

BASIC SCIENCE ARTICLE



Erythropoietin prevented the decreased expression of aquaporin1–3 in ureteral obstructive kidneys in juvenile rats

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BACKGROUND: Urinary tract obstruction is associated with impaired renal urinary concentration; even after the release of the obstruction, patients still suffer from polyuria. It has been reported that the decreased expression of aquaporins (AQPs) is associated with postobstructive polyuria, and erythropoietin (EPO) can promote the recovery of decreased AQP2 expression induced by bilateral ureteral obstruction. However, whether EPO can promote the recovery of the expression of AQP1–3 after the release of unilateral ureteral obstruction (UUO) has not yet been reported.

AIMS: To investigate the effects of EPO treatment on the expression of renal AQP1–3 after the release of UUO.

METHODS: UUO was established in rats by 24-h temporary unilateral obstruction of renal ureters. Three days following EPO treatment, the kidneys were removed to determine the expression levels of AQP1–3, NLRP3, caspase-1, and IL-1 β via semiquantitative immunoblotting and immunohistochemistry.

RESULTS: EPO inhibited the expression of NLRP3, caspase-1, and IL-1 β ; reduced plasma creatinine and urea; and promoted the recovery of AQP1–3 expression in UUO rats.

CONCLUSIONS: EPO treatment prevented the decreased expression of renal AQPs and the development of impaired urinary concentration capacity after the release of UUO, which may partially occur by way of anti-inflammasome effects.

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IMPACT:

- EPO treatment could prevent the decreased expression of renal water transporter proteins AQP1–3 and the development of impaired renal functions, which may be associated with its anti-inflammasome effects.
- EPO regulated the expression of renal water transporter proteins AQP1–3, which could provide the potential for the treatment of postobstructive polyuresis.
- EPO treatment could be one of the effective methods by participating in multiple dimensions for patients with obstructive nephropathy.

INTRODUCTION

Ureteral obstruction is a common reason for impaired renal function in children and infants. Congenital urinary tract obstruction-induced obstructive nephropathy is the second most common cause of end-stage renal disease in children.¹ Surgical operation is the choice for many patients, but even after the release of the obstruction, patients still suffer from postobstructive polyuria and natriuresis, which has been demonstrated in children after the release of the obstruction caused by congenital pelvic ureteral junction obstruction.² Moreover, urinary tract obstruction is also associated with an obvious interstitial inflammatory response, which may contribute to the impaired urine concentration.^{3,4}

Aquaporins (AQPs) compose a membrane protein family, function as water channels, and play an important role in water reabsorption in the kidney. AQP1, located in the proximal tubules

and the descending thin limb of the Henle loop, has been demonstrated to be significantly downregulated in the kidneys of rats within 24 h unilateral ureteral obstruction (UUO) and bilateral ureteral obstruction (BUO).^{5,6} AQP2, located at the apical plasma membrane and intracellular vesicles of the principal cells, and AQP3, located at the basal lateral of the renal collecting duct cells, have also been shown to be markedly downregulated in rat kidneys after 24 h of UUO and BUO.^{5,6} The decreased expression of these water channel proteins was shown to be associated with postobstructive diuresis.⁷ The mechanisms responsible for the alterations in water channel proteins are not yet clear.

Inflammation can be triggered without pathogens, which is referred to as sterile inflammation and is mediated through the inflammasome.^{8,9} Recent studies have suggested that the NLRP3 inflammasome plays a pivotal role in the progression of various kidney diseases, including obstructive nephropathy, and that

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pharmacological or genetic inhibition of NLRP3 could alleviate renal injuries.^{10–12} The NLRP3 inflammasome is a multiprotein complex consisting of the sensor protein NLRP3, the adapter protein ASC and the effector protein caspase-1.^{13,14} Activation of NLRP3 leads to the assembly of the adapter ASC, which can induce the autoactivation and cleavage of pro-caspase-1 into mature caspase-1. Mature caspase-1 can regulate the secretion of IL-18 and IL-1 β , which are proinflammatory cytokines.^{13,14} Ureteral obstruction-induced excessive activation of the NLRP3 inflammasome could be among the potential mechanisms for the downregulation of renal AQPs in ureteral obstructive nephropathy. Inhibition of NLRP3 inflammasome activation might be an effective method to reverse the decreased AQP expression and, at the same time, promote renal function recovery.

Erythropoietin (EPO), originally identified for promoting erythrocyte survival and differentiation, has been demonstrated to be a novel cytoprotective agent that may specifically be useful in protecting the brain, heart, and kidney from injury.¹⁵ Ren CC found that EPO promoted the recovery of the expression of AQP2 in rats after the release of BUO.¹⁶ Gong et al. found that EPO treatment significantly reduced the downregulation of AQP1–3 levels and major renal sodium transporters in rats with bilateral ischemia-induced acute renal failure.¹⁷ However, whether EPO could promote the recovery of AQP1, AQP2, and AQP3 expression after the release of unilateral obstruction is not yet clear.

Therefore, in the present study, we examined whether EPO treatment promotes the recovery of renal AQP1, AQP2, and AQP3 downregulation in rats after the release of UO and explored the relationship between the effects of EPO treatment and NLRP3 inflammasome activation.

MATERIALS AND METHODS

Experimental protocols

All procedures conformed to the Chinese National Guidelines for the Care and Handling of Animals and the published guidelines from the National Institutes of Clinical Medicine, Zhengzhou University, according to the licenses for use of experimental animals issued by the Chinese Ministry of Justice (2020-KY-273). Studies were performed on male (Sprague–Dawley) rats, initially weighing 180–200 g (Henan Experimental Animal Center, Henan, China). The animals were maintained on a standard rodent diet and had free access to water. During the entire experimental period, the rats were kept in individual metabolic cages with a 12/12-h artificial light/dark photoperiod, a temperature of 21 \pm 2°C, and a humidity of 55 \pm 2%.

