# Tetramethylpyrazine alleviates acute kidney injury by activating the Wnt/β-catenin pathway independent of DKK1

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**Abstract.** Acute kidney injury (AKI) is a group of common clinical syndromes characterized by a rapid decline in renal function over a short period of time. At present, the treatment methods are limited, and research is needed to identify drugs that could alleviate renal ischemia-reperfusion (I/R) injury. Tetramethylpyrazine (TMP) is a bioactive alkaloid extracted from the Chinese herbal medicine Chuanxiong. TMP is known to possess various anti-inflammatory and cardiovascular and renal protective effects; however, the therapeutic molecular targets are still unclear. In the present study, using a rat renal I/R model, the effects of TMP on renal injury, dickkopf-1 (DKK1) expression, Wnt/β-catenin signaling and apoptosis were evaluated through morphological examination, renal function tests, western blotting, immunohistochemistry and TUNEL assays. It was determined that TMP ameliorated tubular pathologic injury and improved renal function in rats following renal I/R. In addition, in rats following I/R, TMP promoted the expression of DKK1, an inhibitor of the Wnt/β-catenin signaling pathway, in renal tissues, activated the Wnt/β-catenin signaling pathway in kidney tissues and reduced apoptosis of renal cells. To the best of our knowledge, the present study is the first to investigate the regulatory effects of TMP on DKK1 and the Wnt/β-catenin signaling pathway, revealing that TMP could attenuate AKI by activating Wnt/β-catenin signaling independent of the inhibitory effect of DKK1.

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## Introduction

Acute kidney injury (AKI) is a common clinical condition that is characterized by a rapid decline in renal function (1). AKI can lead to incomplete renal repair, persistent chronic inflammation and progressive fibrosis, all of which can lead to chronic kidney disease and end-stage renal disease (2). Ischemia-reperfusion (I/R) is a major pathogenic factor for AKI (3). However, the mechanism of tissue damage and repair during AKI is still unclear and the immune inflammatory response mediating tissue damage and fibrosis has become an interest in research worldwide (4,5).

The bioactive alkaloid tetramethylpyrazine (TMP) is found in the Chinese herbal medicine Chuanxiong and has been proven to possess several pharmacological properties in previous studies (6). TMP has been shown to have physiological properties, including antioxidative, anti-inflammatory, anti-calcium antagonism and anti-apoptotic effects. Furthermore TMP has been implicated in autophagy regulation, vasodilation, angiogenesis regulation, mitochondrial damage suppression, endothelial protection, reduction of the proliferation and migration of vascular smooth muscle cells, and neuroprotection (6-8). Clinically, TMP is used to treat cardiovascular, cerebrovascular and chronic kidney diseases due to its ability to enhance blood flow and microcirculation (9-11); however, its therapeutic molecular target is not clear. Previous studies from our group have shown that TMP ameliorates AKI to a certain extent by inducing autophagy, which can downregulate nucleotide-binding oligomerization domain-containing protein 2-mediated inflammation; TMP may also attenuate AKI by inhibiting nucleotide-oligomerization domain-like receptor 3 inflammasome (12,13). Furthermore, previous studies have demonstrated that TMP can inhibit the proliferation and infiltration of cancer cells by blocking the Wnt/β-catenin signaling pathway (14,15).

The Wnt/β-catenin signaling pathway is a signaling pathway, that has been relatively well preserved throughout evolution, which is essential for the growth and development of the body (16). The abnormal activation of this signaling pathway can lead to a variety of diseases, including kidney and cardiovascular diseases and tumors (17,18). Activation

of the Wnt/β-catenin signaling pathway has been shown to be involved in kidney injury caused by AKI, glomerular disease, diabetic nephropathy, renal fibrosis and cystic kidney disease (19-21). In AKI, the activation of the Wnt/β-catenin signaling pathway has a dual effect. Moderate activation of the Wnt/β-catenin signaling pathway is beneficial to the regeneration of renal tubular epithelial cells; however, excessive activation can promote the progression of renal injury to chronic fibrosis (22,23).

The Wnt/β-catenin signaling pathway is controlled by a number of proteins, including dickkopf-1 (DKK1) (16). Secretion of the glycoprotein DKK1 inhibits Wnt/β-catenin signaling pathway activity by binding to cell membrane receptors, such as low-density lipoprotein receptor-related protein 5/6 (LRP5/6) and Kremen 1, and mediates endocytosis (24,25). Chronic kidney disease is associated with DKK1 as it promotes accumulation of mesangial cell matrix and renal dysfunction in response to hyperglycemia (26). In lupus nephritis, increased renal Wnt activity is accompanied by elevated DKK1 levels, and DKK1 elevation contributes to renal injury by promoting apoptosis and increasing the extracellular immunogenic chromatin load (27). Furthermore, DKK1 notably inhibits fibrosis in chronic kidney injury, suggesting that this protein might be a therapeutic target for fibrosis (28,29). However, the role of DKK1 in AKI has not yet been discovered.

To the best of our knowledge, the present study was the first to focus on the regulatory effect of the traditional Chinese medicine derivative TMP on DKK1 and the Wnt/β-catenin signaling pathway, and to investigate its effect on preventing AKI. In the present study, a rat model was used to determine the regulatory effect of TMP on DKK1 and the Wnt/β-catenin signaling pathway using cell biology and molecular immunology techniques, with the aim of revealing the mechanism of TMP in alleviating AKI and promoting the regeneration and recovery of renal cells.

