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Kidney fibrosis induced by various irrigation pressures in mouse models of mild and severe hydronephrosis

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Abstract

Objective We want to study whether the degree of fibrosis in the mild and severe hydronephrosis is different, and whether the irrigation pressure will affect the fibrosis of the hydronephrosis.

Methods Animal models of mild and severe hydronephrosis in the left kidney were established: 72 healthy C57BL/6 mice were randomly divided into nine groups (eight in each group). The N group was used as a control group, and 0 mmHg pressure perfusion was given. The M and S groups were used as mild and severe hydronephrosis groups, respectively. The mild and severe hydronephrosis groups were subdivided into eight subgroups, M0–M3 and S0–S3. Among them, groups 0, 1, 2, and 3 were perfused with 0 mmHg, 20 mmHg, 60 mmHg, and 100 mmHg, respectively. We investigated the effects of irrigation pressures on renal fibrosis in mild (group M) and heavy (group S) hydronephrosis by quantitative real-time polymerase chain reaction, Western blot analysis, Masson staining and immunohistochemistry staining in mouse models.

Results Compared with group N, EMT and ECM deposits were significantly aggravated in both the mild and severe hydronephrosis groups, TGF-β signaling pathway-related molecules significantly changed too. In terms of ECM deposition, S2 and S3 are significantly increased compared to S0.The EMT of M2 and M3 changed significantly compared with M0; the EMT of S1, S2 and S3 changed significantly compared with S0.The molecules related to TGF-β signaling pathway also changed: M0 and S0 changed significantly compared with N; M1, M2 and M3 changed significantly compared with M0; compared with S0, S1, S2 and S3 changed significantly.

Conclusion Compared with mild hydronephrosis, renal fibrosis in severe hydronephrosis is more severe and its tolerance to perfusion pressure is lower. These changes may be related to the TGF-β signalling pathway.

Keywords Hydronephrosis · Irrigation pressures · Renal fibrosis · TGF-β1 · Smad

Introduction

With the development of endoscopic urology, percutaneous nephrolithotomy and ureteroscopy have become increasingly widely used in the treatment of urinary calculi due to their small trauma, high stone removal efficiency and rapid postoperative recovery [1, 2]. In the process of percutaneous nephrolithotomy and ureteroscopy, in order to keep the surgical field clear and flush out the stone fragments, a certain pressure of liquid perfusion is required [3, 4], and excessive irrigation pressure can affect kidney function and tissue morphology. In clinical work, patients with upper urinary calculi are often accompanied by varying degrees of hydronephrosis. However, the existing studies on the damage of the kidney caused by high perfusion pressure are mostly based on normal kidneys. Therefore, we studied the effect of irrigation pressure on the damage of kidneys with different degrees of hydronephrosis and proved that the irrigation pressure is closely related to the degree of postoperative injury due to hydronephrosis. The kidneys with severe hydronephrosis are more susceptible to injury due to irrigation pressure [5, 6].

However, based on reports, hydronephrosis is not only related to injury but also related to renal fibrosis [7, 8]. Fibrosis is often thought to be a long-term manifestation of tissue damage, and as the disease progresses, kidney function gradually deteriorates and even leads to kidney failure [9–13]. Therefore, it is necessary to determine whether the

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irrigation pressure is related to the fibrosis of the hydronephrosis and to clarify the possible mechanism under the premise that the irrigation pressure will cause damage due to hydronephrosis.

Materials and methods

Animal and groups

Male C57BL/6 mice (20–25 g) were purchased from the Center of Experimental Animals at Wuhan University Medicine College (Hubei, China). All mice were caged in a standard temperature-controlled room with alternating 12-h light/dark cycles, with free access to water and a standard laboratory diet. The study received approval from the Wuhan University Committee on Ethics for Animal Experiments.

All animals were randomly divided into three groups: the control group and two surgical groups (mild and severe hydronephrosis). The two surgical groups were further divided into four subgroups (M0–M3 and S0–S3) ($n = 8$).

Surgical manipulation

Mice were anaesthetized with 0.3% pentobarbital sodium (30 mg/kg, IP). The mice were supine on a temperature-controlled operating table. After opening the abdominal cavity, ligation of the left ureter, then closing the abdominal cavity and disinfecting. In group N, we only opened the abdominal cavity without ligation of the ureter. Penicillin was injected intraperitoneally every day to prevent infection after surgery.