After 3 days of acclimation to the metabolic cages, experimental UO models were induced by occlusion of the left ureter for 24 h. During surgery, experimental rats were anesthetized with halothane (Sigma-Aldrich) and placed on a heated table to maintain the rectal temperature at 37–38°C. An abdominal median incision exposed the left ureter, and a 5-mm-long piece of bisected polyethylene tube (PE-50) was placed around the importation of the dissected ureter. The ureter was then occluded by tightening the tube with a 5-0 silk ligature. In protocol 1, the obstruction was released after 24 h by inserting a polyethylene tube (PE-35) into the proximal left ureter. A similar catheter was inserted into the proximal right ureter to allow a separate collection of urine from the left and right kidneys. After urine was collected for 2 h, the rats were sacrificed. In protocol 2, the rats were followed for an additional 3 days after the release of 24 h UO, and the urine of both kidneys was collected 2 h before the sacrifice as described in protocol 1.

Control group rats were subjected to sham operations identical to those used for UO rats but without occlusion of the ureter. The sham-operated rats were monitored in parallel with UO rats.

The technique described above that completely occludes the ureters for 24 h was demonstrated without evidence of subsequent functional impairment of ureteral function.⁶

Protocol 1: the rats were divided into a UO group ($n = 8$), UO+EPO group, and sham group ($n = 8$). For the UO and UO+EPO groups, the rats were subjected to UO for 24 h. EPO (2000 IU/kg) (Shanghai Chemo Wanbang Biopharma, China)^{18,19} was given intraperitoneally at the onset of the obstruction for the UO+EPO group, and the rats in the sham group

and the UO group were treated with vehicles (0.9% saline) intraperitoneally. After 24 h of UO, urine was collected as described above, and plasma was collected from the abdominal aorta to examine renal function. Then, the rats were sacrificed.

Protocol 2: after 24-h obstruction, the obstructed ureters were decompressed by removing the silk ligature, after the release of the 24 h UO, the rats were followed for an additional 3 days, during which time EPO was given four times (at the onset of the obstruction, at the time of the release of the obstruction, and at 1 day and 2 days after the release of the obstruction). The groups consisted of a UO-3dR ($n = 8$) group, a UO-3dR+EPO ($n = 8$) group, and a sham ($n = 8$) group. EPO was given intraperitoneally (2000 IU/kg). The rats in the sham group and in the UO group were treated with vehicles intraperitoneally. Because the proximal catheterization could only be performed under anesthesia, to minimize the influence of external factors on the experimental animals, in this protocol, the proximal catheterization was not performed after the release of the obstruction until the third day before the sacrifice of the animals. Three days after the release of UO, urine and plasma were collected as described above, both kidneys were removed and separately prepared for semiquantitative immunoblotting and histochemical studies, and, then, all rats were sacrificed.

Clearance studies

In protocol 1, urine was collected for 2 h after the release of ligation. In protocol 2, urine was collected for 2 h before the collection of the kidney on the third day after the release of the obstruction. During anesthesia and before removal of the kidneys, 2 mL of blood was collected for the examination of plasma electrolytes and osmolality.

Electrophoresis and immunoblotting

Total protein was prepared from whole-kidney samples. Tissues (50 mg) were minced finely and lysed in 500 μ L of Tissue Protein Extraction Reagent (CWBiotech, China) and homogenized by using an electric grinder. The homogenate was centrifuged in an Eppendorf centrifuge at 15,000 \times g for 20 min at 4°C to remove tissue debris, and the supernatant was collected. A BCA protein kit (CWBiotech, China) was used to determine the concentration of the proteins to ensure identical loading.

For each sample, protein extract (30 μ g) was loaded into and separated with sodium dodecyl sulfate–polyacrylamide gels consisting of a 12% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel. The proteins were electrophoretically transferred to a polyvinylidene fluoride membrane. Then, the blots were washed with Tris-buffered saline (TBS) (pH 7.4) containing 0.1% Tween-20 (TBST) and blocked with 5% nonfat milk in TBST for 2 h at room temperature. The membranes were then incubated overnight at 4°C with affinity-purified, anti-rabbit polyclonal antibodies against AQP1 concentration of 1/2000 (Abcam, ab15080, UK), AQP2 concentration of 1/2000 (Abcam, ab15081), AQP3 concentration of 1/500 (Abcam, ab12519), NLRP3 concentration of 1/500 (BA3677, Boster Biological Technology), caspase-1 concentration of 1/1000 (BA0586, Boster Biological Technology), and IL-1 β concentration of 1/500 (GB111103, Servicebio, China). A secondary antibody concentration of 1/7000 (Santa Cruz Biotechnology, Santa Cruz) was used to incubate the membrane for 1 h at room temperature, after which the membranes were then washed in TBST. Labeling was subsequently performed with an enhanced chemiluminescence system (Thermo Fisher Scientific, Rockford), and the labeling density was quantified on the blots.

Immunohistochemistry

Kidneys were fixed with 4% paraformaldehyde buffer, washed three times for 10 min each with phosphate-buffered saline (PBS) buffer, dehydrated, and embedded in wax. The paraffin-embedded tissues were cut into 4- μ m sections via a rotary microtome (RM2016, Leica). The sections were dewaxed and rehydrated. For immunoperoxidase labeling, endogenous peroxidase was blocked by 0.5% H₂O₂ in absolute methanol for 10 min at room temperature. To reveal the antigens, the sections were incubated in 1 mmol/L Tris solution (pH 9.0) supplemented with 0.5 mM ethylenediaminetetraacetic acid and heated in a microwave oven for 10 min. Nonspecific binding of IgG was prevented by incubating the sections in 50 mM NH₄Cl for 30 min, followed by blocking in PBS supplemented with 2% bovine serum albumin (BSA). The sections were incubated overnight at 4°C with rabbit anti-AQP1 (GB11310-1, Servicebio, China), AQP2 (PB9474, Boster Biological Technology), AQP3 (BA1559, Boster Biological Technology), NLRP3 (BA3677, Boster

