

Original Article

Down-regulation of miR-9 promotes VEGF expression in kidney, inhibits inflammatory factor release, and reduces acute kidney injury

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Abstract: Objective: We aimed to investigate the effects of down-regulating miR-9 on the expression of renal vascular endothelial growth factor (VEGF) and inflammatory factor release in rats with acute kidney injury (AKI). Methods: Twenty Sprague-Dawley rats were assigned to the normal group and 120 rats were injected with glycerin (50% 10 mL/kg) to induce rhabdomyolysis for creating AKI model. The targeting relationship between miR-9 and VEGF was predicted by a bioinformatics website and verified by dual luciferase assay. The AKI model rats were randomized into six groups of 20 rats each: model group, NC group (rats were injected with negative control vector), miR-9 mimic group (rats were injected with miR-9 overexpression vector), miR-9 inhibitor group (rats were injected with miR-9 inhibitor), oe-VEGF group (rats were injected with VEGF overexpression vector), and miR-9 mimic + oe-VEGF group (rats were injected with both miR-9 and VEGF overexpression vectors). The levels of miR-9 and VEGF and the pathological changes in the renal tissues were examined. The levels of serum creatinine (sCr), blood urea nitrogen (BUN), tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-10, superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-PX) were detected. TUNEL staining was performed to detect the cellular apoptosis in the renal tissues. The expression levels of apoptosis-associated proteins, Bax, caspase-3, and Bcl-2, were also measured. Results: miR-9 could target and inhibit VEGF expression. Compared with the normal rats, the AKI model rats exhibited renal tubular epithelial cell swelling, renal interstitial edema, and inflammatory cell infiltration in the renal tissues and had higher expression levels of miR-9 and lower levels of VEGF. Meanwhile, the model rats had higher apoptosis rate, higher levels of sCr, BUN, TNF- α , IL-6, MDA, Bax, and caspase-3, and lower levels of IL-10, SOD, GSH-PX, and Bcl-2 than the normal rats (all $P < 0.05$). Inhibiting miR-9 expression or enhancing its target VEGF expression could partially reverse the increased inflammation, oxidative stress, apoptosis, as well as functional deficits (all $P < 0.05$). Conclusion: Down-regulating miR-9 can reduce the levels of the inflammatory factors and upregulate renal VEGF expression, thereby exerting protective effects on the kidney of rats with AKI.

Keywords: Acute kidney injury, inflammatory factor, miR-9, oxidative stress, vascular endothelial growth factor

Introduction

Acute kidney injury (AKI) is a common acute critical illness, which can occur for various reasons. The pathogenesis of this disease remains unclear, and the incidence of AKI has been increasing each year. The disease can be life-threatening and the mortality rate exceeds 50% [1, 2]. Therefore, it is essential to investigate the mechanism of AKI in order to provide a theoretical basis for an effective treatment of this disease.

Vascular endothelial growth factor (VEGF) is a factor that can maintain the endothelial func-

tion and induce the endothelium-dependent dilatation in renal tubule, thereby promoting the survival of renal epithelial cells and exerting protective effects on kidney [3-5]. Currently, many studies have demonstrated that VEGF is significantly downregulated in kidney injuries. VEGF can reduce renal cellular necrosis and apoptosis through suppressing caspase-3 expression, thus reducing kidney injury [6-9]. MicroRNA (miRNA), which can regulate life activities by silencing specific genes at the post-transcriptional level, has been a popular research topic in recent years [10-12]. In order to investigate whether VEGF has an upstream control element to regulate VEGF expression and affect

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AKI rat model, we searched a bioinformatics website and found that miR-9 may target VEGF. Some literature documented that miR-9 is upregulated in rats' renal fibroblasts, and inhibiting the expression of this miRNA can have protective effects on renal fibroblasts and attenuate the injury caused by uric acid [13-15]. Moreover, some scholars performed a microarray analysis on miRNAs expressed in renal tubular epithelium and reported upregulation of miR-9 expression in cisplatin-induced renal tubule injury [16, 17]. Therefore, we speculated that miR-9 expression may be upregulated in AKI and cause inflammatory response and kidney damage via inhibiting VEGF expression.

In the present study, we established a rat model of AKI and injected the rats with miR-9 and VEGF vectors to investigate how miR-9 regulates VEGF and the effect of this miRNA on the inflammatory response and kidney damage in rats with AKI.

Materials and methods

Animal subjects

A total of 140 healthy specific pathogen-free Sprague-Dawley rats were used for the study (6 weeks of age, 178.26±14.28 g, Vital River, China). The rats were raised at 25°C with 45% relative humidity. The experiment was approved by the Ethics Committee of The First Affiliated Hospital of Fujian Medical University.

Grouping and treatment

The rats were equally assigned to seven groups of 20 rats each: normal group (normal rats), model group (AKI model), NC group (AKI model rats injected with negative control vector), miR-9 mimic group (AKI model rats injected with miR-9 overexpression vector), miR-9 inhibitor group (AKI model rats injected with miR-9 inhibitor), oe-VEGF group (AKI model rats injected with VEGF overexpression vector), and miR-9 mimic + oe-VEGF group (AKI model rats injected with both miR-9 and VEGF overexpression vectors). Before model establishment, 50 µg of miR-9 mimic (Thermo Fisher Scientific, USA), miR-9 inhibitor (Thermo Fisher Scientific, USA), negative control vector (Thermo Fisher Scientific, USA), and VEGF overexpression vector (Thermo Fisher Scientific, USA) were injected into the rats in the corresponding groups

through the tail vein. Forty-eight hour after injection, the AKI model was established in the model, NC, miR-9 mimic, miR-9 inhibitor, oe-VEGF, and miR-9 mimic + oe-VEGF groups.

AKI model establishment

AKI model was created by injection of 50% 10 mL/kg glycerin (Acme Biochemical, China) through the bilateral vastus medialis to induce rhabdomyolysis [18]. After grouping, all the rats were raised for one week to help them adapt to their environment and then underwent water fast for 24 h before modeling. The rats with body weight reduced by 10% were chosen and injected with the glycerin. Meanwhile, same volume of normal saline was injected in the same way to the rats in the normal group. The rats were then sacrificed. The whole blood samples were isolated through retro-orbital blood collection, and the renal tissues were taken out for the subsequent experiments [19].

