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The protective effect of miR-377 inhibitor against renal ischemia-reperfusion injury through inhibition of inflammation and oxidative stress via a VEGF-dependent mechanism in mice

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ABSTRACT

MicroRNAs (miRNAs) play important roles in kidney development and maintenance of kidney physiological functions. MiR-377 has been reported to regulate inflammation in cardiac and cerebral ischemia. However, it remains unclear whether it has a similar function in renal ischemia/reperfusion (I/R). Using I/R model mice, miR-377 expression was determined by qRT-PCR in the renal tissues. Renal function was assessed by detection of the concentrations of blood urea nitrogen (BUN) and serum creatinine (Cr). Oxidative stress was evaluated by ELISA analysis of oxidation-related enzymes and molecules. Inflammatory factor concentration and other protein levels were analyzed by the ELISA assay and Western blot, respectively. Our study found that renal I/R stimulated miR-377 expression, while the inhibition of miR-377 attenuated renal I/R injury, and blocked renal I/R-induced oxidative stress and inflammation. Meantime, NF-κB and MAPK signaling were activated by renal I/R, which could also be reversed by miR-377 inhibitor. Furthermore, vascular endothelial growth factor (VEGF) depletion by siRNA completely abrogated the impact of miR-377 on renal I/R-induced oxidative stress, inflammation and renal dysfunction. In conclusion, renal I/R induced miR-377 expression, which upregulated VEGF expression to attenuate renal I/R-induced oxidative stress and inflammation, and finally ameliorated renal dysfunction.

1. Introduction

Ischemic-reperfusion (I/R) injury (IRI) is the tissue damage caused by blood reperfusion to the tissue that suffered for a period of ischemia. IRI is a common pathological phenomena in multiple clinical situations, such as infarction, sepsis as well as organ transplantation (Malek and Nematbakhsh, 2015). Renal ischemia/reperfusion injury (RIRI) which cannot be avoided in situations such as heart infarction, lung injury or kidney transplantation is a main cause of acute kidney injury (AKI) (Bonventre and Yang, 2011). In patients underwent renal transplantation, RIRI affects early functional recovery and graft survival (Venkatachalam et al., 2015). Accumulating data obtained from decades of studies have revealed complicated biological processes involved in RIRI, including generation of reactive oxygen species (ROS) and inflammatory factors (Weight et al., 1996). The blood reperfusion during RIRI induces generation of ROS which results in tissue damage and cell death (Kehrer, 1993). In this process, ROS production is enhanced by downregulation of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase (Singh et al., 1993). Treatment with ROS scavengers was found to prevent ROS-mediated kidney damage from renal I/R (Sahna et al., 2003; Sener et al., 2002). RIRI could also stimulate the expression of inflammatory factors such as interleukin 6 (IL6) and tumor necrosis factor α (TNF-α) which trigger inflammatory reaction that aggravates renal injuries (Malek and Nematbakhsh, 2015). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) signaling pathway has an important role in modulating inflammation (Chen et al., 2009; Guijarro and Egido, 2001). It has been reported that Folic acid-induced AKI can be reduced by limiting the activity of NF-κB signaling (Kumar et al., 2015).

MicroRNAs (miRNAs) are small RNAs with a length of 21–25 nucleotides which could inhibit gene expression by either affecting mRNA degradation or inhibiting translation. miRNAs play important roles in

Abbreviations: miRNAs, MicroRNAs; I/R, ischemia/reperfusion; BUN, blood urea nitrogen; Cr, creatinine; RIRI, Renal ischemia/reperfusion injury; AKI, acute kidney injury; ROS, reactive oxygen species; VEGF, Vascular endothelial growth factor

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kidney development and maintenance of kidney physiological functions, and deletion of the miRNA-processing enzyme in some renal cells was found to cause renal dysfunction (Patel et al., 2012; Sequeira-Lopez et al., 2010). Several miRNAs have been found to play a role in RIRI and kidney injury. For instance, miR-489 induced by hypoxia-inducible factor-1 (HIF-1) attenuated ischemic renal injury after renal I/R (Wei et al., 2016). miR-21 protected kidney from IRI through targeting caspase signaling (Hu et al., 2014). Depletion of miR-150 aggravated AKI triggered by myocardial infarction (Ranganathan et al., 2015).