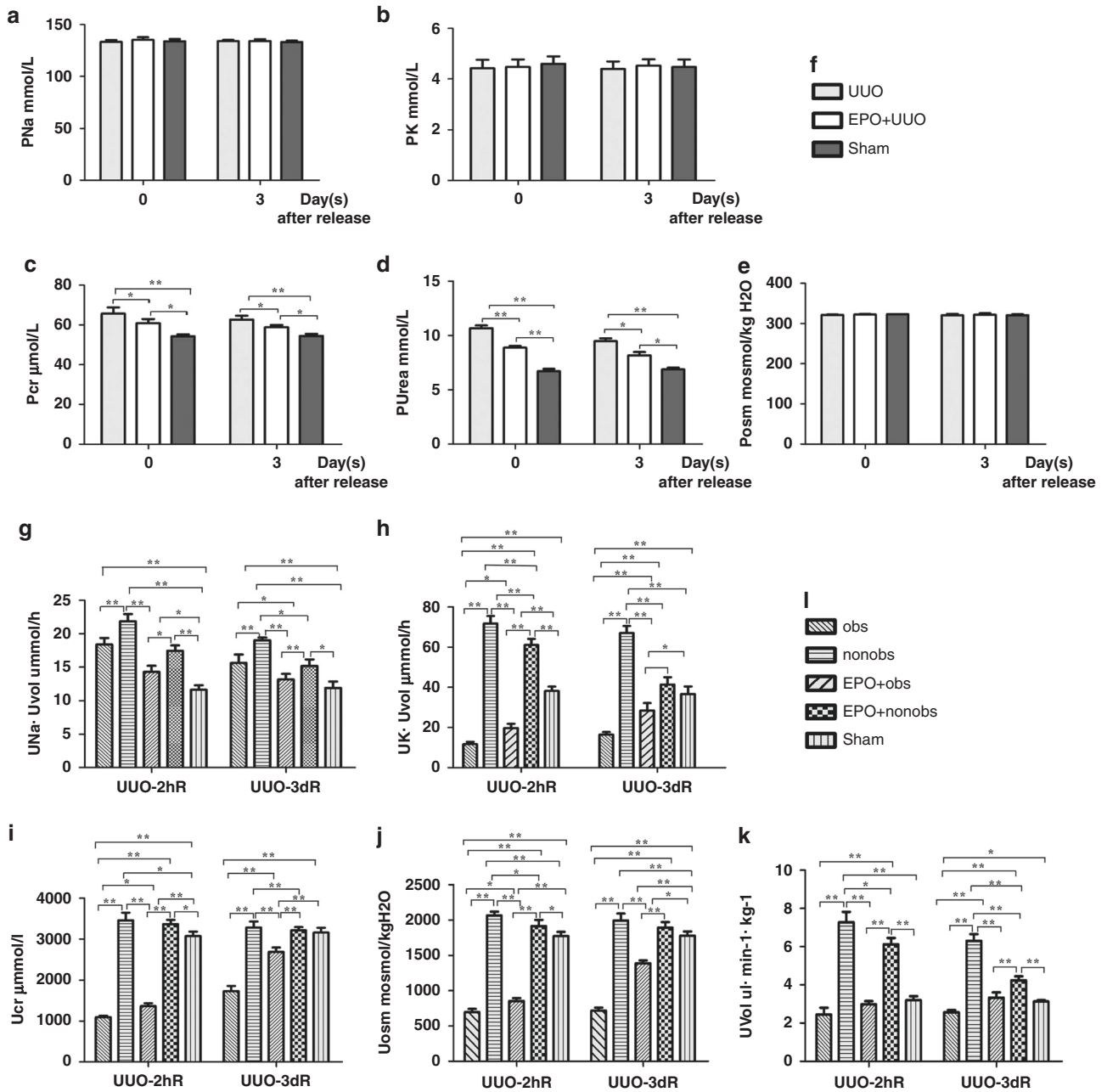


Fig. 1 Renal function changes at different time points after the release of 24-h UUO. **a** Plasma sodium, **b** plasma potassium, **c** plasma creatinine, **d** plasma urea, **e** plasma osmolality, **f** group character for (a–e), **g** urine sodium, **h** urine potassium, **i** urine creatinine, **j** urine osmolality, **k** urine volume, **l** group character for (g–k). UUO unilateral ureteral obstruction rats, UUO+EPO unilateral ureteral obstruction rats treated with EPO, Sham sham-operated control rats, UUO-2hR 2 h after release of UUO, obs obstructed kidneys, nonobs nonobstructed kidneys, obs+EPO obstructed kidneys treated with EPO, nonobs+EPO nonobstructed kidneys treated with EPO, Posm plasma osmolality, PNa plasma sodium, PK plasma potassium, Pcr plasma creatinine, Pura plasma urea, Uosm urine osmolality, UNa urine sodium, UK urine potassium, Ucr urine creatinine, Uvol urine volume. ANOVA followed by the Bonferroni multiple comparison post hoc test for the statistical analyses of the differences among and between groups of each indicator. Values are mean \pm SD; $n = 8$ for each group. * < 0.05 , ** < 0.01 .

Biological Technology), IL-1 β (GB111103, Servicebio), caspase-1 (BA0586, Boster Biological Technology), macrophage/monocyte chemotactic protein (MCP-1) (GB11199, Servicebio), and CD68 (GB113109, Servicebio) diluted in PBS supplemented with 0.3% BSA and 0.3% Triton X-100. The sections were rinsed with PBS three times for 5 min each, after which the sections were incubated with horseradish peroxidase-conjugated secondary antibodies (G23303, goat anti-rabbit immunoglobulin, Servicebio) for 1 h at room temperature. After rinsing with PBS washing buffer, the sites of antibody-antigen reactions were visualized with 0.05% 3,3'-diaminobenzidine dissolved in distilled water supplemented with 0.1% H₂O₂. Light microscopy was carried out with a Leica microscope. The immunohistochemistry quantification was performed

by evaluating the labeling index in 10 consecutive high-power (400 \times) fields of each section with Image pro plus 6.0 analysis software (Media Cybernetics, Inc., Rockville, MD).