Dual-luciferase reporter (DLR) assay

The targeting relationship between miR-9 and VEGF was analyzed through a bioinformatics website (www.targetscan.org) and verified by DLR assay. The VEGF DLR vectors with or without mutant miR-9 binding site were constructed and named as PGL3-VEGF mut and PGL3-VEGF wt, respectively. Two reporter plasmids were transfected into HEK 293T cells with renilla plasmid and miR-9 or NC plasmids. DLR assay was performed after 24 h of transfection. The cells from each group were first lysed and centrifuged at 13,000× g for 1 min followed by removal of precipitation and collection of the supernatant. Detection of luciferase activity was carried out according to the manufacturer's instructions of the kit (E1910, Promega, WI, USA). The lysed cells were placed in Eppendorf tubes, and 100 µL of firefly luciferase solution was added to every 10 µL sample. After measuring the activity of firefly luciferase, 100 µL renilla luciferase solution was added for detecting the activity of renilla luciferase. Relative luciferase activity = firefly luciferase activity/renilla luciferase activity.

Hematoxylin and eosin (HE) staining

The renal tissues from each group were fixed with 4% paraformaldehyde (Zibo Qixing, China) for 24 h and dehydrated in a gradient of alcohol

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(80%, 90%, 100%) and normal butyl alcohol (Sinopharm Chemical Reagent, China). Afterward, the samples were placed in a wax box for paraffin embedment at 60°C and sectioned at a thickness of 5 µM. The sections were flattened at 45°C and picked up and dried at 60°C for 1 h followed by dewaxing in xylene. Next, the samples underwent hydration and were stained with HE. The sections were first dewaxed and hydrated through a gradient of alcohol followed by hematoxylin staining (Solarbio, China) for 2 min and rinse in tap water for 10 s. Subsequently, 1% hydrochloric acid alcohol was applied for differentiation for 10 s before wash in distilled water for 1 min. The sections were then stained with eosin (Solarbio, China) for 1 min, rinsed in distilled water for 10 s, and dehydrated through a gradient of alcohol. Next, the sections were cleared in xylene (Sinopharm Chemical Reagent, China), sealed in neutral balsam, and placed under a light microscope (XP-330, Bingyu Optical Instrument, China) for observing the pathological changes. The tubule dilation was evaluated and scored based on the following criteria: no tubule dilation, 0 point; change <25%, 1 point; change within 25-50%, 2 points; change >50%, 3 points.

Measurement of biochemical markers

The whole blood samples in each group were centrifuged at 1,200× g for 15 min to collect the serum. The serum levels of creatinine (sCr) and blood urea nitrogen (BUN), as markers for renal injury, were measured using the test kits (Yaji Biological, Shanghai, China).

ELISA

Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 in rats' serum were measured according to the manufacturer's instructions of the ELSA kit (Abcam, Cambridge, UK), and the standard concentration curve was plotted. Levels of TNF-α (ab6671, Abcam, UK), IL-6 (ab100772, Abcam, UK), and IL-10 (ab100765, Abcam, UK) in each group were measured at 450 nm according to the protocol of the kits.

Measurement of oxidative stress injury in kidney

Renal cortexes (0.01 g) from each group were obtained for preparing homogenate with normal saline. After centrifugation, the supernatant was collected to measure the levels of

malondialdehyde (MDA), glutathione peroxidase (GSH-PX), and superoxide dismutase (SOD) by colorimetry (test kit supplier: Kang Lang, Shanghai, China).

Terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) staining

TUNEL was performed to detect cellular apoptosis in the renal tissues according to the manufacturer's instructions of the kit (116848-17910, Roche, Basel, Switzerland). The renal tissues from each group were fixed in 10% formaldehyde overnight followed by dehydration and paraffin embedment and then sectioned at a thickness of 4 µm. The samples were placed in numbered glass slides coated with polylysine. The cells stained with green color were TUNEL-positive cells. DAPI was used to stain the cell nuclei. Five paraffin sections were made from each sample for staining. Five fields were randomly picked on each glass slide, and the TUNEL-positive cells and total cells were counted and analyzed using Image-Pro Plus 6.0 software. The ratio of the TUNEL-positive cell number to the total cell number was calculated.

qRT-PCR

The kidneys from each group were collected to make homogenate. Total RNA was extracted from the tissues using Trizol (Invitrogen, USA) and the values of A260/A230 and A260/A280 were measured with a UV-Vis spectrophotometer (Nanodrop 2000, 1011U, Thermo Fisher Scientific, USA) for detecting the quantity and purity of the total RNAs. The RNAs were reverse transcribed into cDNAs according to the manufacturer's instructions of TaqMan MicroRNA Assays Reverse Transcription primer (4427975, Applied Biosystems, USA), and the reverse transcription parameters were as follows: 37°C for 30 min, and 85°C for 5 s. The cDNAs (2 µL) were then diluted to 50 ng/µL. The primers for miR-9, caspase-3, GAPDH, Bax, U6, and Bcl-2 were synthesized by Tsingke, China (**Table 1**). qRT-PCR (PCR device: Bio-Rad Laboratories, USA) was conducted with the following parameters: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, and annealing at 60°C for 20 s for 35 cycles. The total reaction volume was 20 µL including forward primer (10 µM, 0.8 µL), reverse primer (10 µM, 0.8 µL), ROX reference dye II (0.4 µL), SYBR Premix EX Taq™ II (10 µL), cDNA template (2.0 µL), and

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Table 1. Primer sequence

Gene	Primer sequence (5'-3')
miR-9	Forward: GGGATAAAGCTAGATAACC Reverse: GTGCAGGGTCCGAGGT
Caspase-3	Forward: TCTACCGCACCCGGTTACTA Reverse: TCAAATTCGGTGGCCACCTT
Bcl-2	Forward: CTGCTAAGGAGGCTGGTAGC Reverse: ACATGCTCAATGCTGAGGCT
Bax	Forward: CAGGCGAATTGCGATGAAC Reverse: GGGTCCCGAAGTAGGAAAGG
U6	Forward: CGCTTCGGCAGCACATATAC Reverse: TTCACGAATTTGCGTGTCAT
GAPDH	Forward: TGTGAACGGATTTGGCCGTA Reverse: GATGGTGATGGGTTCCCGT

sterile distilled water (6.0 μ L). The relative expression of miR-9 was normalized to the expression of U6, and other mRNAs were normalized to the expressions of GAPDH. The relative expression level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = \Delta Ct_{\text{the study groups}} - \Delta Ct_{\text{GAPDH}}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal control}}$; Ct represented the number of PCR amplification cycles).