miR-377, a miRNA targeting many genes (Motta et al., 2013), was found to be downregulated in cerebral ischemic injury (Liu et al., 2015), suggesting an involvement in I/R. Fan et al. reported that miR-377 depletion blocked cerebral inflammation and enhanced angiogenesis in response to cerebral ischemia (Fan et al., 2018). In addition, miR-377 was able to regulate ROS by regulating the expression of Heme Oxygenase-1 (HO-1) (Beckman et al., 2011). Based on these studies, we speculated that miR-377 might also play a role in RIRI.

2. Materials and methods

2.1. Renal I/R mouse model

Male C57BL/6 mice (20–25 g, 8–12 weeks old) were purchased from Shanghai SLAC (Shanghai, China). To conduct renal I/R surgery, the mice were anesthetized by *i.p.* injection of sodium pentobarbital at a dose of 50 mg/kg. Then the kidneys were exposed by midline laparotomy and subjected to renal pedicle closure for 0.5 h using nontraumatic microaneurysm clamps (Shanghai Medical Instruments, Shanghai, China). Then the microaneurysm clamps were released. The abdomen was sealed after reperfusion was observed. The sham group underwent the same surgery except for renal pedicle closure. Once the surgery was completed, the mice were *i.p.* injected with 0.5 L 0.9% sodium chloride solution. To investigate the function of miR-377 and vascular endothelial growth factor (VEGF), miR-377 mimic (miRIDIANMimic, Thermo Scientific), miR-377 inhibitor (miRCURY LNA microRNA inhibitor, Exiqon), VEGF siRNA (ON-TARGET plus SMARTpool, rat VEGF-A, Thermo Scientific) or negative control were dissolved in saline to 5 mg/ml, and delivered into the mice via tail vein at a dose of 10 mg/kg within 1 h before I/R surgery. The blood and kidney tissues were collected at 4 h, 24 h and 48 h after surgery for further examination. All experiments were ethically approved by Cangzhou Central Hospital of Animal Management and Use Committee.

2.2. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To determine the mRNA expression, total RNA was purified using TRIzol (ThermoFisher, Waltham, MA USA), and then 1 μg RNAs were reverse transcribed into cDNAs by a SuperScript III Reverse Transcriptase kit (ThermoFisher, Waltham, MA USA). A SYBR PrimeScript miRNA RT-PCR kit (Takara, Dalian, China) was applied to determine the levels of miRNAs using the following primers: miR-377, 5′-GAGCAGAGGTTGCCCTTG-3′ and 5′-ACAAAAGTTGCCTTTGTG TGA-3′; U6 small RNA (the internal control), 5′-AACGCTTCACGAATT TGCGT-3′ and 5′-CTCGCTTCGGCAGCACA-3′.

2.3. Assessment of renal function

Renal function was evaluated by determining the concentrations of blood urea nitrogen (BUN) and serum creatinine (Cr) at the Department of Clinical Laboratories of Cangzhou Central Hospital.

2.4. Measurement of oxidation

At 24 h post-I/R, the kidney tissues were collected. The concentrations of malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) were examined by kits for MDA, CAT and SOD assays, respectively (Beyotime Biotechnology, Shanghai, China). O^2- concentration was measured by an O^2- assay kit (Najing Jiancheng Bioengineering Institute, Nanjing, China). These assays were performed according to the manufacturer's instructions.

2.5. Enzyme-linked immunosorbent assay (ELISA) analysis of cytokine concentration

The concentration of cytokines including interferon gamma (IFN-γ), interleukin (IL)-6, tumor necrosis factor alpha (TNF-α) and IL-8 was tested by commercial ELISA kits (R&D System, Minneapolis, MN, USA) following the manufacture's instruction.