Statistics analysis

Statistical analyses were performed with SPSS version 21 (SPSS, Inc., IBM) and GraphPad Prism version 5.0 (GraphPad Software, San Diego). All the values are presented as the mean \pm SD. One-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison post hoc test was used for the statistical analyses of the differences among and between groups, and $P < 0.05$ was considered significant.

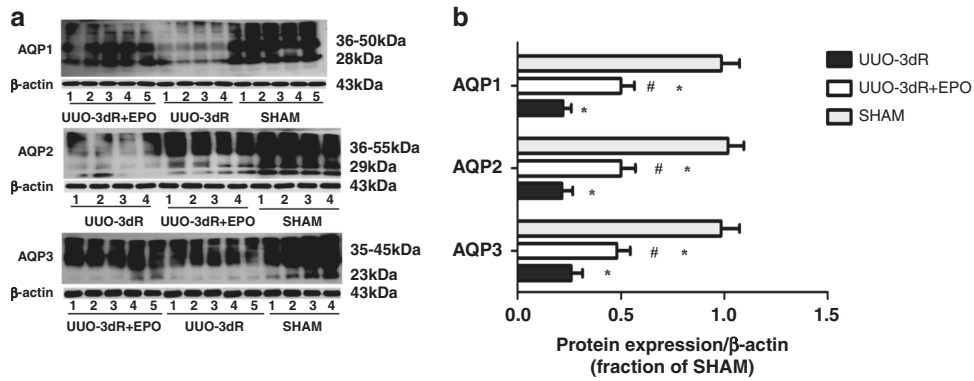


Fig. 2 Semiquantitative immunoblotting of AQP1, 2, and 3 of rat kidney 3 days after the release of UUO. **a** Immunoblots reacted with anti-AQP1, anti-AQP2, anti-AQP3, and anti-β-actin. **b** Densitometric analysis revealed that EPO treatment promoted the recovery expression of AQP1, AQP2, and AQP3. UUO-3dR 3 days after release of UUO group, UUO-3dR+EPO 3 days after release of UUO treated with EPO group. ANOVA followed by the Bonferroni multiple comparison post hoc test for the statistical analyses of the differences among and between groups of each indicator. * < 0.05 when compared with the SHAM group; # < 0.05 when compared with the UUO-3dR.

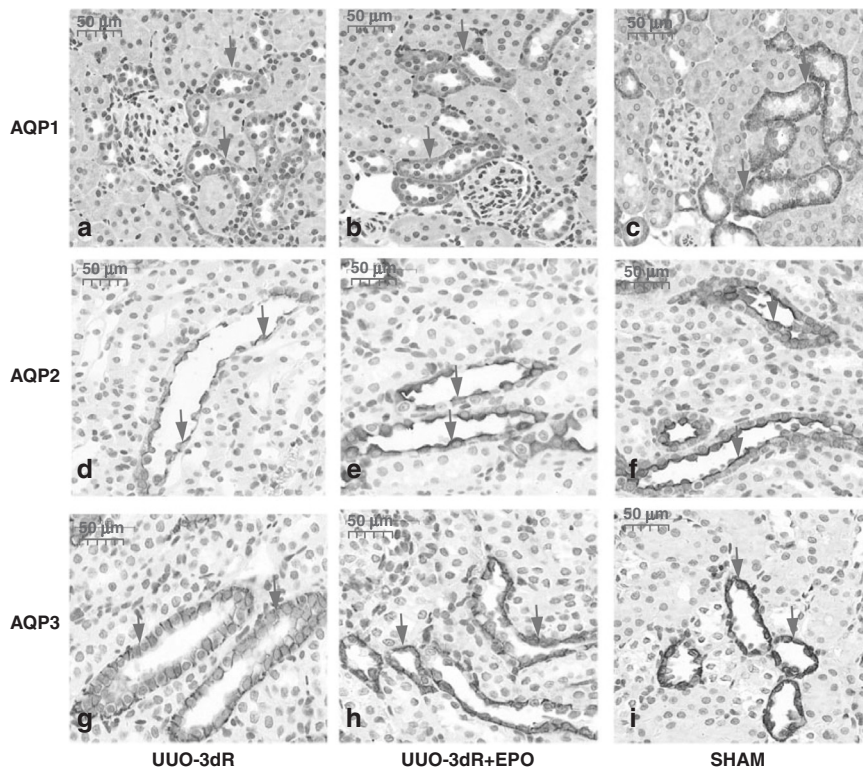


Fig. 3 Immunoperoxidase microscopy of AQP1–3 in rat kidney 3 days after releasing of UUO. **a–c** Representative images for AQP1 staining in the cortex of experimental rat kidney of **a** UUO-3dR group, **b** UUO-3dR+EPO, and **c** SHAM group. **d–f** Representative images for AQP2 staining in the inner medulla of experimental rat kidney of **d** UUO-3dR group, **e** UUO-3dR+EPO, and **f** SHAM group. **g–i** Representative images for AQP3 staining in the inner medulla of experimental rat kidney of **g** UUO-3dR group, **h** UUO-3dR+EPO, and **i** SHAM group. Original magnification: $\times 400$. Scale bar: 50 μm .

RESULTS

UUO-induced renal function insufficiency

Compared with the sham-operated controls, the rats with 24 h of UUO presented significantly increased plasma concentrations of urea and creatinine, and the concentrations of plasma potassium and sodium did not change (Fig. 1a–d). In addition, we examined whether renal excretion of water changed from both the obstructed kidneys and the contralateral nonobstructed kidneys in response to UUO. After 24 h of UUO, the obstruction was released, and urine was collected for 2 h from both kidneys (protocol 1). In the obstructed kidneys, there was a significant decrease in urinary osmolality. In contrast, urinary volume, creatinine, and potassium excretion of the

nonobstructed kidneys were significantly increased compared with those of the sham-operated rats, indicating compensatory changes in contralateral kidneys (Fig. 1h–k).