## Materials and methods

*Animal studies.* Animal experiments were conducted in compliance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (30) and was approved by the Institutional Animal Care and Use Committee of Binzhou Medical University (Yantai, China; approval no. 2020-07). A total of 18 male Sprague-Dawley (SD) rats (weight, 260-300 g; age, 8 weeks) were purchased from the Animal Experimental Center of Shandong University (Jinan, China). All animals were housed at a constant temperature of 24˚C with a humidity of 55%, under a 12-h light/dark cycle, and had unrestricted access to water and food. I/R models were established using rats anesthetized with pentobarbital sodium (40 mg/kg body weight) intraperitoneally (31). Subsequently, the I/R injury model was established in SD rats by clamping the bilateral renal pedicle with non-traumatic microvascular clamps as previously described (12); serum creatinine (SCr), blood urea nitrogen (BUN) levels and morphological examinations were used to assess the success of the model establishment. The rats were randomly divided into the following groups (n=6/group): Sham, kidney I/R and kidney I/R with TMP treatment (I/R + TMP). The sample sizes for the study were determined based on the resource equation method (32). In the sham group, the renal artery was isolated without ischemic treatment. In the I/R + TMP group, TMP hydrochloride (40 mg/kg body weight; Harbin Medisan Pharmaceutical Co., Ltd.) was administered by intraperitoneal injection immediately following reperfusion, at 6 h intervals (12,13). Following completion of the treatment for 24 h, all rats were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (150 mg/kg) and subsequently sacrificed by cervical dislocation.

The following criteria were used to define the humane endpoints for the present study: i) Lack of movement or unresponsiveness to gentle stimuli; ii) respiratory distress (typical symptoms include drooling from the mouth or nose and/or cyanosis); iii) diarrhea or urinary incontinence; iv) weight loss of >20% compared with the pre-experiment body weight; v) inability to eat or drink; vi) persistent seizures or stereotyped behavior (in the absence of external stimuli, rats exhibited spontaneous rotation, digging, jumping and grooming behaviors); and vii) skin lesions covering >30% of the body or signs of purulent infection. The surgical procedure was uneventful, with no rats sacrificed early due to reaching humane endpoints.

Death was verified by loss of corneal reflex, a lack of response to a firm toe pinch, and observation of cardiac and respiratory arrest. Subsequently, kidney tissues and blood samples from the heart were collected. Parts of the kidney tissues were stored at -80˚C for protein detection, whereas others were fixed in 4% paraformaldehyde at 4˚C for 24 h, embedded in paraffin and sectioned at 4-$\mu$m thickness for morphological detection. The blood samples were used for the detection of the biochemical indicators of renal injury.

*Morphological examinations.* Hematoxylin and eosin (H&E) staining was conducted on paraffin-embedded 4-$\mu$m kidney tissue sections. Sections were dewaxed in xylene, stained with hematoxylin for 5 min at room temperature, and then placed in distilled water for 10-30 sec. Following this, the tissue sections were incubated in 1% hydrochloric acid alcohol for 3 sec then washed with running water for 10 min. Finally, the sections were stained with eosin for 10 min at room temperature then dehydrated and sealed with neutral gum. Tubular injury of the cortex or the outer medulla was based on epithelial necrosis, vacuolization or tubular dilatation and was scored as follows: 0, none; 1, 1-10%; 2, 11-25%; 3, 26-45%; 4, 46-75%; 5, >75%. For each section, ≥10 fields were examined under magnification x200. The histological scoring was performed blindly. Visualization was performed using a Leica DM 6000 B light microscope (Leica Microsystems, Inc.).

*Renal function test.* Blood was collected from the heart of the animals after euthanasia and the samples were allowed to stand for 30 min. Subsequently, the samples were centrifuged at 4˚C at 1,000 x g for 10 min and the serum was separated. An automatic biochemical analyzer was used to detect SCr and BUN.

*Immunohistochemical (IHC) staining.* IHC staining was performed on paraffin-embedded 4-$\mu$m kidney tissue sections. Sections were dewaxed and placed in citrate buffer (0.01 M, pH 6.0) for antigen retrieval; briefly, samples were placed in a microwave, boiled on high heat for 4 min then allowed to stand

for 5 min, this was followed by a further 1 min at medium heat then allowed to stand for 5 min. Subsequently, the samples were cooled to room temperature and washed three times with PBS (5 min each). The sections were then incubated with 3% $H_2O_2$ at room temperature for 20 min to remove endogenous peroxidase activity and washed three times with PBS (5 min each). The sections were blocked with 5% normal goat serum (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h and incubated with the following primary antibodies at 4˚C overnight: DKK1 (cat. no. ab109416; 1:250; Abcam), Wnt1 (cat. no. ab15251; 1:100; Abcam), β-catenin (cat. no. WL0962a; 1:200; Wanleibio Co., Ltd.), caspase-3 (cat. no. WL04004; 1:200; Wanleibio Co., Ltd.), Bax (cat. no. ER0907; 1:200; HUABIO) and Bcl-2 (cat. no. ET1702-53; 1:200; HUABIO). The following day, the slides were rewarmed for 45 min at room temperature and washed three times with PBS (3 min each) to remove unbound primary antibodies. The sections were then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse secondary antibody (undiluted; cat. no. PV-6000; OriGene Technologies, Inc.) for 2 h at room temperature, and washed three times with PBS (3 min each) to wash off the unbound secondary antibody. Subsequently, 3,3'-diaminobenzidine staining was performed at room temperature in accordance with the kit's instructions (cat. no. ZLI-9018; OriGene Technologies, Inc.). Tap water was used to terminate the reaction and hematoxylin staining was used to stain the cellular nuclei at room temperature for 5 min followed by rinsing in tap water. Sections were differentiated in 1% hydrochloric acid-alcohol for 1-3 sec, rinsed in tap water for 10 min to achieve bluing, dehydrated and cleared in xylene. The sections were sealed with neutral gum and visualized using a Leica DM 6000 B light microscope (Leica Microsystems, Inc.). ImageJ software (version 1.8.0; National Institutes of Health) was used for semi-quantification.

