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Full Paper

Stimulation of miRNA-1906 restores podocyte function in ischemia-induced renal injury by restoring SLIT2/ROBO1 signalling pathway

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Abstract: The present study evaluates the nephroprotective effect and molecular function of miRNA-1906 stimulation on ischemia/reperfusion (I/R)-induced acute kidney injury (AKI) in mice. Clamping kidney tissues caused I/R-induced renal injury. In this study, 18 mice were divided into three distinct groups (n = 6): control group, AKI group and miRNA-1906 agomir Test group. The miRNA-1906 group received 500 nmol/kg of miRNA-1906 agomir administered intrathecally for 24 hrs before the induction of ischemia. The effects of miRNA-1906 stimulation on kidney function, oxidative stress and mediators of inflammation were evaluated. The expressions of Slit Guidance Ligand 2 (SLIT2), Roundabout homolog 1 (ROBO1), desmin, caspase-3, Transient Receptor Potential Canonical 6 (TRPC6), phosphorylated Akt (p-Akt) and nephrin in renal tissues were measured by western blot to investigate the impact of miRNA-1906 stimulation on podocyte damage. Histopathological changes were assessed with hematoxylin and eosin staining. The miRNA-1906 agomir reduced the serum levels of creatinine and blood urea nitrogen, as well as the excretion of microalbumin, in the AKI group. The treatment with miRNA-1906 agomir also lowered levels of oxidative stress and inflammatory mediators in the kidney tissues of mice that I/R had injured. In comparison to the AKI group, the miRNA-1906 agomir treatment also resulted in a notable upregulation of desmin, caspase-3, and Transient Receptor Potential Cation Channel 6 (TRPC-6), as well as a significant reduction in nephrin. The miRNA-1906 agomir also reduced SLIT2/ROBO1 circuit modification, restoring podocyte function. The I/R-induced AKI was resolved by stimulating miRNA-1906 with miRNA-1906 agomir. This controls podocyte damage by blocking the SLIT2/ROBO1 pathway.

Keywords: miRNA-1906 agomir, podocyte, kidney injury, ischemia, reperfusion, SLIT2/ ROBO1 pathway

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INTRODUCTION

Acute kidney injury (AKI) results in a rapid decline in the ability of the kidneys to filter waste products. Ischemia-reperfusion (I/R) injury of the kidney is the primary cause of AKI [1]. I/R-induced renal injury increases inflammatory mediators and oxidative stress [2]. A previous study [3] suggested that treatment of I/R-induced AKI should focus on reducing apoptosis, renal inflammation and oxidative stress. The glomerular basement membrane, fenestrated capillary endothelial cells and podocytes provide a barrier between plasma proteins and blood cells during glomerular filtration [4]. Protein filtration, one of the most important functions governed by podocytes, occurs in the slit diaphragm semi-porous membrane, where laminin and type-IV collagen proteins are secreted by interconnecting podocytes [5]. Slit diaphragm proteins such as nephrin regulate the function and structure of podocytes [6]. The genetic alteration of these proteins modulates the function of podocytes and causes nephritic syndrome. The Slit Guidance Ligand 2/Roundabout Homolog 1 (SLIT2/ROBO1) signalling pathway modulates nephrin-associated podocyte function [7, 8].

MicroRNAs (miRNAs) are small non-coding RNAs that play a crucial role in the development and progression of various diseases [9]. These miRNAs are involved in cellular processes such as proliferation, differentiation, apoptosis (cell death), inflammation and injury caused by ischemia and toxic stresses [10]. Due to their target specificity and ability to govern cellular processes via target genes, miRNAs are considered promising candidates for renal treatment [11]. It is plausible that miRNAs have a role in the development of AKI caused by ischemia or nephrotoxicity. Various kidney diseases including renal fibrosis, renal carcinoma, AKI and diabetic nephropathy, have been found to exhibit distinct expression patterns of miRNAs [12]. Multiple miRNA expressions that deviate from the norm have been linked to various renal diseases, demonstrating their significant impact on the progression of these conditions [13]. Several distinct miRNAs have been identified as playing a role in the development of AKI, including miR-687, miR-489, miR-494, miR-24, miR-21 and miR-126 [14]. Although the exact targets and functional effects of many miRNAs remain unknown, their potential as therapeutic targets is enormous [15].

miRNA-1906 is one of the leading diagnostic indicators for acute ischemic stroke. An earlier experiment found that miRNA-1906 has the potential to reduce the severity of cerebral stroke [16]. Previous investigation found that miRNA-1906 can reduce the neurological score, infarct volume and brain water content. It can also inhibit neuronal apoptosis and increase the level of inflammatory factors by activating the Janus kinase 2 / phosphorylates signal transducer and activator of transcription 3 (JAK2/STAT3) signalling pathway in cerebral ischemic injury [17]. This investigation demonstrates that miRNA-1906 can protect against cerebral ischemic injury. However, the study of miRNA-1906's function and mechanism in ischemic injury remains incomplete. Therefore, further investigation is needed to understand the complete functions and mechanisms of miRNA-1906 in ischemic injuries [17]. The SLIT-ROBO signalling pathway is significant in several renal diseases. SLIT ligand and its receptor ROBO were first identified for their roles in the guiding of axons during the development of the central nervous system [18, 19]. Over the past several years, research has progressed, resulting in a deeper understanding of the SLIT-ROBO signalling pathway. It is now acknowledged that this route plays a role not only in axonal repulsion but also in cell migration, tumour growth, angiogenesis and bone metabolism [20]. The present investigation aims to examine the nephroprotective effects of the SLIT2/ROBO1

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signalling pathway and the molecular function of miRNA-1906 stimulation on I/R-induced AKI in animals.

