pathogen-free male Sprague-Dawley rats weighing 220–250 g were purchased from Hebei University Laboratory Animal Center (Hebei, China) and were maintained with a standard laboratory diet under controlled indoor temperature ($22\pm1^{\circ}\text{C}$) and humidity (65–70%) conditions. The animal experiments were approved by the Institute of The 252nd Hospital of the PLA (Baoding, China). All male Sprague-Dawley rats were randomly and equally divided into five groups (A–E; $n=10$ rats/group) as follows: Group A, control group; Group B, model group; Group C, 30 mg/kg demethylzeylasteral group (Aburaihan Pharmaceutical Company, Tehran, Iran; Fig. 1); Group D, 60 mg/kg demethylzeylasteral group; Group E, 120 mg/kg demethylzeylasteral group. The male Sprague-Dawley rats were anesthetized with 2% pentobarbitalum natricum (40 mg/kg). Under anesthesia, the left ureter of each rat was exposed and separated through a flank incision. The left ureter was then ligated with 4-0 silk, severed between the ligatures and left undisturbed. Following 8 weeks of treatment with demethylzeylasteral, kidney samples and blood were collected, fixed in 4% neutral-buffered formalin and stored at -80°C .

Laboratory biochemical analysis. Following 8 weeks of treatment with demethylzeylasteral, urine and serum samples from the Sprague-Dawley rats were collected and analyzed at The 252nd Hospital of the PLA. The concentrations of urinary protein and serum creatinine, and blood urea nitrogen, the urinary protein/creatinine (Up/Ucr) ratio, and serum high-density lipoprotein (HDL)-cholesterol (C) and low-density lipoprotein (LDL)-C concentrations were measured.

Histopathological examinations. Following 8 weeks of treatment with demethylzeylasteral, the Sprague-Dawley rats were sacrificed with excess pentobarbitalum natricum. The kidney samples were collected and fixed in 4% neutral-buffered formalin. Subsequently, the paraffin sections (4 μm) were stained with hematoxylin and eosin to reveal histopathological lesions which were visualized using a Zeiss Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany). Kidney samples were evaluated using a semi-quantitative scale from 0 to 3: Score 0, absent; score 1, mild; score 2, moderate; score 3, severe.

Analyses of TNF- α , IL-6, caspase-3/9 activities using ELISA. Following 8 weeks of treatment with demethylzeylasteral, the

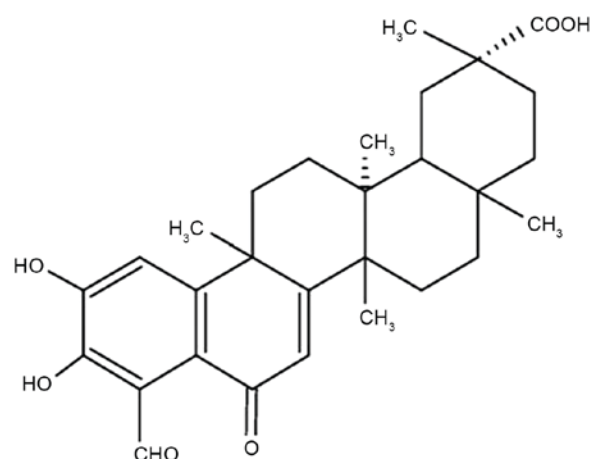


Figure 1. Chemical structure of demethylzeylasteral.

Sprague-Dawley rats were sacrificed with excess pentobarbitalum natricum. Blood samples (500 μl) were collected and centrifuged at $10,000 \times g$ for 10 min at 4°C . The serum was then collected to analyze the activities of TNF- α , IL-6 and caspase-3/9 using ELISA kits.