Western blot

Protein expressions of caspase-3, VEGF, Bcl-2, and Bax in renal tissues of each group were determined by western blot. After preparing the homogenate, total protein was extracted using radioimmunoprecipitation assay buffer (BB-3209, Bebe Bio, China) and separated by SDS-PAGE. The proteins were transferred to polyvinylidene fluoride (PVDF) membrane at a constant voltage of 80 V, and the membrane was incubated in blocking solution for one hour followed by reaction with polyclonal primary antibodies of rabbit anti-VEGF (1:1,000, ab53465, Abcam, UK), caspase-3 (1:500, ab13847, Abcam, UK), Bcl-2 (1:2,000, ab196495, Abcam, UK), Bax (1:5,000, ab32503, Abcam, UK), and GAPDH (1:10,000, ab181603, Abcam UK) in a shaker at 4°C overnight. Subsequently, the membrane was treated with horseradish peroxidase-conjugated goat anti-rabbit polyclonal IgG (1:20,000, ab97051, Abcam, UK) and agitated at 37°C for 2 h. After wash in PBS at room temperature three times (5 min per wash), the PVDF membrane was visualized. The relative protein expression level = the greyscale value of the protein band/the greyscale value of the GAPDH band.

Statistical analysis

Statistical software SPSS 21.0 (SPSS Inc., USA) was applied for data analysis. Measurement data are expressed as mean \pm standard deviation, and comparison between groups was conducted by one-way analysis of variance and Bonferroni post-hoc test. $P < 0.05$ indicated a statistically significant difference.

Results

MiR-9 can target VEGF and inhibit its expression

The bioinformatics website predicted the existence of miR-9 binding site on VEGF in rats (**Figure 1A**). To verify this relationship, we conducted DLR assay and found that the luciferase activity of the group co-transfected with Wt-VEGF and miR-9 mimic was much lower than the group transfected with NC plasmid ($P < 0.05$), which demonstrated that miR-9 can target VEGF (**Figure 1B**). Afterward, we performed qRT-PCR to measure the miR-9 levels in the renal tissues of each group (**Figure 1C**). Compared with the normal groups, the other groups had higher expression levels of miR-9; compared with the model group, the miR-9 expression levels were higher in the miR-9 mimic and miR-9 mimic + oe-VEGF groups and lower in the miR-9 inhibitor group (all $P < 0.05$). These results indicated that we had successfully injected the miR-9 overexpression mimics and miR-9 inhibitors into the rats. Western blot was conducted to determine the VEGF expression levels in the renal tissues of each group (**Figure 1D**). Compared with the normal group, the other groups had lower VEGF protein expression levels; compared with the model group, the VEGF protein expression levels were lower in the miR-9 mimic group and higher in the miR-9 inhibitor, the oe-VEGF, and the miR-9 mimic + oe-VEGF groups (all $P < 0.05$).

Pathological changes in each group

We sectioned the tissues and performed HE staining to examine the pathological changes in the renal tissues of each group (**Figure 2A**). In the normal group, the renal autopsy exhibited normal structure and intact renal tubular epithelial cells, and no evident pathological changes were observed in the renal glomerulus and interstitium. In contrast, the renal tissues in the

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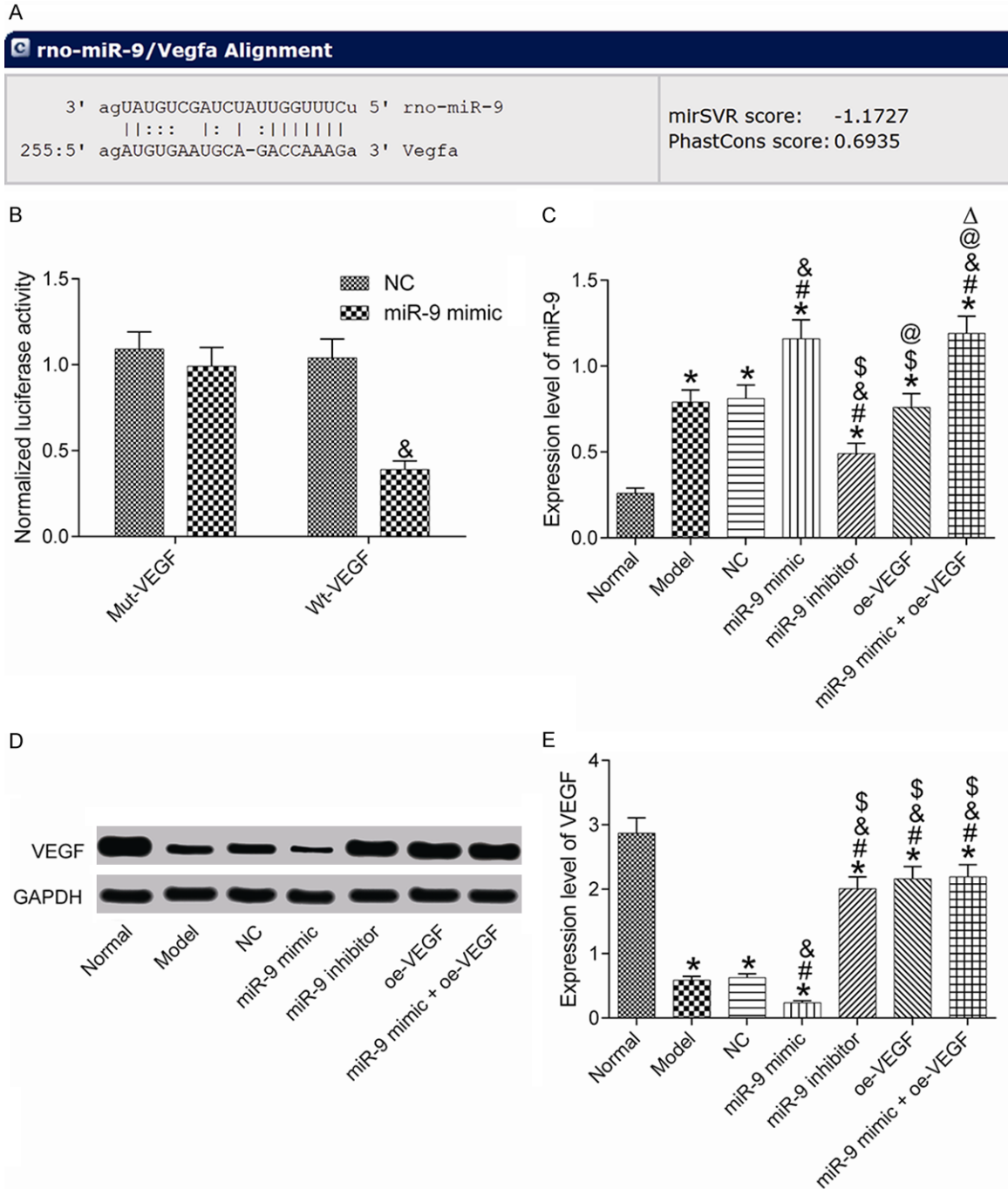