*TUNEL staining*. TUNEL staining was carried out on paraffin-embedded 4-$\mu$m kidney tissue sections. According to the instructions of the TUNEL kit (Roche Diagnostics), the sections were dewaxed, 20 $\mu$g/ml DNase-free proteinase K was added dropwise, and sections were incubated at 37˚C for 15-30 min, and then washed with PBS three times. Subsequently the samples were incubated with 3% $H_2O_2$ at room temperature for 20 min to inactivate the endogenous peroxidase activity, followed by washing with PBS three times. Following this, 50 $\mu$l TdT and 450 $\mu$l fluorescein-labeled dUTP was mixed as biotin labeling solution and added to the sections at 37˚C for 1 h in a dark box. Cellular nuclei were stained with 4',6-diamidino-2-phenylindole (cat. no. C1006; Beyotime Institute of Biotechnology) at room temperature for 5 min. After washing with PBS, slides were incubated with anti-fluorescence quenching reagent to seal the slides. A fluorescence microscope was used to observe the samples.

*Western blot analysis*. The renal cortex was homogenized in ice-cold radioimmunoprecipitation lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology). The total amount of protein extracted was determined with the BCA Protein Assay kit (cat. no. P0010; Beyotime Institute of Biotechnology). In each lane, 40 $\mu$g extracted protein was loaded, separated by 8-10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes (MilliporeSigma). The membranes were blocked using 5% skimmed milk at room temperature for 1 h. Subsequently, the membranes were incubated with primary antibodies overnight at 4˚C, washed and incubated with HRP-conjugated goat anti-mouse IgG (cat. no. SA00001-1; 1:5,000; ProteinTech Group, Inc.) and HRP-conjugated goat anti-rabbit IgG (cat. no. SA00001-2; 1:5,000; ProteinTech Group, Inc.) at room temperature for 2 h. The protein bands were visualized using an enhanced chemiluminescence detection system (Tanon Science and Technology Co., Ltd.) and Millipore Immobilon ECL (MilliporeSigma). Western blot bands were semi-quantified using ImageJ software (version 1.8.0; National Institutes of Health). The following primary antibodies were used in the present study: DKK1 (cat. no. ab109416; 1:1,000; Abcam), Wnt1 (cat. no. ab15251; 1:1,000; Abcam), β-catenin (cat. no. WL0962a; 1:1,000; Wanleibio Co., Ltd.), caspase-3 (cat. no. WL04004; 1:1,000; Wanleibio Co., Ltd.), cleaved caspase-3 (cat. no. WL01992; 1:1,000; Wanleibio Co., Ltd.), Bcl-2 (cat. no. ET1603-11; 1:1,000; HUABIO) and β-actin (cat. no. 66009-1-Ig; 1:5,000; ProteinTech Group, Inc.).

*Statistical analysis*. The renal tubular pathological injury score data are presented as the median (IQR), and were analyzed using Kruskal-Wallis test and Dunn's post hoc test. The other data are presented as the mean ± SEM, and the significance of differences in among the groups was examined by one-way ANOVA followed by Duncan's or Tukey's post hoc test. GraphPad Prism 9 (Dotmatics) was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*TMP protects against AKI by alleviating renal tubular pathological injury and improving renal function following I/R in a rat model*. The results of SCr and BUN quantification indicated that compared with in the sham group, the SCr and BUN levels of the rats in the I/R group were significantly increased (Fig. 1A and B). By contrast, the SCr and BUN levels of the rats in the I/R + TMP group were significantly decreased compared with the levels in the I/R group, indicating that TMP treatment could reduce I/R kidney injury. No significant difference was noted in the body weight of rats in the I/R and the I/R + TMP groups compared with that in the sham group (Fig. 1C). H&E staining indicated that the glomeruli and renal tubules in the sham operation group were intact without apparent morphological abnormalities, whereas H&E staining in the I/R group indicated swelling, vacuolar degeneration, necrosis and shedding, and cast formation in tubular lumen (Fig. 1D). In the I/R + TMP group, the aforementioned pathological changes were alleviated, and the course of the disease was decelerated. Double-blind analysis of renal tubular pathological injury scores by pathologists revealed that TMP could significantly alleviate I/R renal injury in rats.

*TMP promotes the expression of DKK1 in the renal tissues of rats following I/R*. Western blot analysis revealed that the protein expression levels of DKK1 in the I/R group were