In the M group, B-ultrasound examination to determine the formation of hydronephrosis was performed on the 3rd day after surgery, and in the S group, it was performed on the 7th day.

Then, a second operation was performed: after anaesthesia, the left kidney was exposed, a puncture into the collection system with a needle of 0.7 mm diameter was made and fixed; then, it was connected to a physiological recorder (Biopac, CA) and a pressure pump (Laborie, Mississauga, Canada). Using 37 °C physiological saline as a perfusate, the perfusion pressure was set to 0 mmHg (M0, S0), 20 mmHg (M1, S1), 60 mmHg (M2, S2) and 100 mmHg (M3, S3), Group N only exposed the kidneys without suffering from perfusion pressure. Then, there was a pause for 2 min after an 8 min infusion for a total of six cycles. After the end of the perfusion, the ureteral obstruction was removed and the abdominal cavity was closed.

The mice were sacrificed 48 h later and the operated kidneys were harvested. Kidney tissue was stored in two parts: fixed in 4% paraformaldehyde and preserved in liquid nitrogen.

Masson trichrome staining

Masson staining is a common method for staining connective tissue, and it is used to observe the proliferation and distribution of fibrous connective tissue in tissues. Because collagen fibres are an important part of the extracellular matrix, and Masson's staining has a good colour rendering effect on collagen, we were able to evaluate the deposition of the extracellular matrix of the kidney by Masson staining. During this process, 4-μm paraffin sections were used.

Immunohistochemistry staining

The 4-μm paraffin sections were deparaffinised, hydrated and microwave-repaired. We removed endogenous peroxidase with 3% hydrogen peroxide and added the following primary antibodies: α-SMA (1, 200, 19245T, Cell Signaling Technology), E-cadherin (1:400, 3195T, Cell Signaling Technology), TGF-β1 (1:100, ab92486, Abcam), cathepsin S (1:50, Santa Cruz, Sc271619), collagen-I (1:100; ab34710; Abcam) and fibronectin (1:200, ab2413, Abcam). The reaction was carried out in a 37 °C reheating box for 2 h and then allowed to react overnight at 4 °C.

Biotinylated secondary antibody (Zhongshan Golden Bridge Biotechnology) and horseradish peroxidase (Zhongshan Golden Bridge Biotechnology) were added and reacted with 3,30-diaminobenzidine (Zhongshan Golden Bridge Biotechnology). A positive reaction was observed under a microscope, followed by washing with water to stop the reaction.

Ten fields of view (×400) were randomly selected for each slice and analysed with the image analysis software ImagePro-Plus 6.0. Two parameters, the positive area (Area) and integrated optical density (IOD), were measured. The mean optical density (MOD = IOD/Area) (×100%)was used to illustrate the expression of indicators.

Western blotting

Protein from kidney tissue is extracted with RIPA lysate containing PMSF. Protein concentrations were measured using the BCA protein concentration test kit.

Proteins (50 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% skim milk at room temperature for 2 h. The PVDF membranes were incubated with primary antibodies overnight at 4 °C. The next day, the PVDF membranes were rinsed with Tween 2.0-containing Tris–HCl buffer (TBST). Reaction with secondary antibodies was carried out for 1 h at room temperature.

The Odyssey infrared imaging system (Li-Cor, USA) was used to analyse the expression of the target protein. The following antibodies were used in this process: cathepsin S (1:100, Santa Cruz, Sc271619), α-SMA (1:1000, 19245T, Cell Signaling technology), E-cadherin (1:1000, 3195T, Cell Signaling Technology), TGF-β1 (1:500, ab92486, Abcam), fibronectin (1:200, ab2413, Abcam), collagen-I (1:1000, ab34710, Abcam), Smad2 (1:1000, 5339S, Cell Signaling Technology), Smad3 (1:1000, 9513S, Cell Signaling Technology), Phospho-Smad2(1:1000, 18338S, Cell Signaling technology), Phospho-Smad3(1:1000, 9520S, Cell Signaling technology), GAPDH (1:1000, 5174S, Cell Signaling Technology), and anti-mouse/rabbit secondary antibody (1:15,000, 5257P, Cell Signaling Technology), (1:30,000, 5151S, Cell Signaling Technology).