2.6. Western blot

The tissues were homogenized in radioimmunoprecipitation assay buffer supplied with protease inhibitors. Protein concentration was measured with the Bradford assay (Bio-Rad, Hercules, CA, USA). The protein samples were subjected to a sodium dodecyl sulfate polyacrylamide gel electrophores electrophoresis, and transferred onto nitrocellulose membranes (ThermoFisher, Waltham, MA USA). After blocking, membranes were incubated with one of the primary antibodies: anti-ERK (1:1000), anti- phosphorylated ERK (1:1000), anti-JNK2 (1:1000), anti-phosphorylated JNK2(1:1000), anti-IKKα (1:1000), anti-phosphorylated IKKα/β (1:500), anti-β-actin (1:1000), anti-lamin B (1:1000) and anti-p65 (1:500) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), at 4 °C overnight. Then membranes were washed and incubated with secondary antibodies conjugated with horseradish peroxidase (HRP, 1:2,000, Cell Signaling Technology, Danvers, MA, USA). An enhanced chemiluminescent reagent (ThermoFisher, Waltham, MA USA) was used to visualize the bands.

2.7. Statistical analysis

Data were expressed as the mean value ± standard error of mean (SEM). Statistical analysis was conducted with GraphPad Prism version 6 (GraphPad Software; La Jolla, CA, USA). Comparisons between different groups were performed with one- or two-way analysis of variance (ANOVA) followed by a Tukey's post hoc test or general linear model repeated measures. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. The expression of miR-377 is upregulated by renal I/R in mice

To address whether miR-377 plays a role in RIRI, the mRNA expression of miR-377 in kidney was measured at 4, 24 and 48 h after renal I/R surgery. As shown in Fig. 1A, miR-377 expression significantly increased as early as 4 h post renal I/R surgery, and further increased as the time extended. At the time point of 48 h, the miR-377 levels were nearly doubled (Fig. 1B). These results show that renal I/R induced miR-377 expression in mice.

3.2. miR-377 inhibitor alleviates renal I/R-induced injury

Increase of serum Cr and BUN is a hallmark of renal dysfunction (Lyman, 1986). Our results showed that the concentrations of BUN and Cr were elevated at 4 h after renal I/R, and further increased at 24 h and 48 h post-I/R (Fig. 2A and B). Treatment with miR-377 inhibitor attenuated accumulation of BUN and Cr, whereas miR-377 mimic had an opposite function (Fig. 2A and B). These data suggest that miR-377 inhibitor is able to alleviate renal I/R-induced injury.

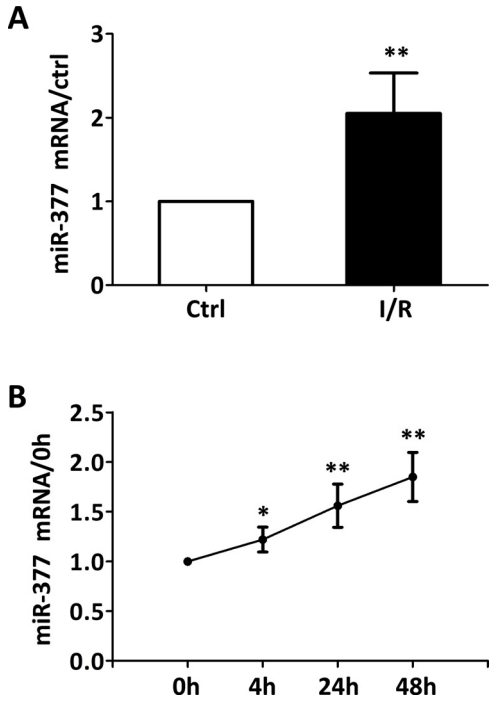


Fig. 1. The expression of miR-377 is upregulated by renal I/R in mice. The mice were subject to renal I/R surgery, and the expression of miR-377 in the kidneys was determined by qRT-PCR at 4 h, 24 h and 48 h post-I/R. miR-377 expressions in I/R group were compared with control (A), and the changes of miR-377 expressions with time were normalized to that at the time point of 0 h (B). n = 8 in each experimental group. Experiments were repeated in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. 0 h.