EPO prevented the development of impaired renal function in both UUO and UUO-R rats

After 24 h of UUO, rats treated with EPO had significantly lower plasma concentrations of urea and creatinine than the sham-operated controls did (Fig. 1c, d). EPO treatment also significantly prevented the decrease in urinary osmolality (Fig. 1j).

Three days after the release of the ligation, rats treated with EPO had lower plasma creatinine levels than the UUO-3dR rats did.

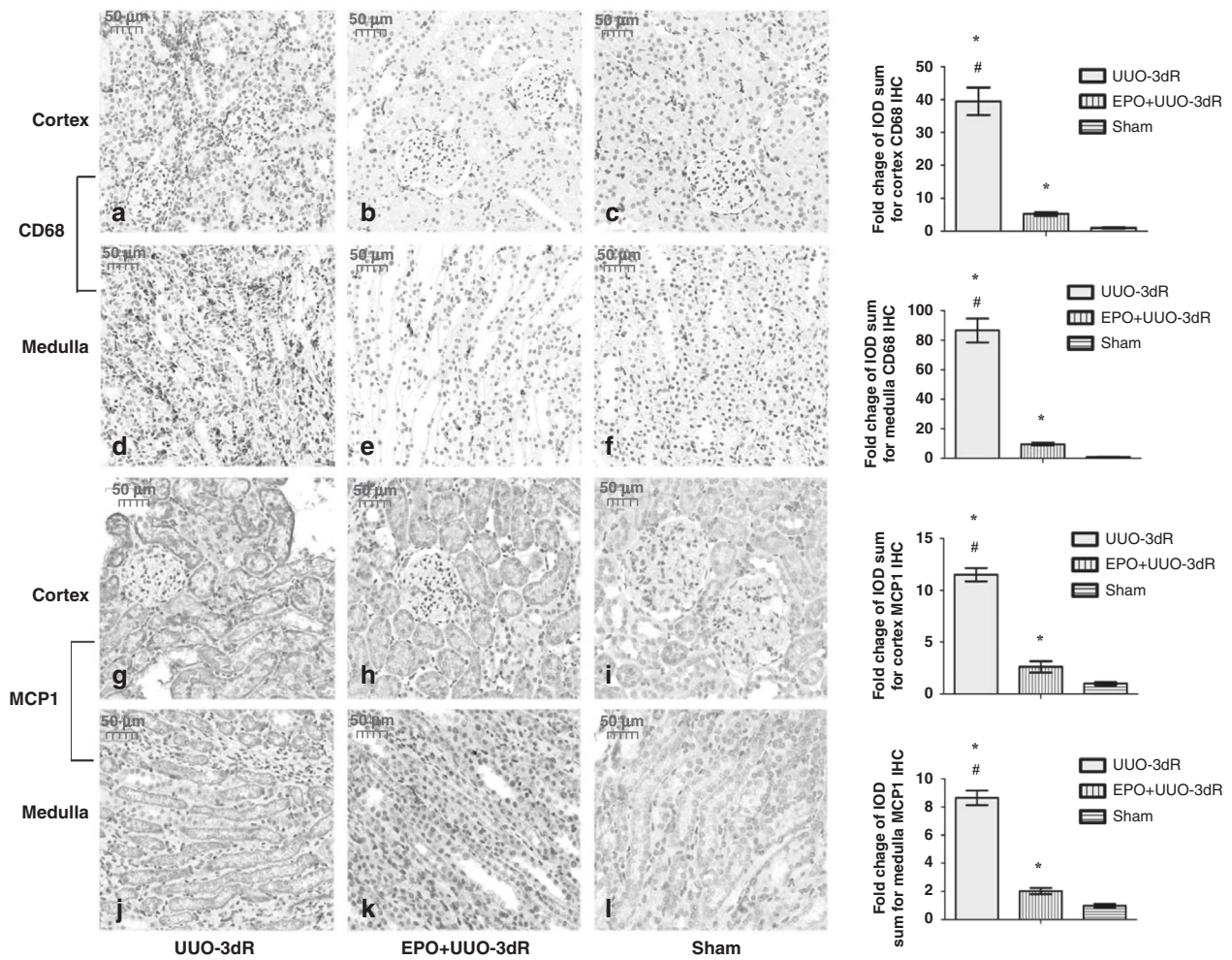


Fig. 4 Immunoperoxidase microscopy of CD68 and MCP-1 in rat kidney 3 days after releasing of UUO. **a–c** Representative images for CD68 staining in the cortex of experimental rat kidney of **a** UUO-3dR group, **b** UUO-3dR+EPO, and **c** SHAM group. **d–f** Representative images for CD68 staining in the medulla experimental rat kidney of **d** UUO-3dR group, **e** UUO-3dR+EPO, and **f** SHAM group. **g–i** Representative images for MCP-1 staining in the cortex of experimental rat kidney of **g** UUO-3dR group, **h** UUO-3dR+EPO, and **i** SHAM group. **j–l** Representative images for MCP-1 staining in the medulla of experimental rat kidney of **j** UUO-3dR group, **k** UUO-3dR+EPO, and **l** SHAM group. CD68 is the marker of macrophages and CD68-positive cells are macrophage cells. MCP-1 is the inflammation-associated factor and is widely expressed in renal interstitial and tubular epithelial cells. Original magnification: $\times 200$. Scale bar: 50 μm . ANOVA followed by the Bonferroni multiple comparison post hoc test for the statistical analyses of the differences among and between groups of each indicator. * < 0.05 when compared with the SHAM group; # < 0.05 when compared with the UUO-3dR.

However, there were no significant differences in plasma osmolality or sodium and potassium concentrations between the groups (Fig. 1a, e). The urine osmolality and potassium concentrations of the obstructed kidneys of UUO-3dR rats were significantly lower than those of the sham group, but the concentrations were higher in the contralateral kidneys of the UUO-3dR rats than in the sham group rats. Compared with the UUO-3dR rats, the rats treated with EPO had significantly higher urine osmolality and potassium concentrations of the obstructed kidneys (Fig. 1g–k).