Quantitative real-time PCR

Total RNA of kidney tissue was extracted using the TRIzol kit (Invitrogen, 155960026). In the process of reverse transcription, we used RevertAid Reverse Transcriptase (Fermentas, EP0442), dNTP (Fermentas, R0191) and RiboLock RNase Inhibitor (Fermentas, E00381). Then, we performed real-time PCR using the Real-time PCR instrument (ABI StepOnePlus). In the process, we used the following reagents: KAPA SYBR FAST qPCR Kit Master Mix (2×) and ABI Prism (KAPA, KR0390). The experimental results were analysed using the $2^{-\Delta\Delta C_T}$ method. The sequences of primers for kidney specimens were as follows: α-SMA, forward, 5′-CCACCGCAAATGCTTCTAAGT-3′, and reverse, 5′-GGCAGGAATGATTTGGAAAGG-3′; E-cadherin, forward, 5′-AGCCAGACACATTCATGGAAC-3′, and reverse, 5′-TCGTTATCCGAGATTGAGA-3′; TGF-β, forward, 5-CTCCCGTGGCTTCTAGTGC-3′, and reverse, 5′-GCCTTAGTTTGGACAGGATCTG-3′; fibronectin, forward, 5′-AGGCTGGATGATGGTGGACT-3′, and reverse, 5′-CGGCTGAAGCACTTTGTAGAG-3′; collagen-I, forward, 5′-CAAGAAGACATCCCTGAAGTC-3′, and reverse, 5′-ACAGTCCAGTTCTTCATTGC-3′ (Invitrogen). The transcript levels of the target genes were normalized with the β-actin gene. The sequences of the β-actin primers were as follows: β-actin, forward, 5′-CTGAGAGGGAAATCGTGCGT-3′, and reverse, 5′-CCACAGGATTCCATACCCAAGA-3′ (Invitrogen).

Statistical analysis

Data are expressed as the mean ± SD, and data were analysed with SPSS 15.0. The data were analysed by analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Kidney fibrosis was observed in hydronephrotic kidneys and perfusion enhanced ECM accumulation

We first analysed the effect of perfusion pressure on renal fibrosis in different degrees of hydronephrotic kidney by Masson staining. The results showed that a significant increase in the degree of fibrosis was observed in the M0 group and S0 group compared with the control group. Although significant changes in fibrosis were not observed in patients with mild hydronephrosis exposed to different perfusion pressures, fibrosis was significantly aggravated in the S2 and S3 groups compared with the S0 group (Fig. 1). Then, COL-I and FN were analysed by WB, IHC and qPCR, respectively (Fig. 2), and the results showed that the expression levels in the M0 and S0 groups were significantly higher than those in the N group. Compared with the M0 group, the expression levels of COL-I and FN in the M1, M2 and M3 groups did not change significantly. A significant increase in the expression of COL-I and FN was observed in the S2 and S3 groups compared with the S0 group.

Pressure perfusion promotes EMT in hydronephrotic kidneys

In this section, we mainly tested α-SMA and E-cadherin by WB, IHC and qPCR, respectively. Compared with the N group, α-SMA increased significantly in the M0 group and S0 group; α-SMA expression in the M1, M2 and M3 groups increased significantly compared with the M0 group; the expression levels of α-SMA in the S1, S2 and S3 groups were significantly higher than those in the S0 group (Fig. 3a, c, d, f, h).

As for E-cadherin, its expression in the M0 group and S0 group was significantly lower than that in the N group; its expression in the S1, S2 and S3 groups was significantly lower than that in the S0 group. Compared with the M0 group, the expression level of E-cadherin did not change significantly in the M1 group, but it decreased significantly in the M2 group and M3 group (Fig. 3b, c, e, g, i).