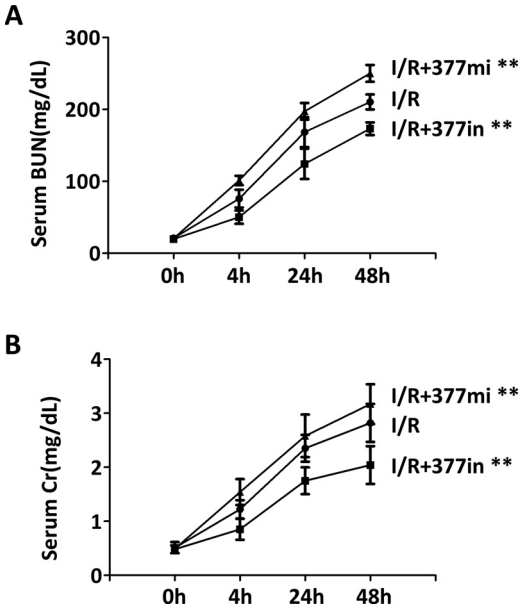


Fig. 2. miR-377 inhibitor alleviates renal I/R-induced injury. miR-377 mimic (377mi) or inhibitor (377 in. were injected into the mice via tail vein prior to I/R surgery, and then the levels of BUN (A) and Serum Cr (B) were measured. n = 8 in each experimental group. Experiments were repeated in triplicate. $^{**}p < 0.01$ vs. 0 h.

3.3. miR-377 inhibitor attenuates renal I/R-induced oxidative stress

Next we investigated the effect of miR-377 on oxidative stress induced by renal I/R. SOD and CAT are two enzymes responsible for scavenging ROS, and MDA is byproducts of lipid peroxidation (Barrera, 2012). As expected, I/R treatment reduced the concentration of SOD and CAT (Fig. 3A and B) and increased the levels of MDA and O2- (Fig. 3C and D). miR-377 inhibitor treatment prevented I/R-induced reduction of SOD and CAT (Fig. 3A and B), and elevation of MDA and O2- (Fig. 3C and D). On the contrary, miR-377 mimic played an opposite effect in these processes (Fig. 3A–D). These results show that miR-377 inhibitor ameliorates renal I/R-induced oxidative stress.

3.4. miR-377 inhibitor downregulates expression of inflammatory factors

To determine whether miR-377 is involved in renal I/R-induced inflammation, the effect of miR-377 on cytokine production was examined. Results showed that the production of cytokines, including IL-6, IFN-γ, IL-8 and TNF-α, was dramatically increased at 24 h post renal I/R surgery (Fig. 4A–D). The renal I/R-induced cytokine productions were significantly reduced by miR-377 inhibitor but further enhanced by miR-377 mimic (Fig. 4A–D), suggesting that miR-377 functions in renal I/R-triggered inflammation.

3.5. miR-377 inhibitor impedes renal I/R-induced activation of NF-κB and MAPK signaling pathways

It was documented that increase of TNF-α and other inflammatory factors stimulated activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling, including ERK, JNK and p38 MAPK, *etc.* (Toledo-Pereyra et al., 2004). Since miR-377 modulated expression of inflammatory factors, we investigated the impact of miR-377 on activation of NF-κB, ERK and JNK signaling pathways. Results showed that renal I/R greatly elevated the phosphorylation levels of ERK and JNK2 (Fig. 5A and B), suggesting activation of ERK and JNK signaling pathways in kidney post I/R. Similarly, renal I/R induced phosphorylation of IκB kinase (IKK) and increased the nuclear p65 protein levels (Fig. 5A and B), indicating activation of NF-κB signaling. miR-377 mimic had no influence on renal I/R induced phosphorylation of ERK and IKK, but enhanced renal I/R induced phosphorylation of JNK2 and protein levels of nuclear p65 (Fig. 5A and B). In contrast, miR-377 inhibitor significantly attenuated renal I/R induced activation of ERK, JNK and NF-κB signaling pathways (Fig. 5A and B). These results indicate that miR-377 is involved in regulating activation of NF-κB and MAPK signaling after renal I/R.

