Renal interstitial fibrosis (RIF) is a common pathological characteristic associated with end-stage renal disease. However, treatment strategies for RIF are still very limited. In this study, we reported that kaempferol, a classic flavonoid, exhibited strong and widely inhibitory effect on the expression of fibrosis related genes in transforming growth factor beta 1 (TGF-β1) treated NRK-52E cells. Further studies revealed that kaempferol inhibited TGF-β1 induced epithelial–mesenchymal transition (EMT) process of NRK-52E cells and improved renal function deterioration and RIF in unilateral ureteral obstruction (UUO) rats. After exploring the underlying mechanisms, we found that kaempferol was able to activate the BMP-7-Smad1/5 pathway, rather than the TGF-β1-Smad2/3 pathway.

Our previous study has shown that Ginkgo biloba extract (GbE) attenuated RIF caused by diabetes. GbE is a multi-component mixture contains flavonoids and terpene lactones. The flavonoids that have been identified mainly include rutin, myricetin, quercetin, luteolin, kaempferol, isorhamnetin and genistein. Among the flavonoid components, the effects of rutin, quercetin and myricetin have been reported in renal fibrosis induced by diabetes and UUO and in EMT process of renal tubular epithelial cells. However, the effects of luteolin, kaempferol, isorhamnetin and genistein on RIF are rarely studied. This study aimed to evaluate the effects of these four compounds on renal fibrosis in vitro and in vivo.

**MATERIALS AND METHODS**

**Reagents** Reagents used in this study are listed in Table S1.

**Cell Culture and Treatment** The rat proximal tubular epithelial cell line NRK-52E was a kind gift from Xuzhou Medical University (Xuzhou, Jiangsu, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (with 10% fetal bovine serum (FBS)). The cells were serum starved for 24h prior to treatment. To induce EMT, the cells were treated with TGF-β1 (5 ng/mL) for 24h. Genistin, isorhamnetin, kaempferol and luteolin were dissolved in 0.1% dimethyl sulfoxide (DMSO) to 0.5, 1, 5, 10, 20, 40, 80, 100, and 200 mM, respectively, and added to the medium at a ratio of 1:1000 (v/v). To inhibit the TGF-β1 or BMP-7 signaling pathways, the cells were treated with LY3200882 (1 µM) or DMH1 (20 µM), respectively.

**Animals and Study Design** Male Sprague-Dawley rats, weighing 180–220g, were purchased from the Laboratory Animal Center of Xuzhou Medical University. The performance of UUO and Sham rats were described previously. For the first animal experiment, the UUO rats were randomly divided into 5 groups with 8 animals in each
was performed through the intensity measurement using thejugated secondary antibodies (1:50,000). The quantification of \( \alpha \)-smooth muscle actin (\( \beta \)-Tubulin) was quantified using the ImageJ software (Version 1.52a, NIH, MD, U.S.A.).

**Histological Analysis** Kidney samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 4\( \mu \)m thickness were cut for histological analysis. The sections were stained with the Masson’s trichrome staining kit or Sirius Red staining kit according to the manufacturer’s instructions, respectively. Then, the sections were observed and the pictures were taken using a BX43F microscope (Olympus, Tokyo, Japan). Percentage of the positive areas was determined by the Image Pro Plus Software (Version 6.0, Media Cybernetics, MD, U.S.A.).

**Immunohistochemistry Analysis** Paraffin embedded kidney sections were deparaffinized in xylene and hydrated in graded alcohol and water, then were treated with 3% \( \text{H}_2\text{O}_2\). After blocking with 5% BSA, the sections were incubated with the primary antibody collagen I (1:200) at 4\( ^\circ \)C overnight. Then the sections were incubated with HRP-labelled secondary antibody (1:1000) at room temperature. A DAB reagent was used to perform visualization. After hematoxylin counterstaining, differentiation, dehydration and transparency, the sections were examined using an Olympus BX43F microscope. The protein expression was quantified by the Image Pro Plus Software.

**Statistical Analysis** Data were presented as the mean ± standard deviation (S.D.) All data were analyzed using the SPSS software (Version 24.0, IBM, NY, U.S.A.). For multiple comparisons, ANOVA was applied with LSD post hoc analysis for data meeting homogeneity of variance or with Dunnett’s T3 analysis for data not assuming equal variances. In all cases, differences were considered significant at \( p<0.05 \).

**RESULTS**

**Kaempferol Inhibits EMT of NRK-52E Cells Induced by TGF-\( \beta 1 \)** We first measured the effects of genistein, isorhamnetin, kaempferol and luteolin on the viability of NRK-52E cells. We first measured the effects of genistein, isorhamnetin, kaempferol and luteolin on the viability of NRK-52E cells. The results showed that the four compounds had little effect on the viability of NRK-52E cells when the concentrations are lower than 40\( \mu \)M (Fig. 1a and Figs. S1a–c). Therefore, three concentrations of the four compounds (5, 10 and 20\( \mu \)M) were selected for subsequent experiments. Cells were treated with TGF-\( \beta 1 \) to induce EMT and PFD was set as a positive control. The results showed that high dose of genistein slightly downregulated the expression of \( \text{Colla}2 \) and \( \text{Ctgf} \) genes induced by TGF-\( \beta 1 \) (Fig. S1d). Treatment with isorhamnetin at a high dose was able to inhibit the gene expression of \( \text{Colla}1 \), \( \text{Colla}2 \), \( \text{Ctgf} \) and \( \text{Fnd}1 \) (Fig. S1e). Likewise, luteolin showed to downregulate the gene expression of \( \text{Colla}2 \) and \( \text{Col3a1} \) (Fig. S1f).