Changes in the TGF-β signalling pathway after perfusion

We first tested TGF-β by WB and qPCR (Fig. 4a, f, g) and found that the expression level was significantly higher in the M0 group and S0 group than in the N group. Its expression levels in the M1, M2 and M3 groups were significantly higher than in the M0 group, and the expression levels in the S1, S2 and S3 groups were significantly

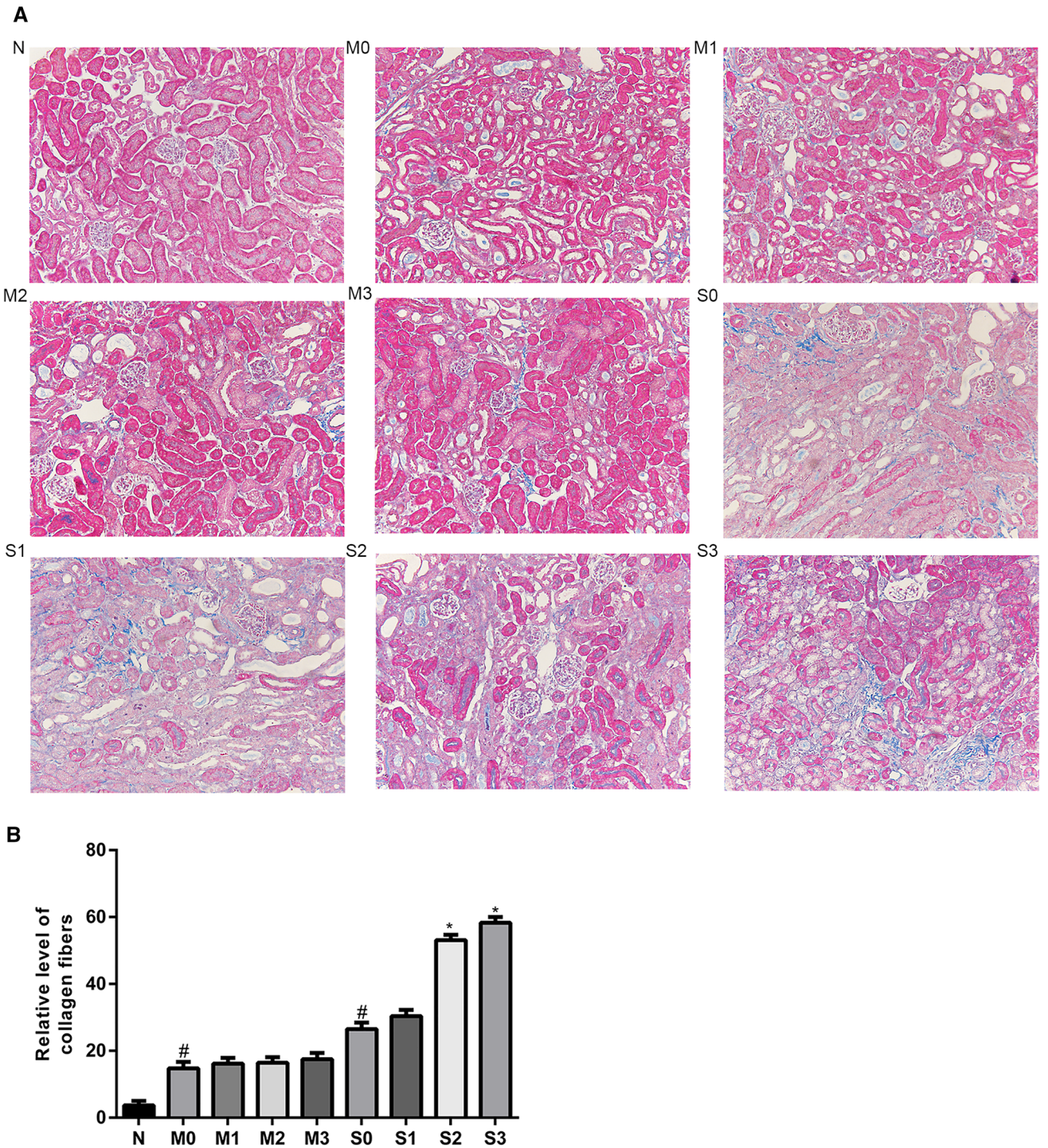

Fig. 1 Not only severe hydronephrosis will increase fibrosis, but the increase of perfusion pressure will also aggravate fibrosis. **a** Masson staining (×200); **b** quantitative analysis of the degree of fibrosis, blue area represents the deposition of collagen. The data are shown as the mean ± s.d; #< 0.05 compared with the N group, *< 0.05 compared with the S0 group

higher than in the S0 group. We then detected Smad2/3 (Fig. 4a–c) and phosphor-Smad2/3 (Fig. 4a, d, e) by WB. The expression levels of Smad2/3 and phosphor-Smad2/3 in the M0 and S0 groups were significantly higher than those in the N group. The expression levels of Smad2/3 and phosphor-Smad2/3 in the M1, M2 and M3 groups were significantly higher than those in the M0 group, while the expression levels of Smad2/3 and phosphor-Smad2/3 in