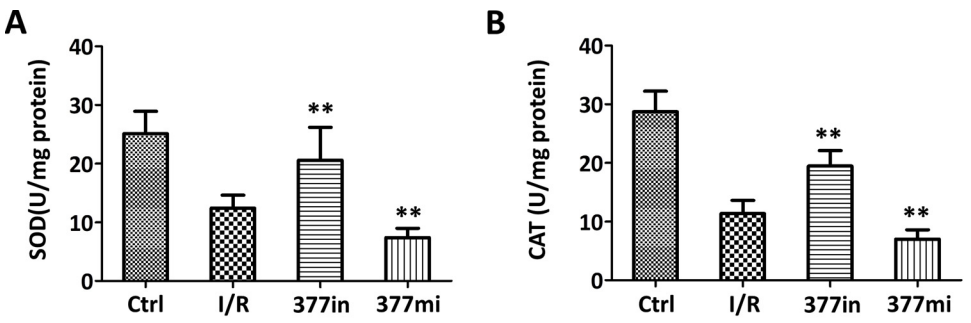
3.6. VEGF depletion abrogates the effect of miR-377 inhibitor in RIRI

Renal ischemic injury reduces microvascular density (Basile et al., 2001), and VEGF administration elicits angiogenesis and maintains renal functions in renal disease (Kang et al., 2001). We then investigated whether VEGF was involved in the effect of miR-377 in RIRI. Renal I/R slightly upregulated the protein level of VEGF, which was enhanced by miR-377 inhibitor while attenuated by miR-377 mimic (Fig. 6A), indicating that miR-377 is capable of regulating VEGF protein levels. We further found that VEGF depletion by VEGF siRNA abrogated the effect of miR-377 inhibitor or mimic on renal I/R-induced production of serum BUN (Fig. 6B), serum Cr (Fig. 6C), IL-6 (Fig. 6D), IFN-γ (Fig. 6E), MDA (Fig. 6F) and O2- (Fig. 6G). These data suggest that the effect of miR-377 in RIRI is mediated by VEGF.

4. Discussion

This study investigated the functions of miR-377 in RIRI. Renal I/R induced expression of miR-377, and inhibition of miR-377 attenuated RIRI. Further study revealed that miR-377 regulated oxidative stress, stimulated inflammation and activated NF-κB and MAPK signaling after renal I/R. Interestingly, knockdown of VEGF completely abolished the effect of miR-377 in RIRI.

miR-377 has been reported to exert a role in heart ischemic injury (Wen et al., 2014) and cerebral ischemic injury (Fan et al., 2018).



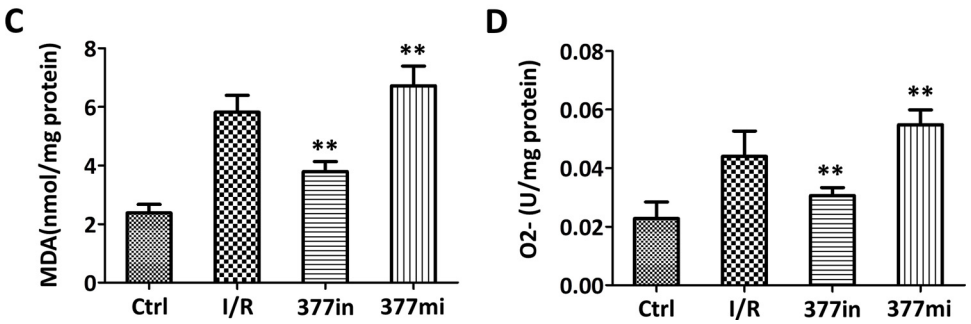
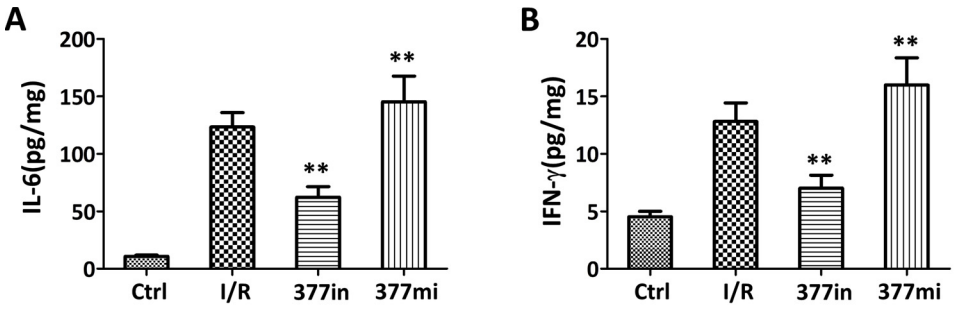