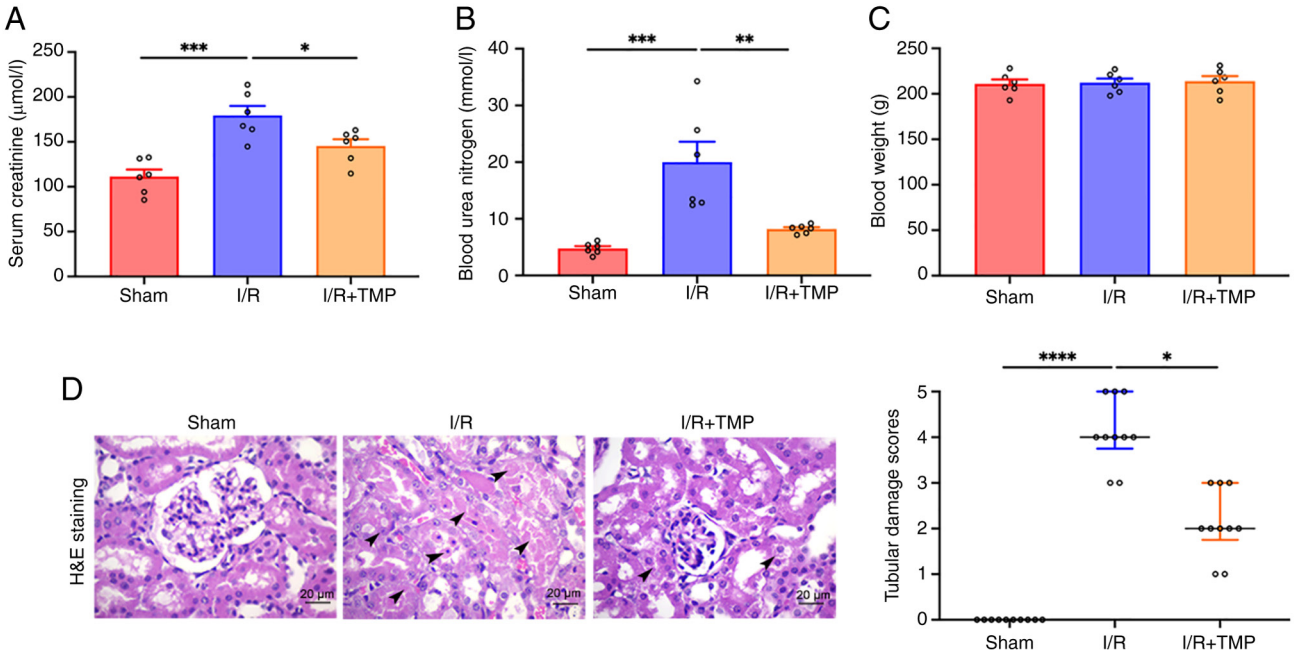


Figure 1. TMP protects against acute kidney injury by alleviating renal tubular pathological injury and improving renal function following I/R in a rat model. (A) Serum creatinine and (B) blood urea nitrogen levels of rats in different groups were measured using a biochemical analyzer. n=6. (C) Body weight in different groups. n=6. (D) Representative H&E images showing the renal morphological changes (black arrows) and renal tubular damage scores of rats in different groups. Scale bars, 20 $\mu$m, magnification, x400. n=10. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001, $^{****}$P<0.0001. I/R, ischemia-reperfusion; TMP, tetramethylpyrazine; H&E hematoxylin and eosin.

significantly lower than those in the sham group, whereas they were significantly increased following treatment with TMP compared with those in the I/R group (Fig. 2A). Furthermore, IHC analysis indicated that the expression levels of DKK1 in the I/R group were significantly lower than those in the sham group, whereas the expression levels of DKK1 were significantly increased following treatment with TMP compared with the expression levels in the I/R group (Fig. 2B). The results of the IHC analysis were consistent with the results of the western blot analysis. TMP significantly increased the expression levels of DKK1 in the kidney tissues of rats following I/R.

*TMP activates the Wnt/β-catenin signaling pathway in the kidney tissues of rats following I/R*. Western blot analysis of Wnt1 expression in the renal tissues of each group indicated that compared with in the sham group, the protein expression levels of Wnt1 were significantly decreased in the renal tissues of the I/R group; however, following treatment with TMP, the protein expression levels of Wnt1 in the renal tissues of the I/R + TMP group were significantly increased compared with those in the I/R group (Fig. 3A). The results of the IHC analysis indicated that the expression levels of Wnt1 in the renal tissues were significantly decreased following renal I/R injury in rats, whereas the expression levels of Wnt1 were significantly increased following treatment with TMP compared with those in the I/R group (Fig. 3B). Furthermore, western blot analysis indicated that the protein expression levels of β-catenin were significantly lower in the renal tissues of the I/R group compared with those in the sham group; by contrast, the protein expression levels of β-catenin were significantly increased in the renal tissues following TMP treatment compared with those in the I/R group (Fig. 3C). In addition, IHC analysis indicated that the expression levels of β-catenin were significantly reduced in the renal tissues following I/R injury in rats; however, the protein expression levels of β-catenin were significantly increased following TMP treatment compared with those in the I/R group (Fig. 3D). Taken together, these results suggested that TMP activated the Wnt/β-catenin signaling pathway.

*TMP ameliorates renal cell apoptosis following I/R in rats*. TUNEL staining indicated that, compared with that in the sham group, the number of apoptotic renal tubular epithelial cells in the I/R group was significantly increased, whereas the number of apoptotic renal tubular epithelial cells was significantly decreased following treatment with TMP compared with that in the I/R group (Fig. 4A). In addition, the apoptotic proteins caspase-3 and Bax, as well as the anti-apoptotic protein Bcl-2, were detected in the renal tissues of rats (Fig. 4B-F). IHC analysis indicated that rats in the I/R group expressed significantly higher levels of the apoptotic protein Bax in renal tissues as compared with rats in the sham group, whereas Bax expression levels decreased significantly following treatment with TMP compared with in the I/R group (Fig. 4B). IHC analysis also indicated that rats in the I/R group expressed significantly higher levels of the apoptotic protein caspase-3 in renal tissues as compared with rats in the sham group, whereas caspase-3 expression levels decreased significantly following treatment with TMP compared with in the I/R group (Fig. 4C). Western blot analysis indicated that the relative ratio of cleaved caspase-3/caspase-3 in the renal tissues of the I/R group was significantly higher than that in the sham group, which was significantly decreased in the renal tissues following TMP