Western blot analysis. Following 8 weeks of treatment with demethylzeylasteral, the Sprague-Dawley rats were sacrificed with excess pentobarbitalum natricum. Kidney tissue samples were collected and lysed in RIPA buffer. Proteins were quantified using a BCA assay according to the manufacturer's protocol (Roche Diagnostics, Basel, Switzerland). The proteins (50 μg) were electrophoresed on a 10% polyacrylamide gel containing SDS and transferred onto nitrocellulose membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked with TBS-0.05% Tween-20 (TBST) containing 5% non-fat milk for 2 h at room temperature, and then incubated with anti-cyclooxygenase-2 (COX-2; 1:3,000; cat. no. 12282; Cell Signaling Technology, Inc., Boston, MA, USA), anti-intercellular adhesion molecule-1 (ICAM-1; 1:3,000; cat. no. ab20; Abcam, Cambridge, UK), anti-NF- κ B p65 (1:3,000; cat. no. 8242; Cell Signaling Technology, Inc.) and β -actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.) overnight at 4°C . The membranes were washed with TBST and then incubated with mouse anti-rabbit IgG-HRP (1:5,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature and observed using an enhanced ECL chemiluminescence system (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Experiments were repeated three times. Quantification was performed using Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are expressed as the mean \pm standard error of the mean. The significance of results obtained from the control and treated groups was determined by one-way analysis of the variance followed by the Newman-Keuls test for multiple comparisons using SPSS version 20.0 software (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

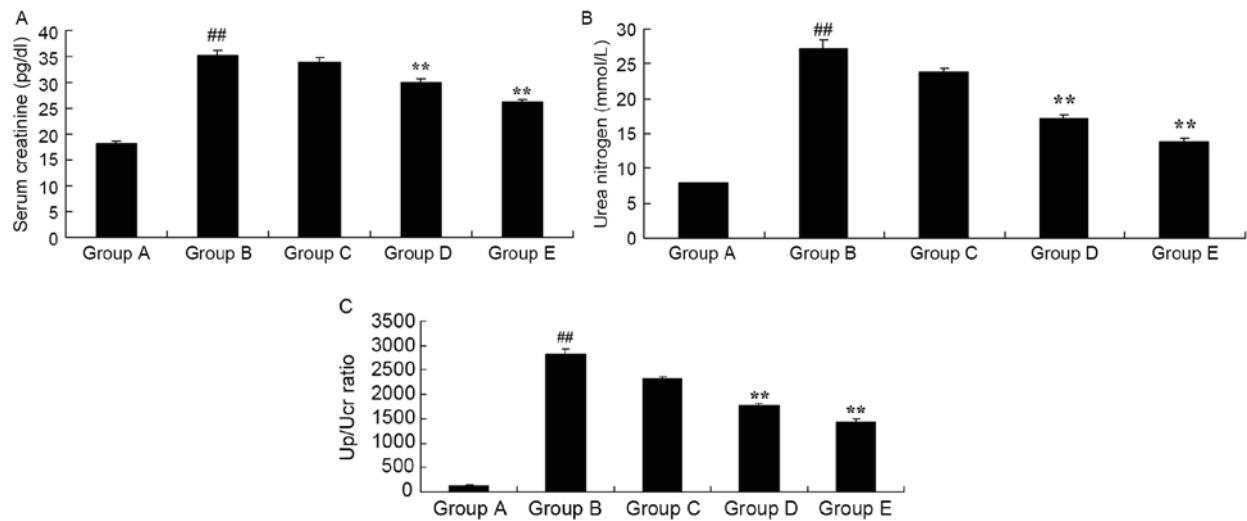


Figure 2. Demethylzeylasteral ameliorates biochemical indicators in urine/serum in a rat model of unilateral ureteral obstruction. Demethylzeylasteral ameliorated levels of (A) serum creatinine and (B) blood urea nitrogen, and (C) decreased the Up/Ucr ratio in the rat model of unilateral ureteral obstruction. Group A, control; Group B, model; Group C, 30 mg/kg demethylzeylasteral; Group D, 60 mg/kg demethylzeylasteral; Group E, 120 mg/kg demethylzeylasteral. ^{##}P<0.01, compared with Group A; ^{**}P<0.01, compared with Group B. Up/Ucr, urinary protein/creatinine.