Fig. 3. miR-377 inhibitor attenuates renal I/R-induced oxidative stress. miR-377 mimic (377mi) or inhibitor (377 in. were injected into the mice via tail vein prior to I/R surgery. The renal tissues were collected at 24 h post I/R surgery, and followed by determination of the concentration of SOD (A), CAT (B), MDA (C) and O2- (D). n = 8 in each experimental group. Experiments were repeated in triplicate. $^{**}p < 0.01$ vs. I/R group.



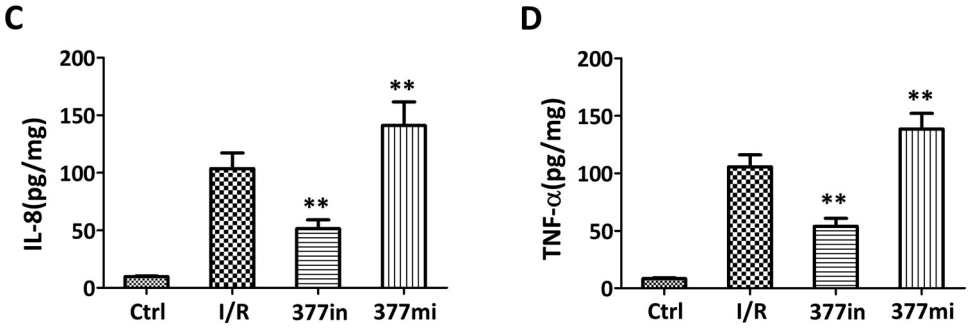


Fig. 4. miR-377 inhibitor downregulates expression of inflammation factors. miR-377 mimic (377mi) or inhibitor (377 in. were injected into the mice via tail vein prior to I/R surgery. The renal tissues were collected at 24 h post I/R surgery, followed by ELISA analysis of IL-6 (A), IFN-γ (B), IL-8 (C) and TNF-α (D). n = 8 in each experimental group. Experiments were repeated in triplicate. $^{**}p < 0.01$ vs. I/R group.

Hypoxia environment in ischemia represses miR-377 expression (Fan et al., 2018; Wen et al., 2014), whereas reperfusion after ischemia induces miR-377 expression in heart (Joladarashi et al., 2015). In line with these results, renal I/R also induced miR-377 expression in this study. Previous studies have revealed VEGF as a direct target of miR-377, and miR-377 played an important role in angiogenesis in a rat model of cerebral ischemia by regulating VEGF (Fan et al., 2018). In this study, we found that the effect of miR-377 on RIRI was totally dependent on VEGF, suggesting that VEGF plays a critical role in this process.

It was shown that miR-377 could directly target VEGF and suppress VEGF expression (Fan et al., 2018). Since miR-377 was induced by renal I/R, VEGF was supposed to be downregulated after renal I/R. However, our study showed that the VEGF level after RIRI was not statistically significantly different from that in the control. This result is consistent with a previous report which found no difference in VEGF mRNA expression among different groups in a rat renal I/R model (Kanellis et al., 2002). One explanation of this consequence is that VEGF expression is regulated by multiple factors, such as HIFα (Legrand et al., 2008), and the final protein abundance level of VEGF is a result of combined effects of miR-377 and other factors. Therefore, as expected, when the homeostasis between miR-377 and other factors was broken by miR-377 inhibitor, VEGF protein level was dramatically increased in this study.