EPO treatment prevented UUO-induced downregulation of renal AQP1–3

Figure 2 shows the effects of EPO treatment on renal AQP1–3 levels 3 days after the release of UUO. EPO treatment prevented the reduction in AQP1 expression, and UUO-3dR rats treated with EPO presented significantly higher AQP1 expression levels than the untreated UUO-3dR rats did (Fig. 2a, b).

We also examined whether the downregulation of AQP2 and AQP3 in UUO-3dR rats was prevented by EPO. Similar to the change in AQP1 expression, the expression of collecting duct AQP2 was higher in the EPO-treated UUO-3dR rats than in the

untreated UUO-3dR rats. AQP3 expression was also markedly increased in the EPO-treated UUO-3dR rats compared with the untreated UUO-3dR rats (Fig. 2a, b).

Immunohistochemistry confirmed that AQP1 labeling in proximal tubules from kidneys of UUO-3dR rats was dramatically reduced (Fig. 3a) compared with the abundant labeling of AQP1 seen in the apical and basolateral plasma membranes of proximal tubules from sham-operated kidneys (Fig. 3c). In contrast, EPO treatment dramatically prevented the downregulation of AQP1 in the proximal tubules of UUO-3dR kidneys (Fig. 3b). Immunohistochemistry also revealed a decrease in AQP2 expression in the apical plasma membrane of the collecting duct cells from UUO3d-R rats (Fig. 3d) in comparison to the sham-operated rats, whose AQP2 labeling was intense (Fig. 3f). In the EPO-treated UUO-3dR rats, AQP2 labeling in the plasma membrane of collecting duct cells was substantially stronger than that in the untreated UUO-3dR rats (Fig. 3e). Moreover, in UUO-3dR kidneys, AQP3 labeling in collecting duct cells was significantly reduced (Fig. 3g), whereas the labeling of AQP3 in collecting duct cells from the EPO-treated UUO-3dR rats was stronger than that from the untreated UUO-3dR rats (Fig. 3i).

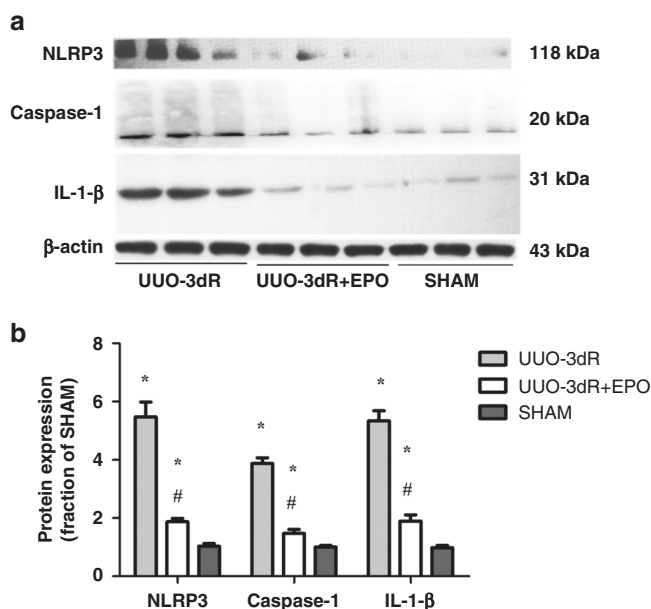


Fig. 5 Semiquantitative immunoblotting of NLRP3, caspase-1, and IL-1 β of rat kidney at day 3 after the release of UUU. **a** Immunoblots were reacted with anti-NLRP3, anti-caspase-1, anti-IL-1 β , and anti- β -actin antibodies. **b** Densitometric analysis revealed that EPO treatment inhibited the expression of NLRP3, caspase-1, and IL-1 β . ANOVA followed by the Bonferroni multiple comparison post hoc test for the statistical analyses of the differences among and between groups of each indicator. * < 0.05 when compared with the SHAM group; # < 0.05 when compared with the UUU-3dR.

EPO treatment significantly attenuated inflammatory cell infiltration and the high expression of NLRP3, caspase-1, and IL-1 β in the kidney

Figure 4 shows that postobstructive kidneys were infiltrated with macrophages and had high expression of inflammation-associated factor MCP-1. EPO treatment significantly inhibited the infiltration of macrophages into the renal cortex and medulla and reversed the high expression of MCP-1.

Figure 5 shows the effects of EPO treatment on renal NLRP3, caspase-1, and IL-1 β 3 days after the release of UUU. NLRP3, caspase-1, and IL-1 β expression were markedly increased in the UUU-3dR rats compared with the sham-operated rats. In contrast, EPO treatment significantly reversed the high expression of NLRP3, caspase-1, and IL-1 β in the postobstructive kidneys (Fig. 5a, b).

The immunohistochemistry results confirmed that NLRP3, caspase-1, and IL-1 β labeling in UUU-3dR group rat kidneys was the strongest, and in the sham group, it was the weakest. NLRP3, caspase-1, and IL-1 β labeling in the EPO treatment groups was substantially weaker than it was in the UUU-3dR group (Fig. 6).

DISCUSSION

The present study demonstrated that complete UUU for 24 h and 3 days after the release of UUU caused impaired urinary concentrating ability of the obstructed kidney. EPO treatment significantly prevented the deterioration of renal function in rats with 24 h UUU and 3 days after the release of UUU: (1) EPO treatment prevented the development of impaired renal functions and normalization of the decreased urine osmolality in UUU and UUU-3dR rats; (2) EPO treatment prevented the downregulation of AQP1–3 three days after the release of ureteral obstruction, which may play a pivotal role in improving UUU-induced urinary concentrating defects; and (3) EPO treatment prevented the infiltration of macrophages and the high expression of NLRP3, caspase-1 and IL-1 β in UUU-3dR rat kidneys, which may be among

the underlying mechanisms for EPO protective effects in UUU-3dR rats.