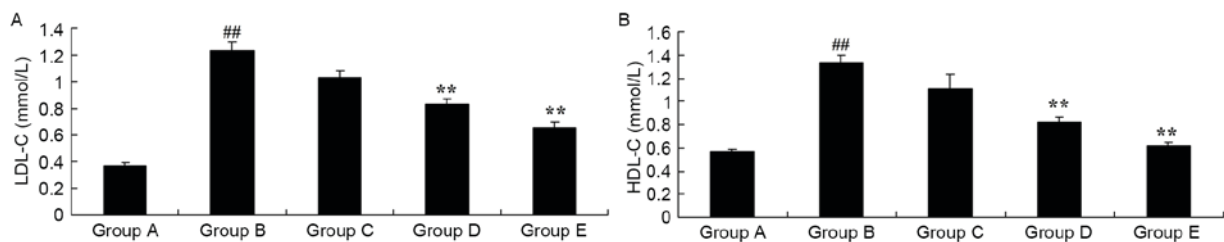


Figure 3. Demethylzeylasteral ameliorates HDL-C and LDL-C concentrations in a rat model of unilateral ureteral obstruction. Demethylzeylasteral ameliorated the concentrations of (A) LDL-C and (B) HDL-C in the rat model of unilateral ureteral obstruction. Group A, control; Group B, model; Group C, 30 mg/kg demethylzeylasteral; Group D, 60 mg/kg demethylzeylasteral; Group E, 120 mg/kg demethylzeylasteral. ^{##}P<0.01, compared with Group A; ^{**}P<0.01, compared with Group B. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

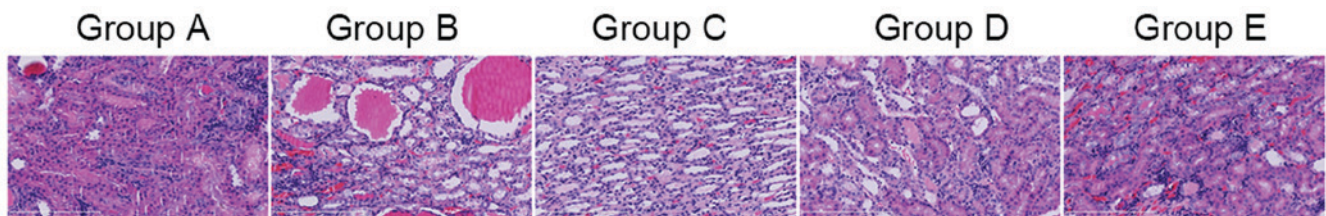


Figure 4. Demethylzeylasteral ameliorates kidney damage in a rat model of unilateral ureteral obstruction (magnification, x40). Group A, control; Group B, model; Group C, 30 mg/kg demethylzeylasteral; Group D, 60 mg/kg demethylzeylasteral; Group E, 120 mg/kg demethylzeylasteral.

Results

Demethylzeylasteral ameliorates the biochemical indicators of urine/serum in a rat model of unilateral ureteral obstruction. The chemical structure of demethylzeylasteral is shown in Fig. 1. Following 8 weeks of treatment with demethylzeylasteral, the levels of serum creatinine, blood urea nitrogen and the Up/Ucr ratio were markedly higher, compared with those in the normal control group (Fig. 2). Demethylzeylasteral treatment (60 or 120 mg/kg) markedly inhibited the increases in serum creatinine, blood urea nitrogen and Up/Ucr ratio in the rat model of unilateral ureteral obstruction (Fig. 2A-C).

Demethylzeylasteral ameliorates HDL-C and LDL-C concentrations in a rat model of unilateral ureteral obstruction. Following 8 weeks of treatment with demethylzeylasteral, the concentrations of HDL-C and LDL-C were examined in the rat model of unilateral ureteral obstruction to evaluate the effect of demethylzeylasteral on unilateral ureteral obstruction. The concentrations of HDL-C and LDL-C were also markedly higher, compared with those in the control normal rat (Fig. 3A and B). Treatment with 60 or 120 mg/kg of demethylzeylasteral significantly inhibited HDL-C and LDL-C concentrations in the rat model of unilateral ureteral obstruction (Fig. 3).