Morphological changes of the cells further confirmed the
results above. The long fusiform morphology induced by TGF-β1 was reversed after kaempferol treatment (Fig. 1c). Moreover, the expression of epithelial phenotype E-cadherin in the cells was downregulated after TGF-β1 treatment. In contrast, the mesenchymal protein α-SMA was upregulated. Medium and high doses of kaempferol treatment showed a strong reversal effect on the protein expression of E-cadherin and α-SMA induced by TGF-β1 (Fig. 1d and Fig. S2).

Kaempferol Improves RIF in Rats Induced by UUO To study the effect of kaempferol on RIF, we prepared a UUO rat renal fibrosis model. The results showed that the left ureteral obstruction induced an enlargement of both the left and right kidneys of the rats. Kaempferol and PFD treatment reversed this phenomenon to varying degrees (Figs. 2a, b). In addition, we also detected the expression of Collagen I in kidney sections of the rats using immunohistochemistry. In accordance with the above results, a large amount of Collagen I was found deposited in the kidney of UUO rats, mainly in the renal interstitium. Both medium and high doses of kaempferol downregulated the content of this protein (Fig. 2h and Fig. S3f).

Kaempferol Prevents EMT of NRK-52E Cells Induced by TGF-β1 through the BMP-7-Smad1/5 Pathway The TGF-β1-Smad2/3 and BMP-7-Smad1/5 are two key pathways in fibrosis regulation. In this study, TGF-β1 significantly induced the phosphorylation of Smad2/3, while inhibited the phosphorylation of Smad1/5. Kaempferol treatment restored the phosphorylation levels of Smad1/5 but had little effect on the activity of Smad2/3. LY3200882 and DMH1 verified the reliability of our results (Fig. 3a and Figs. S4a, b). Besides, kaempferol itself was able to upregulate the protein expression of BMP-7 and the phosphorylation of Smad1/5 slightly. However, it showed little effect on the phosphorylation levels of Smad2/3 (Fig. S4c). These results suggested that the effects of kaempferol are likely to be associated with its regulation on BMP-7 signaling pathway. To test this hypothesis, we first studied the effects of kaempferol on EMT process of NRK-52E cells induced by TGF-β1 when treated with DMH1. The results showed that DMH1 reversed the effect of kaempferol on TGF-β1 induced cell morphological changes and expression of fibrosis related genes (Fig. 3b and Fig. S4d). In addition, DMH1 treatment notably reversed the phenomena that kaempferol prevented the down-regulation of E-cadherin and...
the up-regulation of α-SMA caused by TGF-β1 (Fig. 3c and Figs. S4e, f).

Considering the fact that kaempferol can up-regulate the protein expression of BMP-7 (Fig. 3c and Figs. S4g, h), we studied the roles of BMP-7 interference on protective effects of kaempferol in vitro. It showed that BMP-7 knockdown blocked the improvement of kaempferol on cell morphological changes and gene expression of Col1a1, Col1a2, Col3a1, Ctgf, Fn1 and Acta2 induced by TGF-β1 (Fig. 3d and Fig. S4i). In accordance with the role of DMH1, BMP-7 knockdown blocked kaempferol’s effects on E-cadherin upregulation and α-SMA downregulation (Fig. 3e and Figs. S4j–m).

Protective Effects of Kaempferol on Renal Fibrosis of Rats Induced by UUO Are Dependent on the BMP-7-Smad1/5 Pathway To further investigate the role of BMP-7 pathway in the protective effects of kaempferol on renal fibrosis in vivo, UUO rats were treated with DMH1. The results showed that DMH1 treatment not only antagonized the improvement of kaempferol on kidney hypertrophy and renal function decline in UUO rats (Figs. 4a–d), but also reversed the effect of kaempferol on protein expression of E-cadherin and α-SMA (Fig. 4e and Figs. S5a, b). It is worth mentioning that the combined treatment with DMH1 and kaempferol totally restored the expression Collagen I and Collagen III, which was downregulated after kaempferol treatment (Fig. 4e and Figs. S5c–f). Moreover, administration of DMH1 combined with kaempferol up-regulated the gene expression of Coll1a1, Colla2, Col3a1, Ctgf and Fn1 again compared with the rats treated with kaempferol alone (Fig. 4f). Masson staining revealed that the deposition of collagens in the kidney of UUO rats was notably reduced when treated with a medium dose of kaempferol. However, inhibition of BMP-7 signaling by DMH1 made this amelioration abolished (Fig. 4g and Fig. S5g). The results in Sirius Red staining and Collagen I immunohistochemistry staining were consistent with that in Masson staining (Figs. S5h–k).