Figure 1. miR-9 can inhibit VEGF expression. A: The pairing of miR-9 with VEGF as predicted by the bioinformatics website; B: miR-9 can target VEGF as confirmed by dual-luciferase report assay; C: Expression levels of miR-9 and VEGF determined by qRT-PCR; D: Image of the protein band; E: The protein expression levels of VEGF in each group measured by western blot. Compared with the normal group, *P<0.05; compared with the model group, #P<0.05; compared with the NC group, ^aP<0.05; compared with the miR-9 mimic group ^bP<0.05; compared with the miR-9 inhibitor group, ^cP<0.05; compared with the oe-VEGF group, ^dP<0.05. VEGF: vascular endothelial growth factor.

other groups exhibited different degrees of pathological changes, including renal tubule dilation, glomerular hypertrophy, edema and vacuolar degeneration of renal tubular epithelial cells, and interstitial inflammation. We sco-

red the renal tubule dilation in each group (**Figure 2B**), and the results showed that the normal group had lower scores than the other groups; the model group had a lower score than the miR-9 mimic group and a higher score than

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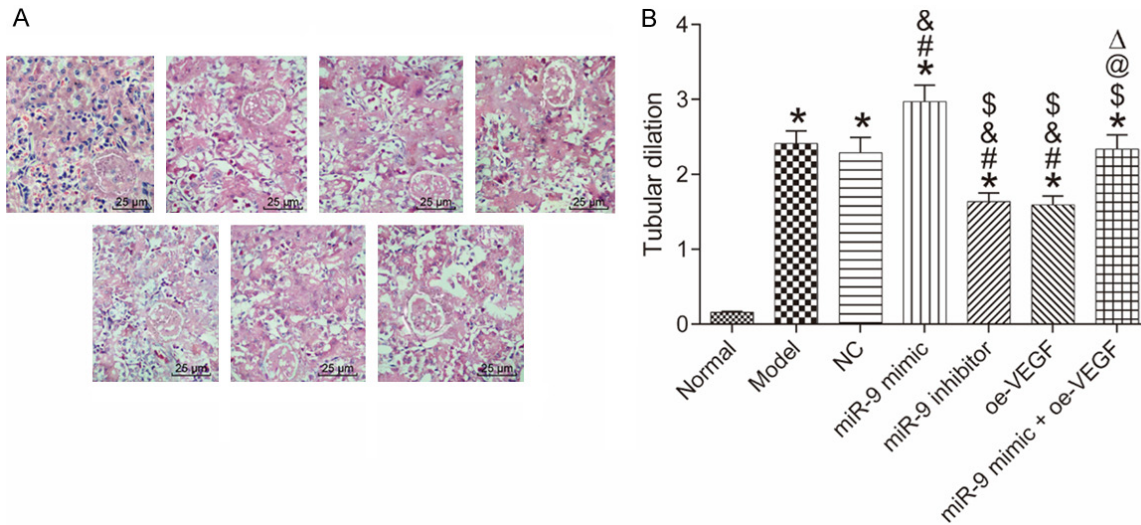


Figure 2. Pathological changes in rats' renal tissues. A: Hematoxylin and eosin; B: Tubule dilation in each group. Compared with the normal group, * $P < 0.05$; compared with the model group, # $P < 0.05$; compared with the NC group, & $P < 0.05$; compared with the miR-9 mimic group \$ $P < 0.05$; compared with the miR-9 inhibitor group, @ $P < 0.05$; compared with the oe-VEGF group, &# $P < 0.05$. VEGF: vascular endothelial growth factor.

the miR-9 inhibitor and the oe-VEGF groups; the miR-9 mimic + oe-VEGF group had a lower score than the miR-9 mimic group (all $P < 0.05$). All these results indicated that silencing miR-9 or overexpressing VEGF can significantly reduce the renal tubule dilation, and VEGF overexpression can partially reverse the tubule dilation caused by miR-9 overexpression.

Measurement of biochemical markers in each group

Based on the phenotype observation, we concluded that miR-9 and VEGF can regulate the pathological features in rat's kidney. To further investigate the effects of miR-9 and VEGF on rat's renal function, we measured the sCr and BUN levels in rats' whole blood samples by colorimetry (Figure 3). The results showed that the normal group had lower levels of sCr and BUN than the other groups (all $P < 0.05$). Compared with the model group, the sCr and BUN levels were higher in the miR-9 mimic group and lower in the miR-9 inhibitor and the oe-VEGF groups (all $P < 0.05$). Meanwhile, the miR-9 mimic + oe-VEGF group had lower levels of sCr and BUN than the miR-9 mimic group (both $P < 0.05$).

Levels of inflammatory factors in each group

ELISA was conducted to measure the levels of TNF- α , IL-6, and IL-10 in rats' serum in each group (Figure 4). The results showed that compared with the normal group, the other groups

had higher levels of TNF- α and IL-6 and lower levels of IL-10 (all $P < 0.05$). Compared with the model group, the miR-9 mimic group had higher levels of TNF- α and IL-6 and lower levels of IL-10 whereas the miR-9 inhibitor and oe-VEGF groups had lower levels of TNF- α and IL-6 and higher levels of IL-10 (all $P < 0.05$). The miR-9 mimic + oe-VEGF group had lower levels of TNF- α and IL-6 and a higher level of IL-10 than the miR-9 mimic group (all $P < 0.05$).

Oxidative stress in each group

In order to investigate the oxidative stress in each group, colorimetry was performed to detect the changes in the levels of SOD, MDA, and GSH-PX in rats' renal tissues (Figure 5). Compared with the normal group, the other groups had higher levels of MDA and lower levels of SOD and GSH-PX (all $P < 0.05$). Compared with the model group, the miR-9 mimic group had higher levels of MDA and lower levels of SOD and GSH-PX, whereas the miR-9 inhibitor and the oe-VEGF groups had lower levels of MDA and higher levels of SOD and GSH-PX (all $P < 0.05$). The miR-9 mimic + oe-VEGF group had a lower level of MDA and higher levels of SOD and GSH-PX than the miR-9 mimic group (all $P < 0.05$).