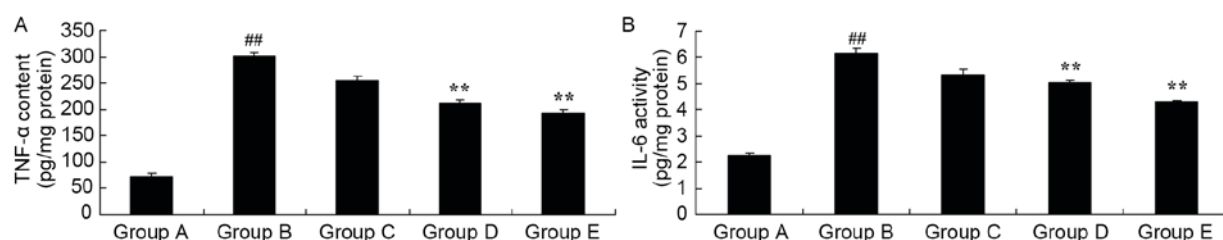


Figure 5. Demethylzeylasteral ameliorates TNF- α and IL-6 activities in a rat model of unilateral ureteral obstruction. Demethylzeylasteral ameliorated the activities of (A) TNF- α and (B) IL-6 in the rat model of unilateral ureteral obstruction. Group A, control; Group B, model; Group C, 30 mg/kg demethylzeylasteral; Group D, 60 mg/kg demethylzeylasteral; Group E, 120 mg/kg demethylzeylasteral. ##P<0.01, compared with Group A; **P<0.01, compared with Group B. TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6.

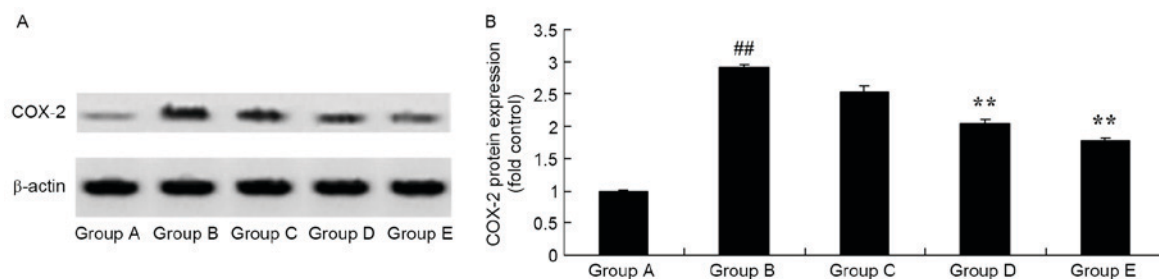


Figure 6. Demethylzeylasteral ameliorates the protein expression of COX-2 in a rat model of unilateral ureteral obstruction. (A) Demethylzeylasteral ameliorated the protein expression of COX-2, determined using western blot analysis. (B) Statistical analysis of the protein expression of COX-2 in the rat model of unilateral ureteral obstruction. Group A, control; Group B, model; Group C, 30 mg/kg demethylzeylasteral; Group D, 60 mg/kg demethylzeylasteral; Group E, 120 mg/kg demethylzeylasteral. ##P<0.01, compared with Group A; **P<0.01, compared with Group B. COX-2, cyclooxygenase-2.