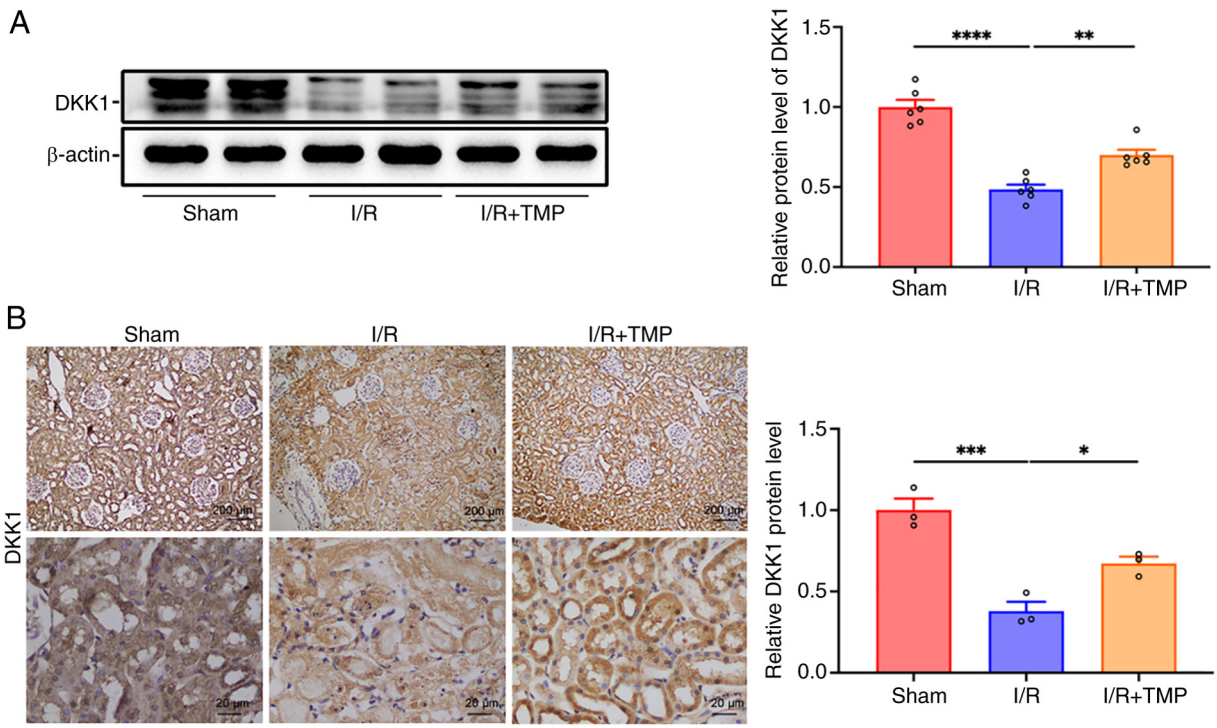


Figure 2. TMP promotes the expression of DKK1 in the renal tissue of rats following I/R. (A) Representative western blots and semi-quantified data showing the expression of DKK1 in the renal tissue of rats in the sham, I/R and I/R + TMP groups. n=6. (B) Representative IHC images of DKK1 in the renal tissue of rats in the sham, I/R and I/R + TMP groups, and semi-quantification. n=3. IHC images: Top row, scale bars, 200 $\mu$m, magnification, x40; bottom row, scale bars, 20 $\mu$m, magnification, x400. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001, $^{****}$P<0.0001. I/R, ischemia-reperfusion; TMP, tetramethylpyrazine; DKK1, dickkopf-1; IHC, immunohistochemistry.

treatment compared with in the I/R group (Fig. 4D). IHC analysis indicated that compared with those in the sham group, the expression levels of the anti-apoptotic protein Bcl-2 were significantly increased in the renal tissues of the I/R group (Fig. 4E). However, the expression levels of Bcl-2 were further increased following treatment with TMP compared with in the I/R group. Western blot analysis also indicated that the protein expression levels of Bcl-2 in the renal tissues of the I/R group were significantly higher than those in the sham group, and the protein expression levels of Bcl-2 were further increased in the renal tissues following TMP treatment compared with those in the I/R group (Fig. 4F). These results suggested that TMP could alleviate the apoptosis of renal tissue following I/R by inhibiting the cleavage of caspase-3 and the expression of Bax, and increasing the expression of the anti-apoptotic protein Bcl-2.

## Discussion

The pathological mechanism of AKI is complex, including inflammation, ischemia and nephrotoxic injury (33). To the best of our knowledge, the present study was the first to discover the regulatory effect of TMP on DKK1 and the Wnt/β-catenin signaling pathway in AKI renal tissues, revealing that TMP attenuated AKI by activating the Wnt/β-catenin signaling independent of DKK1. AKI is a group of clinical syndromes in which renal function suddenly declines sharply in a short period of time, and may result in chronic kidney disease, end-stage renal disease or death (1). Notably, AKI is a common clinical critical illness; it affects 10-15% of hospitalized patients, and >50% of patients in the intensive care unit (34). The pathological changes of AKI are mainly apparent in the renal tubular morphology, including loss of renal tubular epithelial cell polarity, swelling, vacuolar degeneration, necrosis, brush border shedding, cast formation in the tubular lumen and inflammatory cell infiltration in the interstitium. The main pathological mechanisms of AKI include generation of free radicals, intracellular calcium overload (35), inflammatory reactions and apoptosis (36).

The Wnt/β-catenin signaling pathway is a common developmental signaling pathway that plays a crucial role in embryonic development, morphogenesis and organogenesis, tissue regeneration, and the pathogenesis of various diseases (17). A number of studies have indicated that the Wnt/β-catenin signaling pathway is involved in multiple organ I/R injuries, such as cardiac, cerebral and renal I/R injuries (37-40). Studies have shown that in AKI transient activation of the Wnt/β-catenin signaling pathway is beneficial in reducing the development of this disease, and promoting kidney repair and regeneration, whereas sustained activation of the Wnt/β-catenin signaling pathway promotes the progression of AKI to chronic kidney disease (41). It is currently considered that the Wnt/β-catenin signaling pathway promotes renal fibrosis in chronic kidney disease (18,42). Inhibition of the Wnt/β-catenin signaling pathway may maintain the integrity of podocytes, reduce proteinuria and attenuate kidney injury (43). Wnt/β-catenin signaling pathway serves as a regulatory network that promotes or inhibits apoptosis/autophagy under certain circumstances (44-46). Therefore, activation of the Wnt/β-catenin signaling pathway could attenuate AKI