Cellular apoptosis in each group

Results of TUNEL test showed that compared with the normal group, the other groups had

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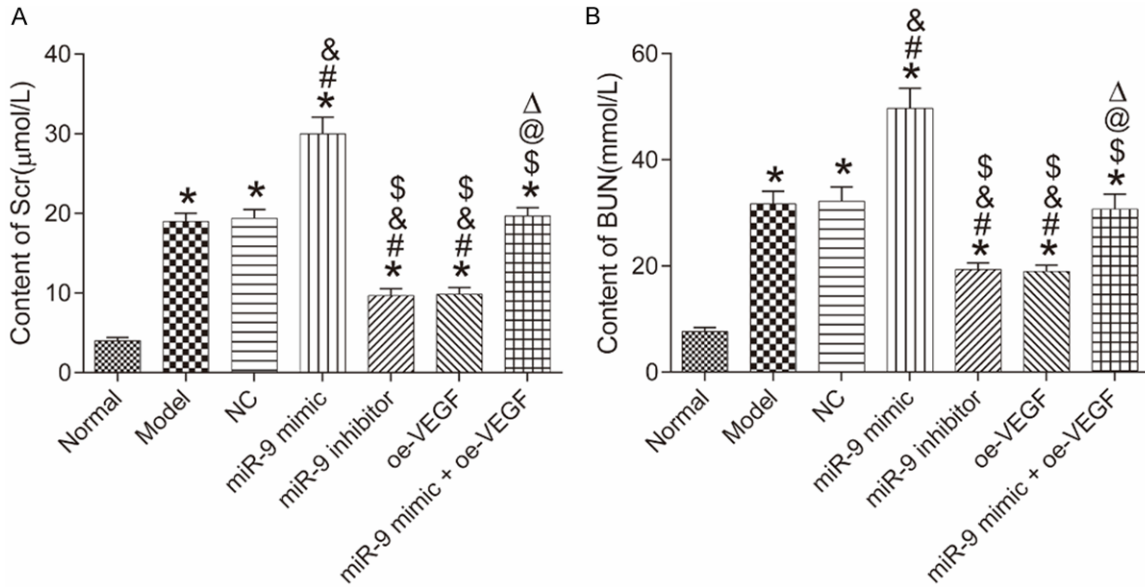


Figure 3. Levels of sCr and BUN in rats' whole blood samples. A: sCr content; B: BUN content. Compared with the normal group, *P<0.05; compared with the model group, #P<0.05; compared with the NC group, &P<0.05; compared with the miR-9 mimic group \$P<0.05; compared with the miR-9 inhibitor group, @P<0.05; compared with the oe-VEGF group, ΔP<0.05. sCr: serum creatinine; BUN: blood urea nitrogen; VEGF: vascular endothelial growth factor.

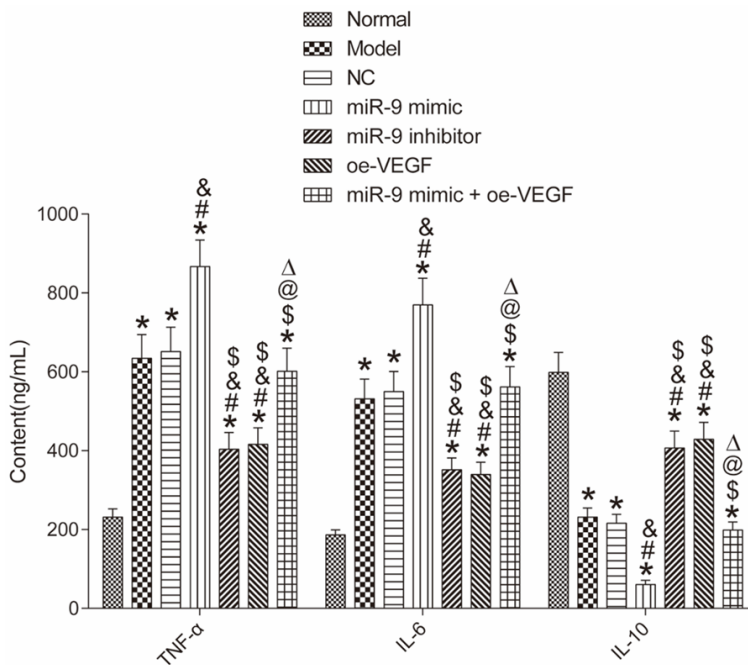


Figure 4. Levels of TNF-α, IL-6, and IL-10 in rats' serum detected by ELISA. Compared with the normal group, *P<0.05; compared with the model group, #P<0.05; compared with the NC group, &P<0.05; compared with the miR-9 mimic group \$P<0.05; compared with the miR-9 inhibitor group, @P<0.05; compared with the oe-VEGF group, ΔP<0.05. TNF: tumor necrosis factor; IL: interleukin; ELISA: enzyme-linked immunosorbent assay; VEGF: vascular endothelial growth factor.

mimic group had higher apoptosis rate and the miR-9 inhibitor and the oe-VEGF groups had lower apoptosis rates (all P<0.05). The miR-9 mimic + oe-VEGF group had lower apoptosis rate than the miR-9 mimic group (P<0.05). See **Figure 6**.

Expressions of apoptosis-associated genes in each group

qRT-PCR and western blot were performed to measure the mRNA and protein expressions of Bax, caspase-3, and Bcl-2 in rats' renal tissues (**Figure 7**). Compared with the normal group, the other groups had higher mRNA and protein expression levels of Bax and caspase-3 and lower mRNA and protein expression levels of Bcl-2 (all P<0.05). Compared with the model group, the miR-9 mimic group had higher mRNA and protein expression levels of Bax and caspase-3 and lower mRNA and protein expression levels of Bcl-2, whereas the miR-9 inhibitor and the

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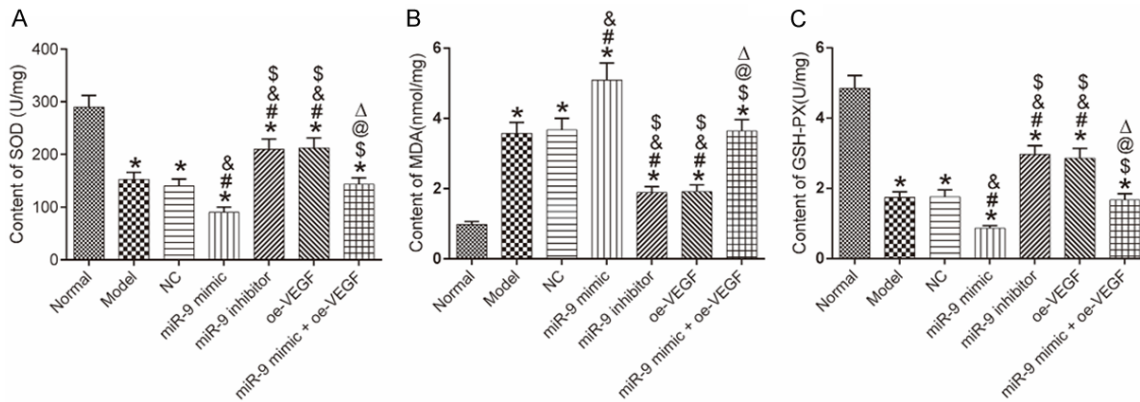


Figure 5. Oxidative stress in rats' renal tissues. A: SOD content; B: MDA content; C: GSH-PX content. Compared with the normal group, * $P < 0.05$; compared with the model group, # $P < 0.05$; compared with the NC group, & $P < 0.05$; compared with the miR-9 mimic group, \$ $P < 0.05$; compared with the miR-9 inhibitor group, @ $P < 0.05$; compared with the oe-VEGF group, ^ $P < 0.05$. MDA: malondialdehyde; SOD: superoxide dismutase; GSH-PX: glutathione peroxidase; VEGF: vascular endothelial growth factor.

oe-VEGF groups had lower mRNA and protein expression levels of Bax and caspase-3 and higher mRNA and protein expression levels of Bcl-2 (all $P < 0.05$). The miR-9 mimic + oe-VEGF group had lower mRNA and protein expression levels of Bax and caspase-3 and higher mRNA and protein expression levels of Bcl-2 than the miR-9 mimic group (all $P < 0.05$).