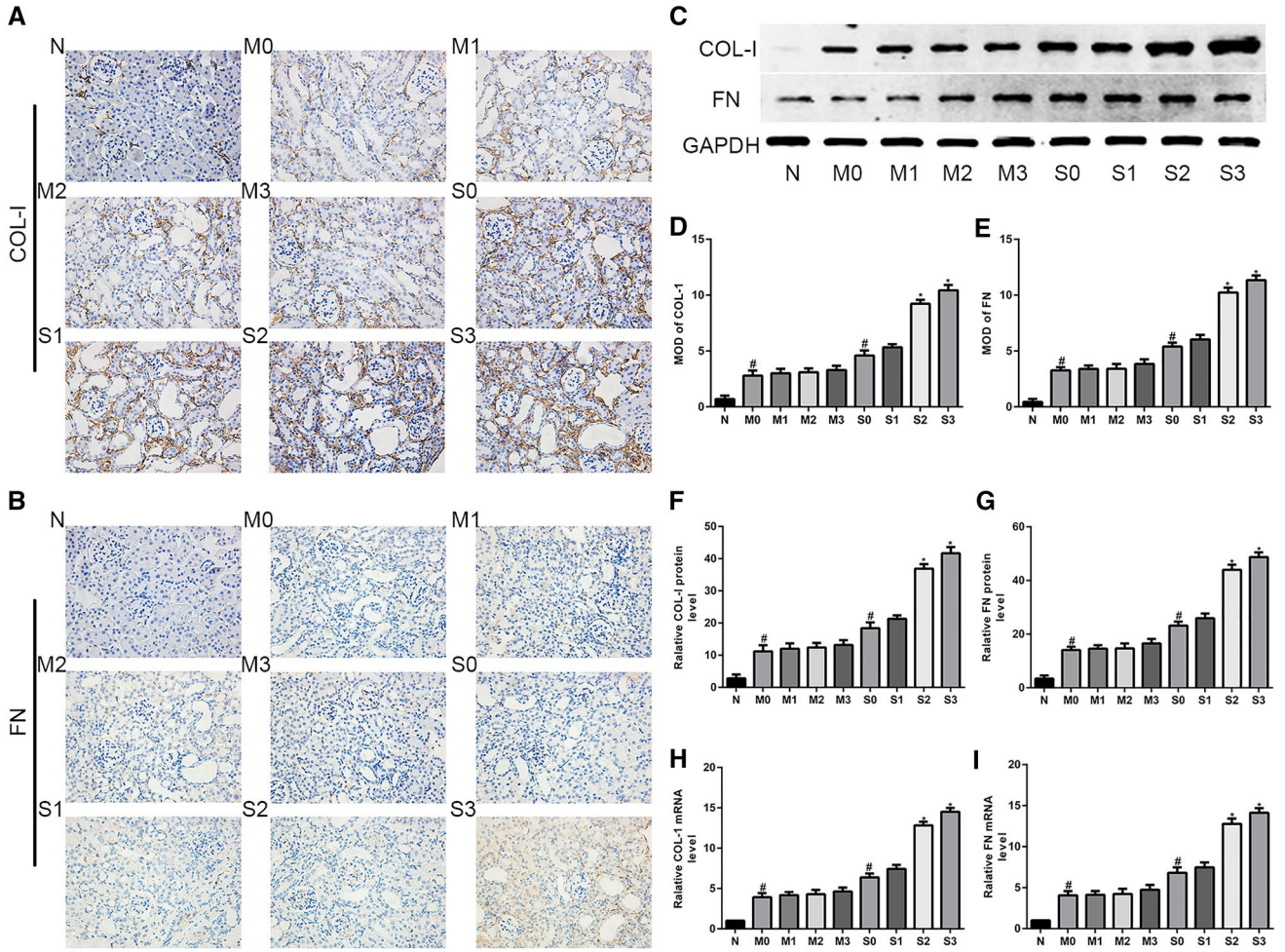

Fig. 2 Not only severe hydronephrosis will increase ECM deposition, but the increase of perfusion pressure will also aggravate ECM deposition. **a, b, d** COL-1 and FN measured by IHC (×400) in mice with mild and severe hydronephrosis exposed to different perfusion pressures; **c, e** COL-1 and FN measured by WB in mice with mild and severe hydronephrosis exposed to different perfusion pressures; **f** COL-1 and FN measured by qPCR. The data are shown as the mean ± s.d; #< 0.05 compared with the N group, *< 0.05 compared with the S0 group

the S1, S2 and S3 groups were significantly higher than those in the S0 group.