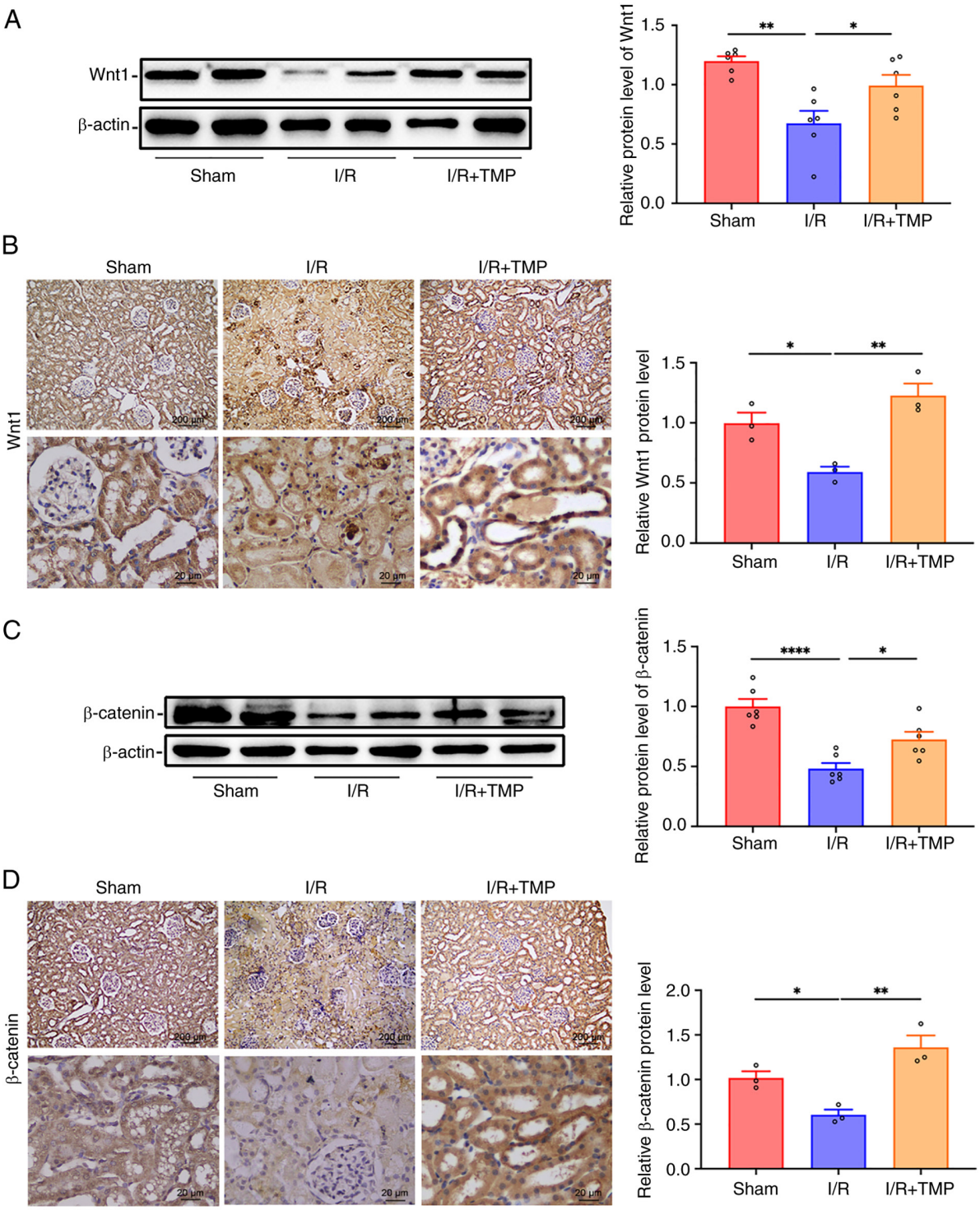


Figure 3. TMP activates the Wnt/β-catenin signaling pathway in the kidney of rats following I/R. (A) Representative western blots and semi-quantified protein levels showing the expression levels of Wnt1 in the renal tissue of rats in different groups. n=6. (B) Representative IHC images and protein semi-quantification of Wnt1 in the renal tissue of rats in the sham, I/R and I/R + TMP groups. n=3. (C) Representative western blots and semi-quantified protein levels showing the expression levels of β-catenin in the renal tissue of rats in different groups. n=6. (D) Representative IHC images and protein semi-quantification of β-catenin in the renal tissue of rats in the sham, I/R and I/R + TMP groups. n=3. IHC images: Top row, scale bars, 200 $\mu$m, magnification, x40; bottom row, scale bars, 20 $\mu$m, magnification, x400. $^{*}$P<0.05, $^{**}$P<0.01, $^{****}$P<0.0001. I/R, ischemia-reperfusion; TMP, tetramethylpyrazine; IHC, immunohistochemistry.

caused by I/R (47). Recent studies have demonstrated that TMP can block the Wnt/β-catenin signaling pathway, and inhibit the proliferation and infiltration of cancer cells (14,15).

The findings of the present study differ from those of previous studies (19,48) and indicated that the expression levels of Wnt1 and β-catenin were significantly decreased in rats following I/R, while the expression levels of Wnt1 and β-catenin were significantly increased following treatment with TMP. Western blotting results showed that Wnt1 and β-catenin expression in the I/R + TMP group exceeded the expression observed in the I/R group, which was consistent with the results of IHC analysis. The IHC analysis results