Discussion

AKI has high morbidity and mortality and can occur for various reasons. The disease can be life-threatening with a mortality over 50%, and the treatment cost is high. In order to reduce mortality and save medical resources, it is essential to investigate the pathogenesis of AKI in order to provide a theoretical basis for effective treatment of this disease [19-22].

Some studies have demonstrated that VEGF can promote the proliferation of vascular endothelial cells and has protective effects on renal tubular endothelium, thereby protecting kidney. Reduction or loss of VEGF can disrupt the balance between pro-VEGF and anti-VEGF in the body, leading to inflammation [23-27]. In the present study, we created a rat model of AKI and injected the rats with VEGF overexpression vectors. The model group had lower VEGF expression level than the normal group, and the oe-VEGF group had higher VEGF expression level than the model group. In contrast to the rats in the normal group, the rats with AKI exhibited pathological features including renal tubular epithelial cell swelling and renal interstitial edema. Moreover, the AKI rats had lower

levels of IL-10, SOD, GSH-PX, VEGF, and Bcl-2, and higher levels of TNF- α , IL-6, sCr, BUN, MDA, caspase-3, and Bax. Compared with the model group, the oe-VEGF group had lower degrees of tubule dilation, lower levels of sCr, BUN, TNF- α , IL-6, MDA, caspase-3, and Bax, and higher levels of IL-10, SOD, GSH-PX, and Bcl-2. These results demonstrated that VEGF is downregulated in rats with AKI and its overexpression can markedly attenuate kidney injury, protect kidney function, and suppress the progression of inflammation.

MiR-9 is a miRNA. Some studies have reported that miR-9 overexpression can induce renal fibroblast apoptosis by promoting caspase-3 expression, which further damages the renal fibroblasts [28-30]. In this study, we created a rat model of AKI with miR-9 overexpression or miR-9 silencing and found that miR-9 overexpression can aggravate tubule dilation, increase the levels of sCr, BUN, TNF- α , IL-6, MDA, caspase-3, and Bax, and decrease the levels of Bcl-2, IL-10, SOD, and GSH-PX. After silencing miR-9, both tubule dilation and levels of renal function markers and inflammatory factors improved markedly in the rats, and the expression levels of caspase-3 and Bax declined and the Bcl-2 expression level elevated in the rats. These findings showed that miR-9 expression can aggravate the renal function of AKI rats and promote inflammation, causing damage to the kidney. To investigate the relationship between miR-9 and VEGF, we searched the bioinformatics website and found the existence of miR-9 binding site on VEGF. DLR assay was performed to confirm that miR-9 can target VEGF.

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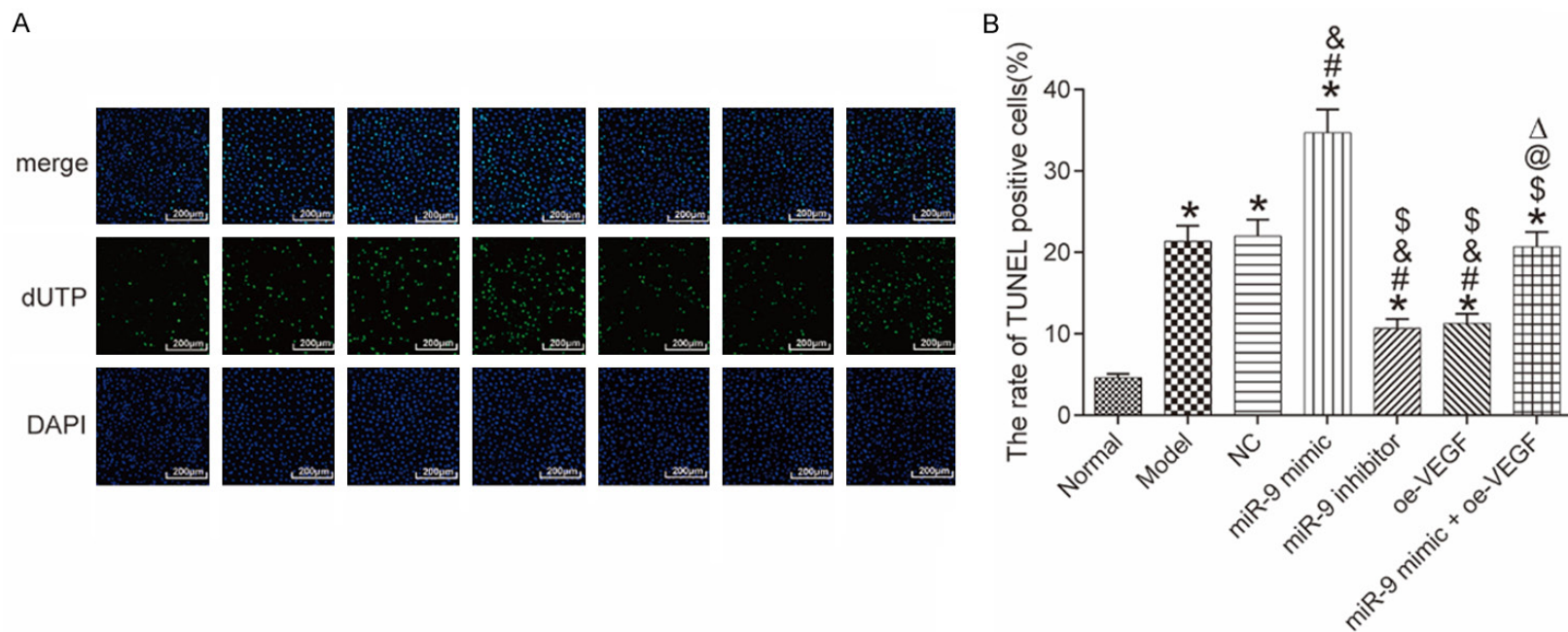
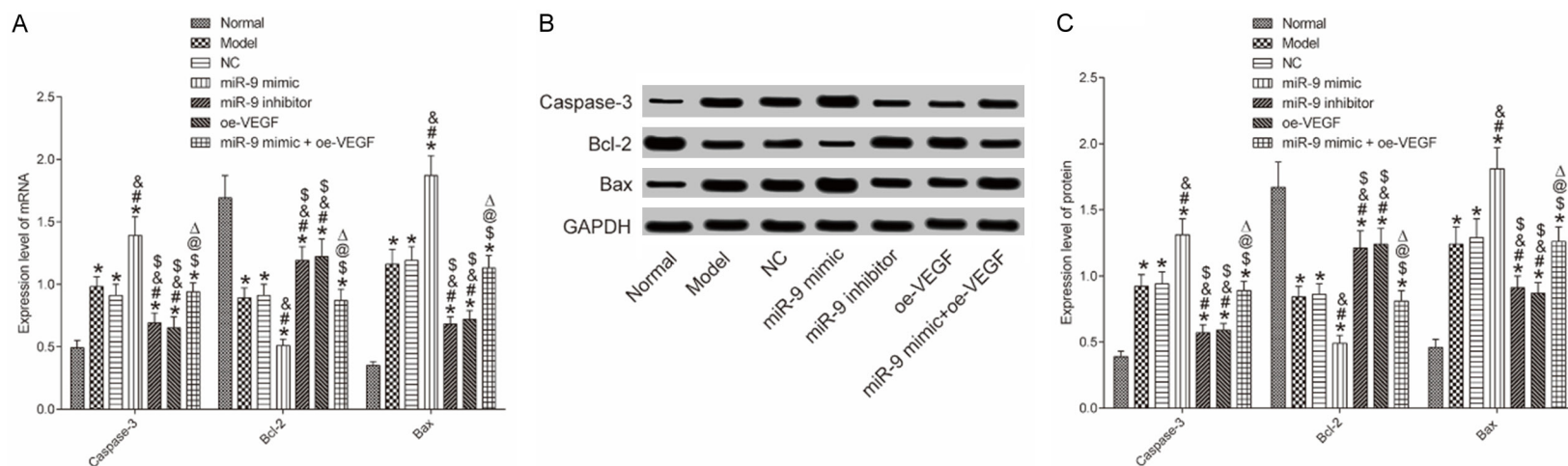