Discussion

In the course of clinical treatment of urinary calculi, it is often necessary to give a certain degree of perfusion [3], and patients with upper urinary calculi are often accompanied by varying degrees of hydronephrosis. Our previous studies have confirmed that irrigation pressures can cause varying degrees of damage due to hydronephrosis [5, 6]. On this basis, we further studied the relationship between irrigation pressures and renal fibrosis. Our experimental results showed that the degree of renal fibrosis varies with different levels of hydronephrosis. Compared with mild hydronephrosis, severe hydronephrosis is more likely to undergo significant fibrosis after perfusion. In addition, the TGF-β signalling pathway may be involved in the regulation of this series of changes.

At present, there are many studies on the mechanisms related to renal fibrosis, and many possible conclusions have been made [14–17], and even some of them have conflicting situations [18–21]. However, regardless of whether there is a unified view on the study of the mechanism of renal fibrosis, it is clear that renal fibrosis is a pathological change in which a large number of ECM accumulate after a series of changes in the kidney after being stimulated by trauma, inflammation, infection and other factors [22, 23]. As an important component of the ECM, the expression levels of COL-I and FN are often used to reflect the deposition of ECM [24, 25]. In this experiment, we administered pressure perfusion to mice with different degrees of hydronephrosis and then tested the expression of COL-I and FN. The experimental