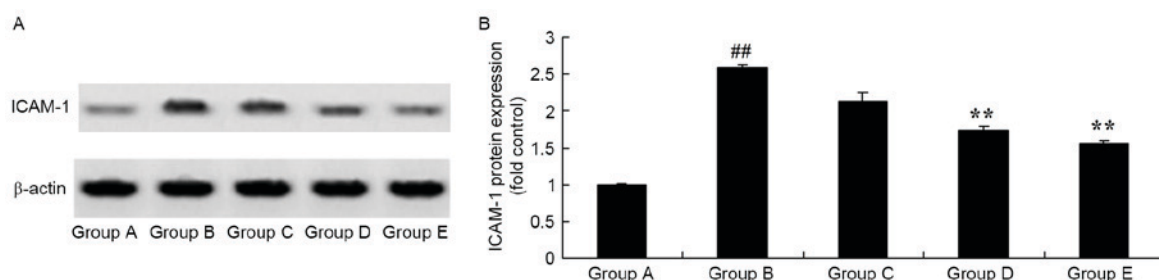


Figure 7. Demethylzeylasteral ameliorates the protein expression of ICAM-1 in a rat model of unilateral ureteral obstruction. (A) Western blot analysis revealed that demethylzeylasteral ameliorated the protein expression of ICAM-1. (B) Statistical analysis of the protein expression of ICAM-1 in the rat model of unilateral ureteral obstruction. Group A, control; Group B, model; Group C, 30 mg/kg demethylzeylasteral; Group D, 60 mg/kg demethylzeylasteral; Group E, 120 mg/kg demethylzeylasteral. ##P<0.01, compared with Group A; **P<0.01, compared with Group B. ICAM-1, intercellular adhesion molecule-1.

Demethylzeylasteral ameliorates kidney damage in a rat model of unilateral ureteral obstruction. Following 8 weeks of treatment, histopathological examinations were performed to evaluate the protective effect of demethylzeylasteral on kidney damage in the rat model of unilateral ureteral obstruction. The kidney damage score of the unilateral ureteral obstruction model group was higher, compared with that of the control group (Fig. 4). Treatment with 60 or 120 mg/kg demethylzeylasteral significantly prevented kidney damage in the rat model of unilateral ureteral obstruction (Fig. 4).

Demethylzeylasteral ameliorates TNF- α and IL-6 activities in a rat model of unilateral ureteral obstruction. To investigate whether demethylzeylasteral has an anti-inflammatory effect in a rat model of unilateral ureteral obstruction, the present study examined the activities of TNF- α and IL-6 using ELIST

kits. As shown in Fig. 5, there were significant increases in the activities of TNF- α and IL-6 in the unilateral ureteral obstruction model, compared with activities in the normal control. Pretreatment with 60 or 120 mg/kg demethylzeylasteral significantly reduced the activities of TNF- α and IL-6 in the unilateral ureteral obstruction group (Fig. 5A and B).

Demethylzeylasteral ameliorates the protein expression of COX-2 in a rat model of unilateral ureteral obstruction. To assess the protein expression of COX-2 in the protective effect of demethylzeylasteral on unilateral ureteral obstruction, the protein expression of COX-2 was examined using western blot analysis. The results of the western blot assay showed that the protein expression of COX-2 in the rats of the unilateral ureteral obstruction group was higher, compared with that in the rats in control group (Fig. 6A and B). However, 60

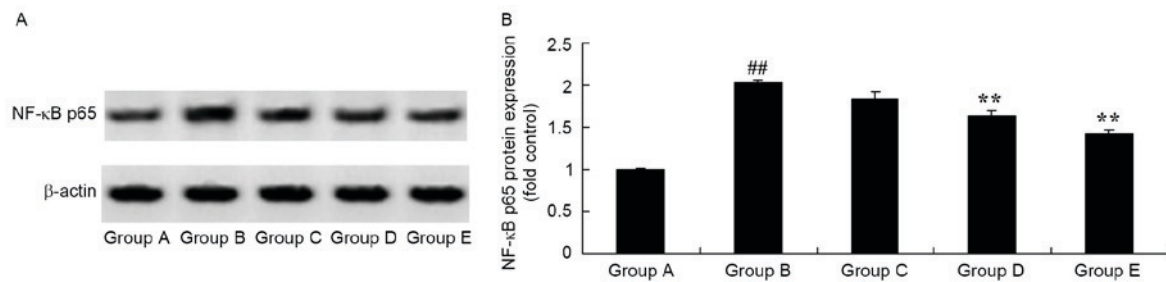


Figure 8. Demethylzeylasteral ameliorates the protein expression of NF-κB p65 in a rat model of unilateral ureteral obstruction. (A) Western blot analysis revealed that demethylzeylasteral ameliorated the protein expression of NF-κB p65. (B) Statistical analysis of the protein expression of NF-κB p65 in a rat model of unilateral ureteral obstruction. Group A, control; Group B, model; Group C, 30 mg/kg demethylzeylasteral; Group D, 60 mg/kg demethylzeylasteral; Group E, 120 mg/kg demethylzeylasteral. ^{##} $P < 0.01$, compared with Group A; ^{**} $P < 0.01$, compared with Group B. NF-κB, nuclear factor-κB.