EPO treatment prevented the development of impaired renal function in UUU-3dR rats

In the clinic, urinary tract obstruction is a life-threatening severe disease for children and infants, and patients with obstructive nephropathy always have renal dysfunction.²⁰ Ureteral obstruction can result in the reduction in renal blood flow and glomerular filtration rate, inducing tubular abnormalities; as a result, the urine-concentrating ability is hindered.²¹ The pathophysiology of UUU is complex and not well defined. Structural and biochemical changes in all segments of the renal tubule are involved.⁶ In this study, EPO was found to have protective effects against UUU-induced renal injury, including the normalization of the increased plasma creatinine and decreased urine osmolality of the obstructed kidney and inhibition of the downregulation of AQP1–3. However, the underlying mechanisms behind these results are not clear. Previous studies have demonstrated that acute ureteral obstruction and after the release of the obstruction is characterized by infiltration of macrophages and lymphocytes into the kidney.^{22–24} Like in previous studies, in this study, we also found infiltration of macrophages in the renal cortex and medulla of the obstructed kidneys, and the inflammation-involved factor MCP-1 was strongly expressed in the obstructed kidney, which indicates that UUU is associated with a severe inflammatory response. Thus, it is possible that infiltrating inflammatory cells may play a potential role in impaired renal function. It is also well known that ureteral obstruction is associated with a progressive loss of renal mass through apoptotic renal cell death.²⁵ In this study, we found that EPO could inhibit the infiltration of macrophages and reverse the high expression of inflammasome NLRP3 and its downstream targets caspase-1 and IL-1 β . The NLRP3 inflammasome was associated with the development and maintenance of inflammation and apoptosis, which indicates that EPO treatment in protecting tubular functions in the postobstructed kidneys could be due to its anti-inflammatory and antiapoptotic effects, but this needs further detailed study.

Notably, it is not known whether the effects of EPO treatment are mediated by other mechanisms, in addition to anti-inflammatory and antiapoptotic actions, that prevent the decline in renal function. Further studies are needed to clarify the underlying mechanisms of the protective effects of EPO treatment after ureteral obstruction.

EPO treatment inhibits the downregulation of AQP1–3

AQPs compose a family of membrane proteins that function as water channels and have been demonstrated to play an important role in the physiology and pathophysiology of renal regulation of body water balance.²⁶ In humans, Wen et al. found that the expression of AQP1–3 was decreased in children with congenital hydronephrosis,²⁷ and our previous study also found that the expression of AQP1–3 was decreased in human fetal hydronephrotic kidneys.²⁸ In animals, Li et al. demonstrated that the reduced expression of these proteins was associated with persistent polyuria after the release of UUU in rats.⁷ Like in previous studies, we found that the expression levels of AQP1, AQP2, and AQP3 were markedly decreased in rat obstructive kidneys 3 days after the release of UUU.

Gong et al. demonstrated that EPO could prevent the downregulation of AQP1, AQP2, and AQP3 and major sodium transporters induced by renal I/R injury.¹³ Ren CC found that EPO promoted the recovery of the expression of AQP2 in rats after the release of 24 h BUO.¹⁶ Like in previous studies, we found that EPO significantly prevented the downregulation of AQP1, AQP2, and AQP3 levels 3 days after the release of UUU. Nevertheless, the underlying mechanism through which EPO treatment prevented the decreased expression of AQPs in response to UUU is unknown.