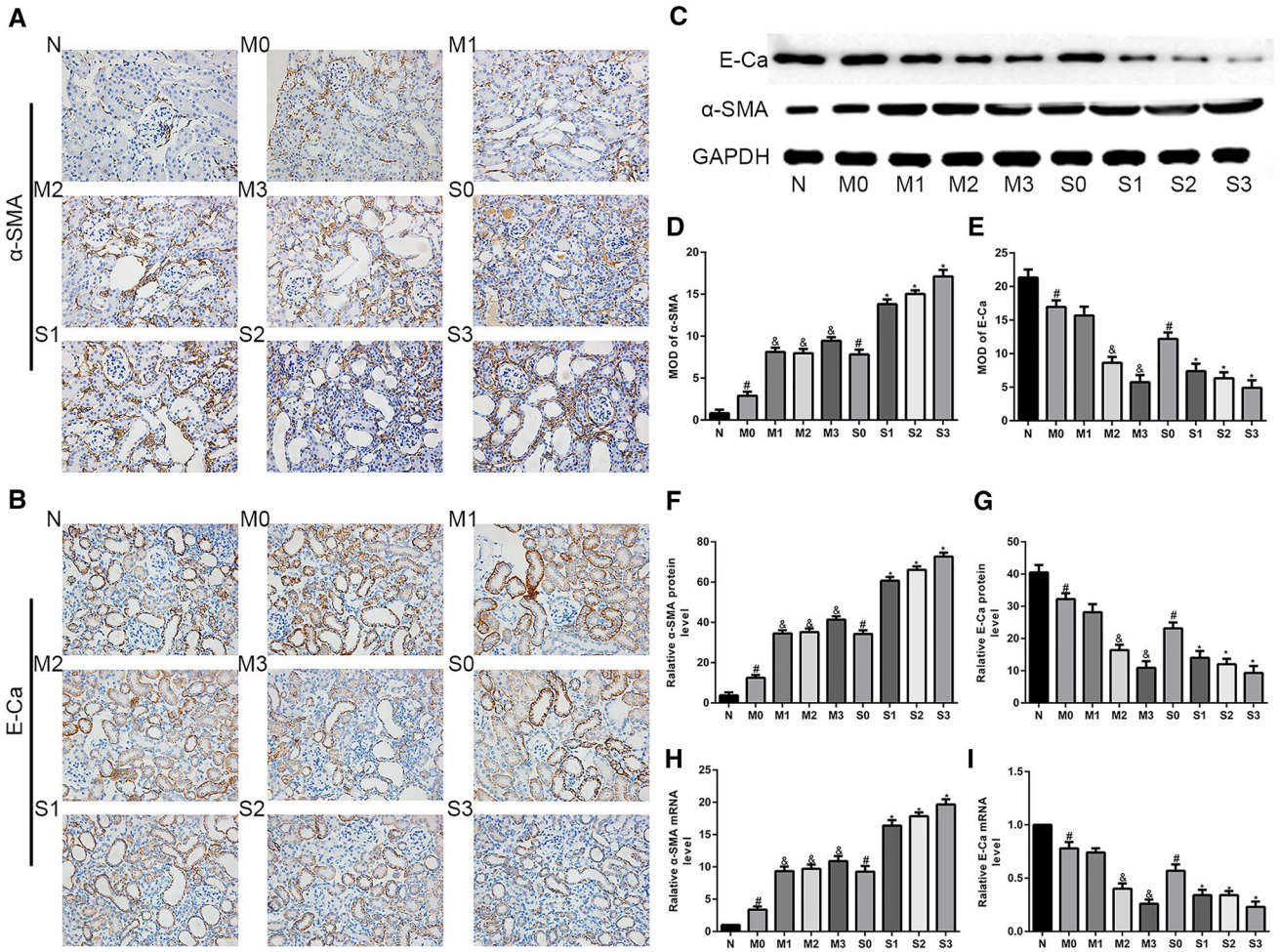

Fig. 3 Not only severe hydronephrosis will increase EMT, but the increase of perfusion pressure will also aggravate EMT. **a, b, d** α-SMA and E-Ca measured by IHC (×400) in mice with mild and severe hydronephrosis exposed to different perfusion pressures; **c, e** α-SMA and E-Ca measured by WB in mice with mild and severe hydronephrosis exposed to different perfusion pressures; **f** α-SMA and E-Ca measured by qPCR. The data are shown as the mean ± s.d; #< 0.05 compared with the N group, &< 0.05 compared with the M0 group, *< 0.05 compared with the S0 group

results showed that, compared with the control group, a significant increase in the expression of COL and FN was observed in both the mild and severe hydronephrosis groups. The expression of COL-I and FN in the mild hydronephrosis group did not change significantly under different irrigation pressures. Under the irrigation pressure of 100 mmHg, the expression of COL-I and FN in the severe hydronephrosis group increased significantly. This result is also consistent with the results of Masson staining. This indicates that hydronephrosis is accompanied by renal fibrosis, and the degree of renal fibrosis is aggravated as the degree of hydronephrosis increases.

It is currently known that the main cell that promotes renal fibrosis is myofibroblasts, which function by secreting ECM [26, 27]. One of the controversies surrounding kidney fibrosis research revolves around the relative contribution of EMT to myofibroblast populations. Traditionally, EMT of renal tubular epithelial cells is a major source of myofibroblasts in addition to the fibroblasts inherent in the renal interstitial [28]. Humphreys et al. believe that renal tubular epithelial cells are not a source of myofibroblasts, and they believe that interstitial pericytes or perivascular fibroblasts are the main sources of myofibroblasts [19]. LeBleu's research indicates that bone marrow cells and endothelial-mesenchymal transition are the main sources of myofibroblasts [29]. Both questioned the role of EMT in renal fibrosis, or to some extent negated it. Grande and colleagues and Lovisa and colleagues provided new insights into how EMT mechanisms of damaged epithelial cells promote the development and progression of renal fibrosis [30]. They proposed the concept of partial EMT, suggesting that although the dedifferentiation of renal tubular epithelial cells does not directly affect the number of myofibroblasts, the occurrence of partial EMT is accompanied