Figure 6. TUNEL staining result. A: TUNEL staining result; B: Cellular apoptosis rate. Compared with the normal group, *P<0.05; compared with the model group, #P<0.05; compared with the NC group, &P<0.05; compared with the miR-9 mimic group, \$P<0.05; compared with the miR-9 inhibitor group, @P<0.05; compared with the oe-VEGF group, ΔP<0.05. TUNEL: terminal deoxynucleotidyl transferase-dUTP nick end labeling; VEGF: vascular endothelial growth factor.



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Figure 7. mRNA and protein expression levels of apoptosis-associated factors in rats' renal tissues measured by qRT-PCR and western blot. A: mRNA expression levels of apoptosis-associated factors in rats' renal tissues measured by qRT-PCR; B: Image of the protein band; C: Protein expression levels of apoptosis-associated factors measured by western blot. Compared with the normal group, *P<0.05; compared with the model group, #P<0.05; compared with the NC group, &P<0.05; compared with the miR-9 mimic group \$P<0.05; compared with the miR-9 inhibitor group, ®P<0.05; compared with the oe-VEGF group, ^P<0.05. VEGF: vascular endothelial growth factor.

MiR-9 overexpression can decrease VEGF expression, whereas VEGF overexpression cannot affect miR-9 expression. Meanwhile, we created a rat model of AKI with both miR-9 and VEGF overexpression and observed that compared with the miR-9 mimic group, the miR-9 mimic + oe-VEGF group had a lower degree of tubule dilation, higher levels of IL-10, SOD, GSH-PX, and Bcl-2 and lower levels of sCr, BUN, TNF- α , IL-6, MDA, caspase-3, and Bax. Therefore, miR-9 overexpression in AKI rats can inhibit VEGF expression and thus aggravating the renal function and causing inflammation and injury in the kidney.

In conclusion, the present study has demonstrated the effects of miR-9 and VEGF on AKI model rats and revealed that miR-9, as an upstream control element, can negatively regulate the VEGF expression. These findings may provide some theoretical basis for understanding the pathogenesis of AKI. However, we need to note that although the rats in the miR-9 mimic + oe-VEGF group had much less renal damage than the rats in the miR-9 mimic group, the results of the various markers in the rats treated with both miR-9 and VEGF overexpression were not as good as those in the rats treated with VEGF alone, suggesting that VEGF overexpression cannot fully reverse the kidney damage caused by miR-9. The reasons behind this finding are yet to be clarified, and the molecular mechanism of miR-9 targeting VEGF needs to be further investigated in the future.

Disclosure of conflict of interest

None.

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References

[1] Zou H, Hong Q and Xu G. Early versus late initiation of renal replacement therapy impacts

mortality in patients with acute kidney injury post cardiac surgery: a meta-analysis. *Crit Care* 2017; 21: 150.

- [2] Mavrakanas TA, Aurian-Blajeni DE and Charytan DM. Early versus late initiation of renal replacement therapy in patients with acute kidney injury: a meta-analysis of randomised clinical trials. *Swiss Med Wkly* 2017; 147: w14507.
- [3] Majumder S and Advani A. VEGF and the diabetic kidney: more than too much of a good thing. *J Diabetes Complications* 2017; 31: 273-279.
- [4] Roskoski R Jr. Vascular endothelial growth factor (VEGF) and VEGF receptor inhibitors in the treatment of renal cell carcinomas. *Pharmacol Res* 2017; 120: 116-132.
- [5] Stevens M, Neal CR, Salmon AHJ, Bates DO, Harper SJ and Oltean S. VEGF-A165 b protects against proteinuria in a mouse model with progressive depletion of all endogenous VEGF-A splice isoforms from the kidney. *J Physiol* 2017; 595: 6281-6298.
- [6] Beaini S, Saliba Y, Hajal J, Smayra V, Bakhos JJ, Joubran N, Chelala D and Fares N. VEGF-C attenuates renal damage in salt-sensitive hypertension. *J Cell Physiol* 2019; 234: 9616-9630.
- [7] Yoshida M, Nakamichi T, Mori T, Ito K, Shimokawa H and Ito S. Low-energy extracorporeal shock wave ameliorates ischemic acute kidney injury in rats. *Clin Exp Nephrol* 2019; 23: 597-605.
- [8] Olsson AK, Dimberg A, Kreuger J and Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* 2006; 7: 359-371.
- [9] Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z and Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 1999; 5: 623-628.
- [10] Baker MA, Davis SJ, Liu P, Pan X, Williams AM, Iczkowski KA, Gallagher ST, Bishop K, Regner KR, Liu Y and Liang M. Tissue-specific microRNA expression patterns in four types of kidney disease. *J Am Soc Nephrol* 2017; 28: 2985-2992.
- [11] Zou YF, Wen D, Zhao Q, Shen PY, Shi H, Zhao Q, Chen YX and Zhang W. Urinary microRNA-30c-5p and microRNA-192-5p as potential biomarkers of ischemia-reperfusion-induced kidney injury. *Exp Biol Med (Maywood)* 2017; 242: 657-667.