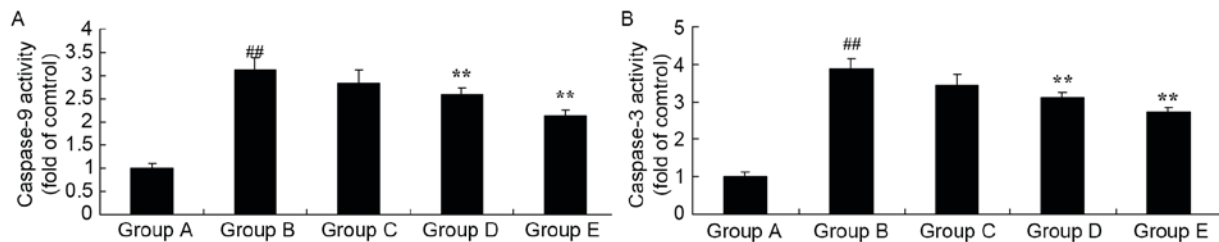


Figure 9. Demethylzeylasteral ameliorates the activities of caspase-3/9 in a rat model of unilateral ureteral obstruction. Demethylzeylasteral ameliorated the activities of (A) caspase-9 and (B) caspase-3 in the rat model of unilateral ureteral obstruction. Group A, control; Group B, model; Group C, 30 mg/kg demethylzeylasteral; Group D, 60 mg/kg demethylzeylasteral; Group E, 120 mg/kg demethylzeylasteral. ^{##} $P < 0.01$, compared with Group A; ^{**} $P < 0.01$, compared with Group B.

or 120 mg/kg demethylzeylasteral treatment significantly suppressed the protein expression of COX-2 in the unilateral ureteral obstruction group (Fig. 6A and B).

Demethylzeylasteral ameliorates the protein expression of ICAM-1 in a rat model of unilateral ureteral obstruction. To investigate whether ICAM-1 is involved in the protective effect of demethylzeylasteral on unilateral ureteral obstruction; western blot analysis was used to analyze the protein expression of ICAM-1. As shown in Fig. 7A and B, an increase in the protein expression of ICAM-1 was observed in the unilateral ureteral obstruction rats, compared with the normal rats. Treatment with 60 or 120 mg/kg demethylzeylasteral significantly reduced the protein expression of ICAM-1 in the unilateral ureteral obstruction model (Fig. 7).

Demethylzeylasteral ameliorates the protein expression of NF-κB p65 in a rat model of unilateral ureteral obstruction. To elucidate the mechanism underlying the anti-inflammatory effect of demethylzeylasteral in unilateral ureteral obstruction, the protein expression of NF-κB p65 was examined using western blot analysis. Unilateral ureteral obstruction significantly induced the protein expression of NF-κB p65 in the unilateral ureteral obstruction rats (Fig. 8A and B). Treatment with 60 or 120 mg/kg of demethylzeylasteral significantly inhibited the protein expression of NF-κB p65 in the rat model of unilateral ureteral obstruction (Fig. 8).

Demethylzeylasteral ameliorates the activities of caspase-3/9 in a rat model of unilateral ureteral obstruction. To further elucidate the mechanism of demethylzeylasteral on unilateral

ureteral obstruction, cell apoptosis and caspase-3/9 activities were examined in the present study. The activities of caspase-3/9 were significantly increased in the unilateral ureteral obstruction model, compared with normal control group (Fig. 9A and B). Treatment with 60 or 120 mg/kg demethylzeylasteral significantly reduced the activities of caspase-3/9 in the unilateral ureteral obstruction rats (Fig. 9A and B).