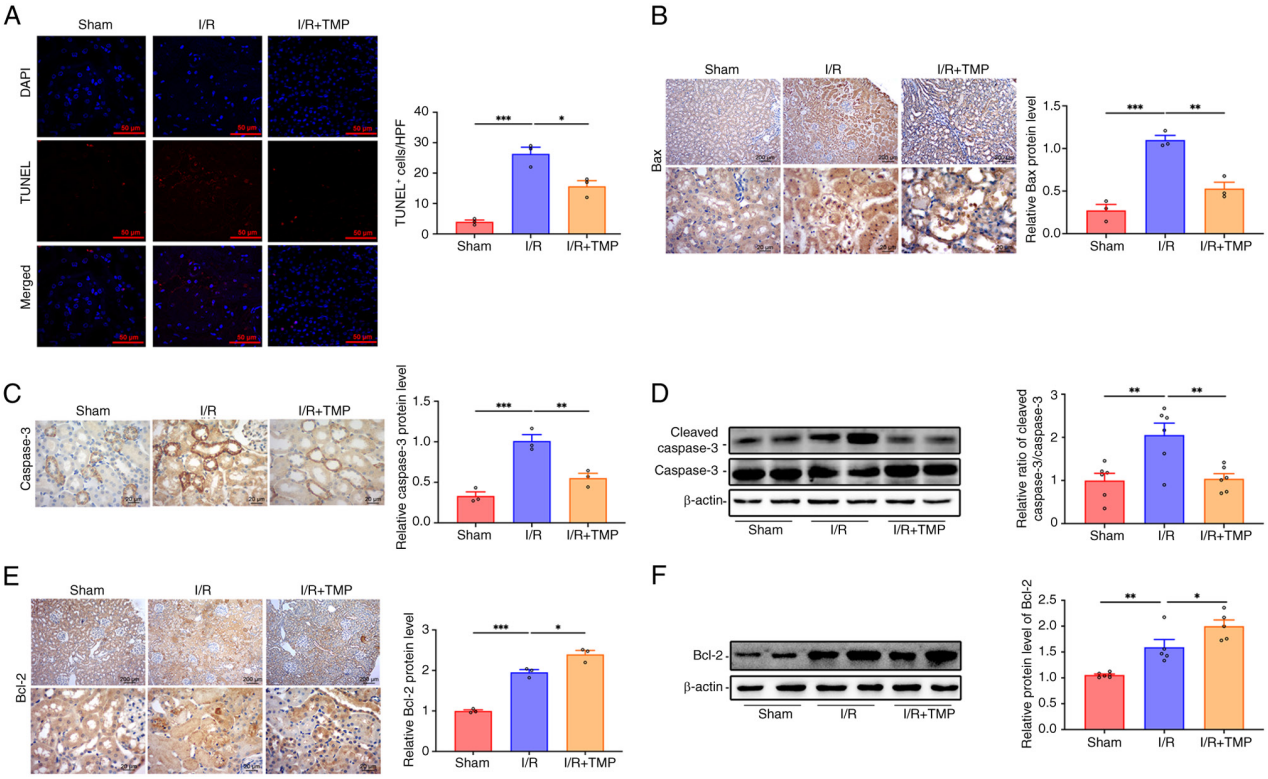


Figure 4. TMP ameliorates renal cell apoptosis after I/R in rats. (A) TUNEL staining showing the renal cell apoptosis levels in different groups. Scale bars, 50 $\mu$m. n=3. Representative IHC images and protein semi-quantification of (B) Bax and (C) caspase-3 in renal tissue of rats in different groups. n=3. (D) Relative ratio of cleaved caspase-3/caspase-3 determined by western blotting. n=6. (E) Representative images and protein semi-quantification of Bcl-2 in the renal tissue of rats in different groups. n=3. (F) Representative western blots and semi-quantified protein levels showing the expression levels of Bcl-2 in the renal tissue of rats in different groups. n=6. IHC images: Top row, scale bars, 200 $\mu$m, magnification, x40; bottom row, scale bars, 20 $\mu$m, magnification, x400. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001. I/R, ischemia-reperfusion; TMP, tetramethylpyrazine; IHC, immunohistochemistry.

showed increased protein expression in the I/R + TMP group to a greater extent than that in the western blotting results; however, there was no direct comparison between western blotting and IHC analysis results. This may be due to the fact that, compared with western blotting, IHC analysis may produce a certain non-specific staining. In addition, following I/R, the necrotic renal tissue had a higher background of non-specific staining than non-necrotic tissue, which may have further exacerbated the discrepancy between IHC analysis and western blotting results. Therefore, there are limitations in relying on a single experimental method to verify experimental conclusions. Since IHC analysis has the advantages of observing morphology and cell localization, both western blotting and IHC analysis were used to verify the conclusions. Taken together, the results of the present study suggested that TMP may promote the regeneration and repair of renal cells by activating the Wnt/β-catenin signaling pathway.

The DKK family includes the following four members: DKK1, 2, 3 and 4. DKK1 is one of the classic inhibitors of the Wnt/β-catenin signaling pathway, which functions by blocking the binding of Wnt to LRP5/6 co-receptors to form dimers (49,50). Studies have shown that DKK1 is involved in the occurrence and development of chronic kidney disease (29,51); however, whether it promotes the progression or alleviation of chronic kidney disease has not been clearly determined and its effects on AKI have not been reported. The histone demethylase inhibitor GSK-J4 has been reported to reduce the expression levels of DKK1, thereby attenuating renal dysfunction, glomerulosclerosis, inflammation and fibrosis in diabetic mice (52). In addition, DKK1 has been shown to aggravate cardiac I/R injury by weakening the effect of pifithrin-α, which alleviates acute cerebral I/R injury via the Wnt/β-catenin pathway (53). DKK1 exacerbates ischemic heart injury mainly by inducing LRP5/6 endocytosis and degradation (54). Although insulin-like growth factor binding protein 4 (IGFBP-4) and DKK1 are inhibitors of the Wnt/β-catenin pathway, IGFBP-4 inhibits β-catenin to protect the ischemic heart, whereas DKK1 aggravates ischemic heart injury mainly by inducing LRP5/6 endocytosis and degradation (54). Furthermore, DKK1 can decelerate vascular calcification by promoting the degradation of phospholipase D1, thereby reducing the high morbidity and mortality caused by vascular calcification in atherosclerosis, chronic kidney disease and diabetes (55). However, an accumulating number of studies has shown that DKK1 can participate in cellular activities independently of the Wnt/β-catenin signaling pathway. For example, DKK1 could suppress JNK-mediated apoptosis and activate the NF-κB-dependent cell survival mechanisms, independently of the canonical Wnt/β-catenin signaling pathway (56). In lupus nephritis, renal Wnt signaling activity is increased, accompanied by an increase in renal and serum levels of DKK1 (27). In the present study, it was shown that TMP treatment increased the expression of DKK1, which is inconsistent with the proposed function of DKK1 as an inhibitor of Wnt/β-catenin signaling pathway. Therefore, it was

considered that the activation of the Wnt/β-catenin signaling pathway by TMP was independent of DKK1.