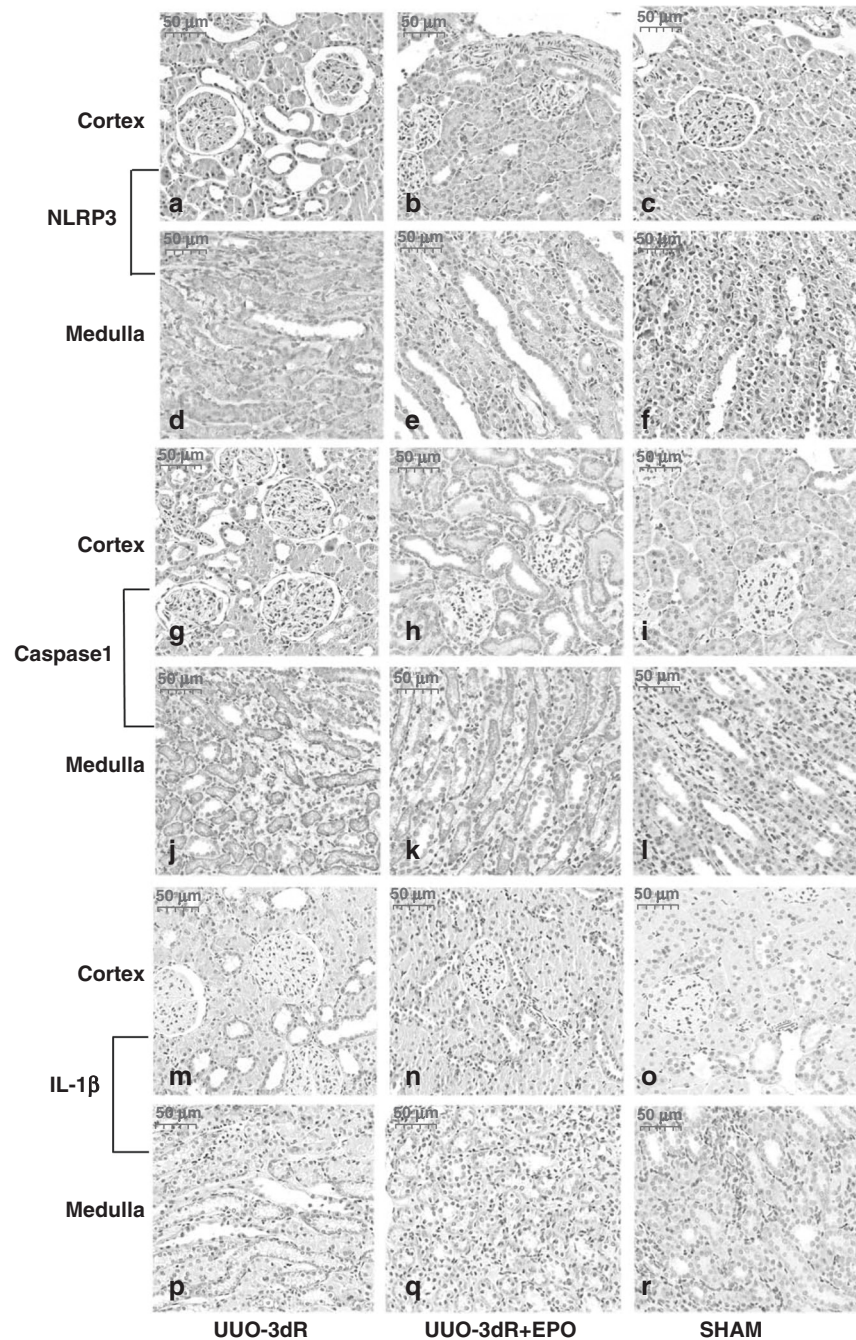


Fig. 6 Immunoperoxidase microscopy of NLRP3, caspase-1, and IL-1 β in rat kidney 3 days after releasing of UUU. **a–c** Representative images for NLRP3 staining in the cortex of experimental rat kidney of **a** UUU-3dR group, **b** UUU-3dR+EPO, and **c** SHAM group. **d–f** Representative images for NLRP3 staining in the medulla of experimental rat kidney of **d** UUU-3dR group, **e** UUU-3dR+EPO, and **f** SHAM group. **g–i** Representative images for caspase-1 staining in the cortex of experimental rat kidney of **g** UUU-3dR group, **h** UUU-3dR+EPO, and **i** SHAM group. **j–l** Representative images for caspase-1 staining in the medulla of experimental rat kidney of **j** UUU-3dR group, **k** UUU-3dR+EPO, and **l** SHAM group. **m–o** Representative images for IL-1 β staining in the cortex of experimental rat kidney of **m** UUU-3dR group, **n** UUU-3dR+EPO, and **o** SHAM group. **p–r** Representative images for IL-1 β staining in the medulla experimental of rat kidney of **p** UUU-3dR group, **q** UUU-3dR+EPO, and **r** SHAM group. Original magnification: $\times 200$. Scale bar: 50 μm .

The present study demonstrated that EPO could inhibit the expression of IL-1 β . Recent studies have revealed that chronic administration of IL-1 β induced significantly reduced AQP2 expression in medullary collecting ducts²⁹ and increased urine output in mice.³⁰ Wang et al. also found that IL-1 β could directly inhibit AQP2 expression in rat inner medullary suspensions.³¹ Crowley et al. found that inhibition of IL-1R signaling within renal myeloid cells could reduce NO production.³² NO is a chemical form of

endothelium-derived relaxing factor that has important roles in renal hemodynamics and renal sodium and water metabolism. Importantly, NO has been demonstrated to inhibit renal collecting duct water transport through a reduction in AQP2 expression.^{33,34} Thus, we speculate that EPO might promote AQP recovery through the inhibition of IL-1 β and NO expression in UUU-3dR rats.

IL-1 β production during kidney injury is known to be regulated by the inflammasome, in which ASC recruits and activates

caspase-1. Caspase-1 cleaves pro-IL-1 β into its mature form and induces the release of IL-1 β .¹¹ It has also been demonstrated that the NLRP3 inflammasome plays a pivotal role in the progression of various kidney diseases, and the pharmacological or genetic inhibition of NLRP3 could alleviate renal injuries.^{10–12} Like in a previous study,³⁵ the expression of NLRP3, caspase-1, and IL-1 β was increased in obstructed kidneys. We speculate that the increased NLRP3 after UO may activate the cleavage of pro-caspase-1 into mature caspase-1. In parallel, more mature IL-1 β is released, which mediates the downregulation of AQPs in renal tubules. Importantly, EPO prevented the increased expression of NLRP3, caspase-1, and IL-1 β in obstructed kidneys. These findings suggest that the EPO-induced suppression of IL-1 β expression via the NLRP3 inflammasome has potential as a mechanism of renal protection after UO injury. Moreover, chemokines and endothelin have also been shown to play a role in urinary concentrating defects associated with ureteral obstruction.

However, until now, there have been few detailed studies on the direct effect of EPO treatment on renal AQP expression. Therefore, further investigations, such as studies of agonists or RNA interference of AQPs *in vitro*, are needed to understand the underlying mechanisms of EPO-mediated regulation of AQP expression, along with the protective effects of EPO treatment.

Summary

Our study demonstrated that EPO could prevent the development of impaired renal function and the decreased renal AQPs expression and renal water handling ability after the release of UO in young rats, which may be partly associated with the inhibition of macrophage infiltration and the expression of caspase-1 and IL-1 β as well as NLRP3 inflammasome activation. However, whether there is a causal association between these changes or whether other pathways are involved still needs further study.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

J.F., J.W., Y.Z., B.D., J.T., S.Yu, S.Yan, E.L., L.L., and X.Z. made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. J.F., J.W., B.D., Y.Z., J.T., S.Yu, and S.Yan made substantial contributions to make animal model. J.F., X.Z., J.W., Y.Z., and B.D. drafted the article or revised it critically for important intellectual content. X.Z. made the final approval of the version to be published. J.F., J.W., and Y.Z. made equal contribution to the research.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

All procedures conformed to the Chinese National Guidelines for the Care and Handling of Animals and the published guidelines from the National Institutes of Clinical Medicine, Zhengzhou University, according to the licenses for use of experimental animals issued by the Chinese Ministry of Justice (2020-KY-273).

CONSENT FOR PUBLICATION

The submission of this manuscript is approved by all authors for publication.

ADDITIONAL INFORMATION

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