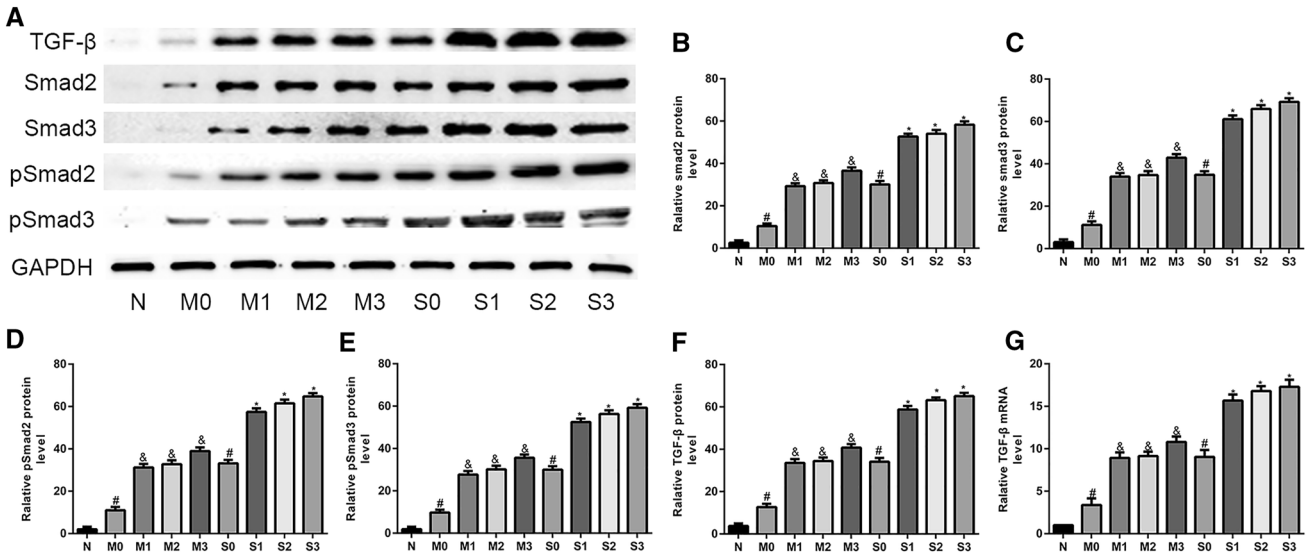

Fig. 4 The degree of hydronephrosis and perfusion pressure affect the expression of TGF-β signaling pathway-related molecules in mouse kidney. **a, b, c, d**, TGF-β, Smad2,Smad3,pSmad2 and pSmad3 measured by WB in mice with mild and severe hydronephrosis exposed to different perfusion pressures; **d** TGF-β measured by qPCR. The data are shown as the mean ± s.d; #< 0.05 compared with the N group, &< 0.05 compared with the M0 group, *< 0.05 compared with the S0 group

by an increase in renal fibrosis, and inhibition of EMT leads to a reduction in renal fibrosis. This suggests that EMT plays an important role in renal fibrosis and works in a non-cellular autonomic manner. Therefore, we tested α-SMA and E-cadherin to assess changes in EMT [31]. In the mild hydronephrosis group, significant changes in α-SMA and E-cadherin were observed when the perfusion pressure reached 60 mmHg, while in the severe hydronephrosis group, significant changes were observed when the perfusion pressure was 20 mmHg. This indicates that hydronephrosis is also accompanied by the dedifferentiation of epithelial cells and an increase in the number of interstitial phenotype cells; the addition of pressure perfusion exacerbates this change, and the severe hydronephrosis group is also more susceptible to significant changes.

TGF-β is a secreted protein that regulates EMT and promotes the proliferation and activation of interstitial fibroblasts [18, 32]. Although Grande and colleagues and Lovisa and colleagues have different conclusions about how TGF-β regulates renal fibrosis, both studies have shown that TGF-β plays an important role. The TGF-β signalling pathway has also been mentioned in many studies [33]. In our study, the expression levels of Smad2/3 and p-Smad2/3 in the hydronephrosis groups were significantly higher than those in the normal group, and significant changes were observed when the perfusion pressure reached 20 mmHg. In previous studies, changes in EMT were thought to be consistent with changes in signalling pathways [34, 35], and our experiments found that changes in EMT and TGF-β signalling pathways were not completely consistent. The partial EMT perspective proposed by Grande et al. explains this very well.

In conclusion, this study showed that the degree of renal fibrosis in severe hydronephrosis is heavier than that of mild hydronephrosis; after the pressure perfusion, the fibrosis of the kidney with severe hydrops is more likely to be aggravated, and these changes are closely related to the TGF-β signalling pathway. The above conclusions indicate that it is necessary to control the irrigation pressure during endoscopic treatment of patients with obstructive nephropathy.

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Compliance with ethical standards

Conflict of interest All authors declare no conflicts of interest.

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