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- [12] Yao J, Wang J, Yao Y, Wang K, Zhou Q and Tang Y. miR133b regulates proliferation and apoptosis in highglucoseinduced human retinal endothelial cells by targeting ras homolog family member A. *Int J Mol Med* 2018; 42: 839-850.
- [13] Khalid U, Newbury LJ, Simpson K, Jenkins RH, Bowen T, Bates L, Sheerin NS, Chavez R and Fraser DJ. A urinary microRNA panel that is an early predictive biomarker of delayed graft function following kidney transplantation. *Sci Rep* 2019; 9: 3584.
- [14] Chen LL and Xu Y. Epigallocatechin gallate attenuates uric acid-induced injury in rat renal interstitial fibroblasts NRK-49F by up-regulation of miR-9. *Eur Rev Med Pharmacol Sci* 2018; 22: 7458-7469.
- [15] Muralidharan J, Ramezani A, Hubal M, Knoblich S, Shrivastav S, Karandish S, Scott R, Maxwell N, Ozturk S, Beddhu S, Kopp JB and Raj DS. Extracellular microRNA signature in chronic kidney disease. *Am J Physiol Renal Physiol* 2017; 312: F982-F991.
- [16] Wu J, Li DD, Li JY, Yin YC, Li PC, Qiu L and Chen LM. Identification of microRNA-mRNA networks involved in cisplatin-induced renal tubular epithelial cells injury. *Eur J Pharmacol* 2019; 851: 1-12.
- [17] Wu J, Pan X, Fu H, Zheng Y, Dai Y, Yin Y, Chen Q, Hao Q, Bao D and Hou D. Effect of curcumin on glycerol-induced acute kidney injury in rats. *Sci Rep* 2017; 7: 10114.
- [18] Narvaez A, Guiteras R, Sola A, Manonelles A, Morote J, Torras J, Grinyo JM and Cruzado JM. siRNA-silencing of CD40 attenuates unilateral ureteral obstruction-induced kidney injury in mice. *PLoS One* 2019; 14: e0215232.
- [19] Forni LG, Darmon M, Ostermann M, Oudemans-van Straaten HM, Pettita V, Prowle JR, Schetz M and Joannidis M. Renal recovery after acute kidney injury. *Intensive Care Med* 2017; 43: 855-866.
- [20] Bagshaw SM, Darmon M, Ostermann M, Finkelstein FO, Wald R, Tolwani AJ, Goldstein SL, Gattas DJ, Uchino S, Hoste EA and Gaudry S. Current state of the art for renal replacement therapy in critically ill patients with acute kidney injury. *Intensive Care Med* 2017; 43: 841-854.
- [21] Plewes K, Kingston HWF, Ghose A, Maude RJ, Herdman MT, Leopold SJ, Ishioka H, Hasan MMU, Haider MS, Alam S, Piera KA, Charunwatthana P, Silamut K, Yeo TW, Faiz MA, Lee SJ, Mukaka M, Turner GDH, Anstey NM, Jackson Roberts L 2nd, White NJ, Day NPJ, Hossain MA and Dondorp AM. Cell-free hemoglobin mediated oxidative stress is associated with acute kidney injury and renal replacement therapy in severe falciparum malaria: an observational study. *BMC Infect Dis* 2017; 17: 313.
- [22] Li Q, Zhao M, Du J and Wang X. Outcomes of renal function in elderly patients with acute kidney injury. *Clin Interv Aging* 2017; 12: 153-160.
- [23] Thomas AA, Feng B and Chakrabarti S. ANRIL: a regulator of VEGF in diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2017; 58: 470-480.
- [24] Falkevall A, Mehlem A, Palombo I, Heller Sahlgren B, Ebarasi L, He L, Ytterberg AJ, Olauson H, Axelsson J, Sundelin B, Patrakka J, Scotney P, Nash A and Eriksson U. Reducing VEGF-B signaling ameliorates renal lipotoxicity and protects against diabetic kidney disease. *Cell Metab* 2017; 25: 713-726.
- [25] Lin Y, Zhai E, Liao B, Xu L, Zhang X, Peng S, He Y, Cai S, Zeng Z and Chen M. Autocrine VEGF signaling promotes cell proliferation through a PLC-dependent pathway and modulates Apatinib treatment efficacy in gastric cancer. *Oncotarget* 2017; 8: 11990-12002.
- [26] Ni X, Ou C, Guo J, Liu B, Zhang J, Wu Z, Li H and Chen M. Lentiviral vector-mediated co-overexpression of VEGF and Bcl-2 improves mesenchymal stem cell survival and enhances paracrine effects in vitro. *Int J Mol Med* 2017; 40: 418-426.
- [27] Zhang B, Wang D, Ji TF, Shi L and Yu JL. Overexpression of lncRNA ANRIL up-regulates VEGF expression and promotes angiogenesis of diabetes mellitus combined with cerebral infarction by activating NF-kappaB signaling pathway in a rat model. *Oncotarget* 2017; 8: 17347-17359.
- [28] Cai Y, Wang W, Guo H, Li H, Xiao Y and Zhang Y. miR-9-5p, miR-124-3p, and miR-132-3p regulate BCL2L1 in tuberous sclerosis complex angiomyolipoma. *Lab Invest* 2018; 98: 856-870.
- [29] Bezerra DP, Keasey M and Oliveira JRM. Erratum to: miR-9-5p down-regulates PIT2, but not PIT1 in human embryonic kidney 293 cells. *J Mol Neurosci* 2017; 62: 34.
- [30] Beresneva EV, Loginov VI, Khodyrev DS, Pronina IV, Kazubskaya TP, Karpukhin AV, Braga EA and Kushlinskii NE. The hyper-methylated genes microRNA as potential markers of clear-cell carcinoma of kidney. *Klin Lab Diagn* 2017; 62: 13-18.