Discussion

According to reliable data, the diagnosis of CKD based on the current method of assessment showed that, between 2009 and 2010, the morbidity rate in China was 10.8% and rates worldwide were 10-13% (3). Regardless of the causes of kidney disease, progressive CKD causes the formation of large scars, and finally result in the complete destruction of renal parenchyma and kidney failure at the terminal stage (15). In this state, renal dialysis or renal transplantation is required. CKD has become an extensive public health issue, which threatens the lives of individuals worldwide (16), and the substantial costs involved in prevention, diagnosis and treatment affect individuals worldwide. In the present study, it was found that demethylzeylasteral treatment markedly inhibited the increased concentrations of serum creatinine, blood urea nitrogen and the Up/Ucr ratio, inhibited the concentrations of HDL-C and LDL-C, prevented kidney damage, and reduced the activities of TNF-α and IL-6 in a rat model of unilateral ureteral obstruction. These observations further supported the hypothesis that demethylzeylasteral alleviates unilateral ureteral obstruction through downstream pro-inflammatory mediators.

Comprising the two isoenzymes of COX-1 and COX-2, COX is regarded as a key rate-limiting enzyme in the metabolic link of prostaglandin (17). COX-1 is involved in normal physiological processes whereas COX-2 can mediate pathological alterations (18). Studies have revealed that the proliferation of COX-2 is important in renal tubule interstitial fibrosis caused by ureteral obstruction, and overexpression of COX-2 in the renal cortex may be a promoting factor for inflammatory cell infiltration and accumulation (19,20). In the present study, demethylzeylasteral treatment significantly suppressed the protein expression of COX-2 in the unilateral ureteral obstruction rats (14).

When renal inflammatory injury occurs, adhesion molecules mediate the adhesion of inflammatory cells on vascular walls and move to regions of inflammation, which promotes injuries (21). The continuous invasion of inflammatory cells is an important factor for the expansion of the renal inflammatory response and renal function injury. The two adhesion molecules, ICAM-1 in the immunoglobulin superfamily and P-selectin in the selectin family (21,22), were also examined. ICAM-1 is essential in early renal tubular interstitial fibrosis. P-selectin is present on the surface of activated endothelial cells and an early initiator of the inflammatory reaction (23). The present study found that demethylzeylasteral significantly reduced the protein expression of ICAM-1 in the unilateral ureteral obstruction rats. Hu *et al* (14) also suggested that demethylzeylasteral exhibits reno-protective effects through inhibiting the activation of COX-2 and ICAM-1.

NF- κ B can form a tripolymer by combining with inhibitor of NF- κ B (I κ B). When I κ B degrades and is lost, a dipolymer with transcriptional activity is formed (9). The activated dipolymer has the following functions in renal interstitial fibrosis (8): Firstly, it is associated with fibroblast growth factors generated by fibroblasts and intrinsic renal cells. Secondly, it is involved in forming chemokines and cell adhesion molecules. Thirdly, it facilitates the proliferation and division of fibroblasts (24). TNF can stimulate the separation of NF- κ B with I κ B and is involved in genetic regulation, inflammatory mediators and cytokines, including TNF (25). TNF primarily assembles inflammatory cells and integrates with receptors on the cell surface. Previous studies have found that, following unilateral urethral obstruction, the expression of NF- κ B in the renal interstitium is increased and the expression of renal interstitial collagen is upregulated (26). Peach kernels can also inhibit inflammatory mediators and associated cytokines, including TNF- α and transforming growth factor- β (27). The present study demonstrated that demethylzeylasteral markedly inhibited the protein expression of NF- κ B p65 and the activities of caspase-3/9 in a rat model of unilateral ureteral obstruction. Hu *et al* (14) also suggested that demethylzeylasteral exhibits reno-protective effects through inhibiting the activation of NF- κ B.

In conclusion, the present study demonstrated that demethylzeylasteral treatment markedly inhibited the increased serum creatinine, blood urea nitrogen and Up/Ucr ratio, and the concentrations of HDL-C and LDL-C. It also prevented kidney damage, and reduced the activities of TNF- α and IL-6 in the rat model of unilateral ureteral obstruction. These effects were accompanied by inhibition of the activation of COX-2, ICAM-1 and NF- κ B p65, and suppression of the

activities of caspase-3/9 in the kidney of the rats with unilateral ureteral obstruction. Therefore, these results indicated that demethylzeylasteral may be a novel therapeutic agent for unilateral ureteral obstruction.

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