NF-κB and MAPK signaling are two important signaling pathways in inflammation. NF-κB is a family of transcription factors, and is involved

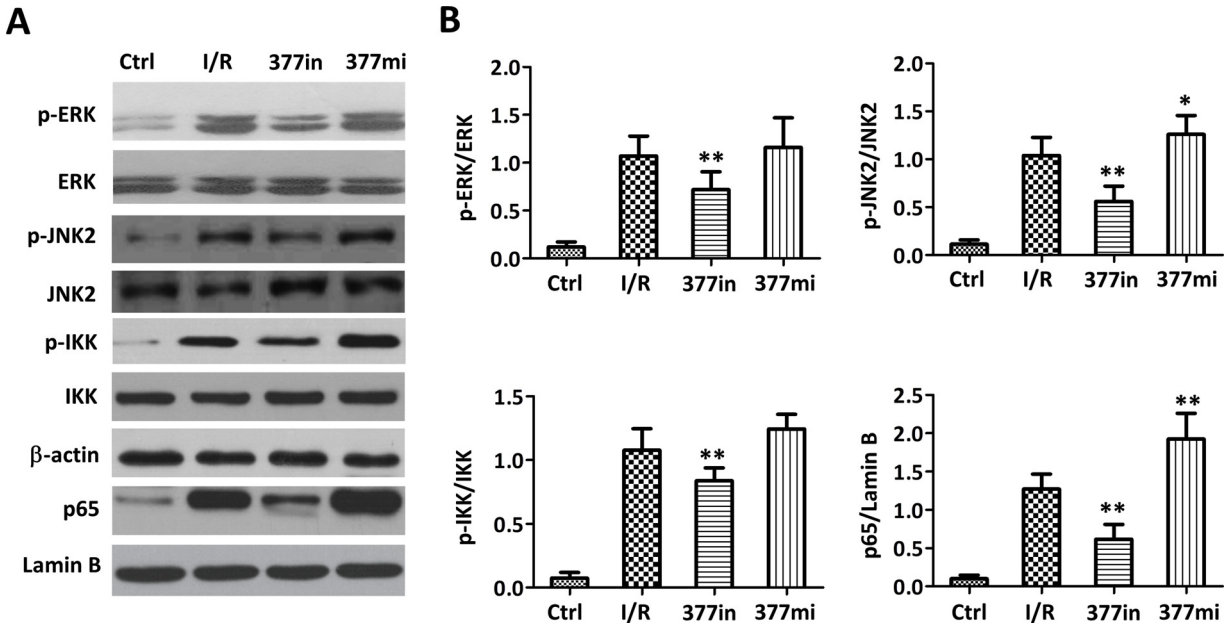


Fig. 5. miR-377 inhibitor impedes renal I/R-induced activity of NF-κB and MAPK signaling. miR-377 mimic (377mi) or inhibitor (377 in. were injected into the mice via tail vein prior to I/R surgery. The renal tissues were collected at 24 h post I/R surgery, followed by Western blot analysis of indicated proteins (A), and quantification analysis of Western blot results (B). Experiments were repeated in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. I/R group.

in multiple biological processes, including inflammation and cell proliferation (Hayden and Ghosh, 2008). The family encompasses five members, c-Rel, RelB, p65, NF-κB2 p52 and NF-κB1 p50. In the absence of stimulation, NF-κB proteins are retented in the cytoplasma by its inhibitors such as IκBα. In the presence of stimulation, IκBα is phosphorylated by IKK and degraded. Then NF-κB proteins are translocated from the cytoplasma into the nuclei to activate the target gene expression (Sun, 2011). In the study we found renal I/R led to IKK phosphorylation and p65 accumulation in nuclei, indicating activation of NF-κB signaling. Activation of NF-κB signaling could further promote