Since the activation of the Wnt/β-catenin signaling pathway by TMP was independent of DKK1, the signaling pathways or specific molecular interactions of the Wnt/β-catenin signaling pathway activated by TMP need to be explored. Known regulators of the Wnt/β-catenin pathway include: R-spondins, Norrin, TSPAN12, secreted frizzled-related protein, Wnt inhibitory factor, glycogen synthase kinase-3β (GSK3β), axis inhibition protein 1, adenomatous polyposis coli protein (APC), disheveled 1, sclerostin, ring finger protein 43, zinc and ring finger 3, Cerberus, Klotho, IGFBP, Shisa, APC down-regulated 1 and Tiki1 (57-65). Among them, it has been reported that an analog of TMP could inhibit the phosphorylation of GSK3β and participate in the regulation of the Wnt/β-catenin pathway (57). Therefore, our future studies will focus on testing whether TMP can regulate the activation of Wnt/β-catenin pathway by regulating GSK3β phosphorylation.

Previous studies have shown that TMP has cardiovascular protection, anti-platelet, anti-ischemic, anti-Alzheimer's disease, neuroprotective and anticancer effects (66). TMP has been used in the treatment of cardiovascular, cerebrovascular, nervous, digestive system and kidney diseases, as well as in cancer (6). For example, a previous study has shown that TMP protects against congestive heart failure induced by myocardial infarction by downregulating the TGF-β1/Smad signaling pathway, suppressing the activation of the renin-angiotensin-aldosterone system, inhibiting the synthesis of pro-inflammatory factors and reducing oxidative stress (67). It has also been suggested that TMP reduces cell apoptosis and inflammation caused by cerebral I/R injury by targeting the circ_0008146/microRNA-709/Cx3cr1 axis (68). Other studies have suggested that TMP may alleviate diabetic nephropathy in rats (69,70). Avila-Carrasco *et al* (71) reported that TMP can increase the expression of natural inhibitors of TGF-β, hepatocyte growth factor, bone morphogenetic protein-7 and other natural inhibitors, and reduce the risk of renal interstitial fibrosis.

TMP exhibits anti-apoptosis effects. Liu *et al* (72) indicated that TMP may ameliorate the apoptosis of H9C2 cardiomyoblasts by negatively regulating hypoxia inducible factor-1α-induced Bcl-2 interacting protein 3 expression, thereby reversing the hypoxic effects induced by hyperglycemia. A previous study has shown that TMP can alleviate cognitive impairment by suppressing oxidative stress, neuroinflammation and apoptosis in type 2 diabetic rats (73). Another study has also shown that TMP alleviates neural apoptosis in the injured spinal cord via the downregulation of miR-214-3p (74). However, there have been limited studies assessing the effect of TMP on apoptosis in AKI. Our previous research showed the anti-apoptosis effect of TMP in AKI (12,13); however, at the time there were less data available on the effect of TMP on apoptosis-related molecules. In the present study, multiple experiments, including detection of the number of apoptotic cells by TUNEL staining, assessment of the expression levels of cleaved caspase-3/caspase-3 by western blotting, and analysis of the expression levels of apoptosis-related molecules Bcl-2 and Bax by western blotting and IHC staining, were performed to prove that TMP could reduce renal cell apoptosis in AKI. The present study demonstrated that the renal cell apoptosis in rats following I/R was significantly reduced after TMP treatment, which was in accordance with the results of the other study conducted by our group (13). Combined with the results that both DKK1 upregulation and Wnt/β-catenin signaling pathway activation by TMP could alleviate AKI renal injury, the protective effect was most likely through the regulation of apoptosis.

The clinical value of TMP in AKI is supported by the high incidence of AKI, its poor prognosis and the absence of approved specific medical therapies other than general supportive care (75). Several preclinical studies have shown that TMP has therapeutic potential for AKI (76,77). If possible, clinical trials combining TMP with the current standard of care could be conducted to further confirm the efficacy of TMP in human AKI.

The limitations of the present study include the lack of *in vitro* cell experiments for mechanistic investigation and the absence of direct molecular targets involving TMP. To verify whether DKK1 and the Wnt/β-catenin pathway were involved in the protective effect of TMP, the Wnt/β-catenin signaling pathway and DKK1 expression need to be verified in *in vitro* experiments. The relevant mechanistic studies that should be conducted in future include animal experiments and cell experiments using Wnt inhibitors, such as small interfering (si)RNA-Wnt and/or siRNA-DKK1. Molecular docking between TMP and regulators or key molecules of the Wnt/β-catenin pathway should also be conducted to preliminarily screen possible direct targets of TMP and for experimental validation. The mechanism in which DKK1 and the Wnt/β-catenin pathway affects apoptosis should also be a point of focus. Furthermore, the sample size was not determined through power analysis; it was determined based on the resource equation method (32). The present animal experiment was an exploratory study. When designing experiments, more consideration should be given to other elements that can be tested rather than sample size estimation to ensure the quality of scientific research (78).

Currently, the only effective treatment strategy for AKI is renal replacement therapy. Therefore, it is imperative to develop more effective treatment strategies. The research results of the *in vivo* rat model of AKI treated with TMP demonstrated in the present study suggested that TMP targets DKK1 and the Wnt/β-catenin signaling pathways, and therefore could effectively treat AKI.

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## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Authors' contributions

XW, XC and DY were involved in conceptualization, conducted the experiments, and were involved in visualization and writing of the original draft. LZ, ZG, XS and AL helped to conduct the experiments, data analysis and draft writing. YN and PD were involved in conceptualization, funding acquisition, project administration, supervision, review and editing. XW and PD confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of Binzhou Medical University (approval no. 2020-07; Yantai, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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