DISCUSSION

RIF is one of the main features of progressive renal injury, in which EMT of the renal tubular epithelial cells is the central event.25) The myofibroblasts can break through the basement membrane and transfer to the renal interstitium, secreting a large amount of ECM and eventually leading to renal function decline.25) There are various animal models of RIF, including UUO, 5/6 nephrectomy, drug induced nephrotoxic-
ity and diabetic nephropathy, etc. Among them, UUO is a simple and reproducible model which can reflect the characteristics of RIF in human obstructive nephropathy and chronic progressive kidney diseases.

Genistein, isorhamnetin, kaempferol and luteolin are four flavonoids in GbE. Among the four compounds, kaempferol has attracted our attention because of its broad-spectrum and strong inhibitory effect on the six fibrosis related genes. Therefore, we continued to study the anti-fibrotic effects of kaempferol in the subsequent experiments and tried to explore the mechanisms preliminarily. It was demonstrated that kaempferol not only ameliorated the EMT process of renal tubular epithelial cells induced by TGF-β1, but also improved renal function decline and RIF in the UUO rats in a dose-dependent manner.

TGF-β1 has been identified as a key regulator of fibrosis and its role in RIF has been extensively studied. Therefore, TGF-β1 is becoming an important target for many studies to design and develop new drugs that prevent RIF. In addition to the TGF-β1 signaling pathway, we concentrated on another protein, BMP-7. BMP-7 is an anti-fibrotic protein and its anti-fibrotic effect is largely achieved by antagonizing the TGF-β signaling pathway. For example, BMP-7 showed to inhibit the production of ECM in mesangial cells and retain the pheno-
To understand the mechanism by which kaempferol attenuates RIF, we examined the effects of kaempferol on TGF-β1 and BMP-7 signaling pathways. Kaempferol treatment reversed the activity of Smad1/5, while had little effect on the phosphorylation levels of Smad2/3. These results indicated that the anti-fibrotic effects of kaempferol may be related to the activation of BMP-7-Smad1/5 signaling pathway. To further validate our hypothesis, DMH1 was utilized to inhibit the BMP-7-Smad1/5 signaling pathway in vitro and in vivo. As a result, effects of kaempferol on TGF-β1 induced EMT in NRK-52E cells and UUO caused renal fibrosis in rats were greatly antagonized. Moreover, we found that kaempferol treatment obviously upregulated protein expression of BMP-7 in TGF-β1 treated cells, which may be responsible for the activation of BMP-7-Smad1/5 signaling pathway. BMP-7 knockdown in NRK-52E cells further confirmed this hypothesis.

It is worth noting that DMH1 treatment or BMP-7 knockdown cannot completely reverse the improvement of renal fibrosis by kaempferol. We believe that the targets of natural compounds are extensive, and kaempferol is no exception. The BMP-7-Smad1/5 pathway is likely to be one of the targets of kaempferol. A recent study reported that kaempferol was able to attenuate renal fibrosis in diabetic nephropathy by inhibiting the RhoA/Rho-kinase.

In conclusion, we confirmed the strong potential of kaempferol in preventing RIF in vitro and in vivo. Through the initial exploration of the underlying mechanisms, we believed that the protective effects of kaempferol on RIF is closely related to the upregulation of BMP-7 protein and subsequent activation of Smad1/5 but not directly associated with the type of renal tubular epithelial cells induced by TGF-β1. To understand the mechanism by which kaempferol attenuates RIF, we examined the effects of kaempferol on TGF-β1 and BMP-7 signaling pathways. Kaempferol treatment reversed the activity of Smad1/5, while had little effect on the phosphorylation levels of Smad2/3. These results indicated that the anti-fibrotic effects of kaempferol may be related to the activation of BMP-7-Smad1/5 signaling pathway. To further validate our hypothesis, DMH1 was utilized to inhibit the BMP-7-Smad1/5 signaling pathway in vitro and in vivo. As a result, effects of kaempferol on TGF-β1 induced EMT in NRK-52E cells and UUO caused renal fibrosis in rats were greatly antagonized. Moreover, we found that kaempferol treatment obviously upregulated protein expression of BMP-7 in TGF-β1 treated cells, which may be responsible for the activation of BMP-7-Smad1/5 signaling pathway. BMP-7 knockdown in NRK-52E cells further confirmed this hypothesis.

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In conclusion, we confirmed the strong potential of kaempferol in preventing RIF in vitro and in vivo. Through the initial exploration of the underlying mechanisms, we believed that the protective effects of kaempferol on RIF is closely related to the upregulation of BMP-7 protein and subsequent activation of Smad1/5 but not directly associated with the...
TGF-β1-Smad2/3 pathway. However, the molecular mechanism of kaempferol on BMP-7 protein regulation was not elucidated and is worthy of further deep study. This research expands the boundary of kaempferol in disease treatment and provides a theoretical basis for the development of novel drugs for RIF therapy.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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