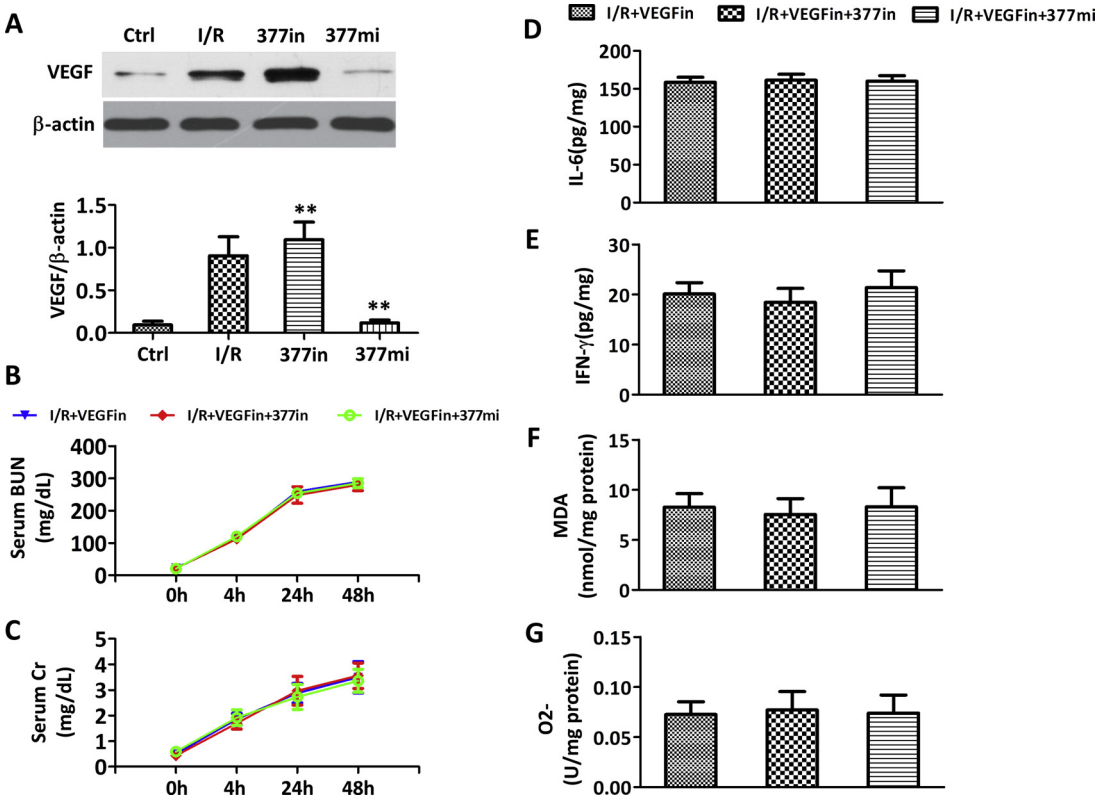


Fig. 6. VEGF depletion abrogates miR-377 inhibitor protecting kidney from RIRI. miR-377 mimic (377mi) or inhibitor (377 in. alone (A, B) or combined with VEGF inhibitor (VEGFin) (B–G) were injected into the mice via tail vein prior to I/R surgery. The renal tissues were collected at 24 h after I/R surgery, and followed by Western blot analysis of VEGF expression (A), determination of levels of serum BUN (B), serum Cr (C), IL-6 (D), IFN- (E), MDA (F) and O2- (G). n = 8 in each experimental group. Experiments were repeated in triplicate. $^{**}p < 0.01$.

inflammation. Our results showed that miR-377 inhibitor suppressed renal I/R induced inflammation and NF-κB signaling activation, and this effect was abrogated by VEGF depletion. These results indicated that VEGF played a key role in inflammation and NF-κB activation after RIRI. Previous study has revealed an involvement of VEGF in inflammation (Shibuya, 2015), and depending on the cellular context, VEGF could either activate or inhibit NF-κB (DeNiro et al., 2013). Therefore, further studies should be carried out to investigate the detailed mechanism underlying the effect of VEGF in these processes.

In conclusion, miR-377 inhibitor upregulated VEGF expression after renal I/R, attenuated inflammation and oxidative stress, and eventually ameliorated renal dysfunction. Thus, miR-377 inhibitor might be a potential drug for treating patients suffered from renal I/R.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

None.

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