MATERIALS AND METHODS

Animals

Eighteen male C57BL/6J mice (22-23 g, 7-8 weeks old) were obtained from the Animal House of Shanghai Xinhua Hospital, Chongming Branch, China. Maintained in the regular conditions (60 ± 5% humidity, 24 ± 3°C temperature, 12:12 h light cycle), typical food and tap water were provided for all animals. The entire experimental protocol (Registration No. IACUC/SXX-TCXSA/2019/06), was reviewed and approved by the Research Review and Ethics Board of Chongming Hospital Affiliated to Shanghai University of Medicine and Health Sciences, (Chongming Branch of Shanghai Xinhua Hospital), Shanghai 202150, China.

Chemicals

miRNA-1906 agomir, xylazine, tiletamine/zolazepam, malondialdehyde, thiobarbituric acid reactive species and glutathione were acquired from Sigma-Aldrich (USA). Creatinine, microalbumin and blood urea nitrogen (BUN) assessment kits were acquired from ERBA Diagnostics (Germany). The enzyme-linked immunosorbent test kit was procured from Thermo Fisher Scientific (USA).

Renal I/R-Induced Injury

The animals were split into three distinct groups (n = 6): control group, AKI group and miRNA-1906 agomir Test group. Before the development of ischemia, the miRNA-1906 agomir group was administered 500 nmol/kg of miRNA-1906 agomir intrathecally using the MaxSuppressor In Vivo RNA-LANCEr II RNA delivery system, as instructed by PerkinElmer (USA), for 24 hr. The AKI and miRNA-1906 agomir groups of animals underwent an intraperitoneal injection of xylazine (10 mg/kg) and tiletamine/zolazepam (30 mg/kg) to induce anaesthesia. Subsequently, a midline incision was performed to reveal the kidneys. A homothermic pad was used to maintain core body temperature. Non-traumatic microaneurysm clamps were used to produce an occlusion in the bilateral kidneys for 23 min, after which the abdomen was closed with sutures. One day after reperfusion, blood was taken from the back of the orbit and kidney tissue samples were isolated after sacrificing the animals via cervical dislocation. Ice-cold saline was used to rinse the kidney tissue before homogenisation in Tris buffer (50 mM, pH 7.4). The homogenised renal tissues were subjected to centrifugation at 3,000 rpm for 15 min. The liquid portion was obtained for further studies.

Assessment of Kidney Function

Serum creatinine and BUN concentrations were obtained from both serum and urine samples. The enzyme-linked immunosorbent assay was used to evaluate serum creatinine levels, BUN concentrations and microalbumin, assessed according to the manufacturer's instructions provided by the manual from ERBA Diagnostics (Germany).

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Estimation of Markers of Oxidative Stress

The kidney homogenate was utilised to assess the levels of malondialdehyde, glutathione and superoxide dismutase (SOD) as indicators of oxidative stress. The activity of SOD was assessed by estimating the absorbance of a solution of 0.2 mL of nicotinamide adenine dinucleotide, 0.3 mL of nitro blue tetrazolium and 0.1 mL of the kidney homogenate. The final tissue mixture was assessed at an absorbance of 560 nm. The level of malondialdehyde in the tissue homogenate was estimated by determining the concentration of thiobarbituric acid reactive substances at 534 nm. Ellman's reaction was used for measuring the level of glutathione and absorbance was determined at 412 nm.

Estimation of Inflammatory Markers

The concentrations of prostaglandin E2 (PGE-2), interleukin-1β (IL-1β), and tumour necrosis factor-α (TNF-α) in the tissue mixture were measured using the enzyme-linked immunosorbent assay, following the manufacturer's instructions from Thermo Fisher Scientific (USA).

Western Blot Assay

Proteins were extracted from kidney tissues by utilising the radioimmunoprecipitation assay buffer [50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM phenylmethylsulfonyl fluoride]. A bicinchoninic acid test was performed to measure the total protein content [21]. The absorbance was measured at 562 nm using a UV-VIS spectrophotometer (L9 double-beam Visible / UV spectrophotometer, Nanbei Instruments, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and nitrocellulose membrane filtration were used to separate the protein sample. The precipitates were blotted with Anti-rabbit caspase-3 (1:500), desmin (1:200) (R&D Systems Ltd., China), nephrin (1:500), roundabout guidance receptor 1 (ROBO1) (1:500), phosphorylated Ak strain transforming protein (1:1,000), SLIT2 (1:500) (Santa Cruz Biotechnology, USA) and transient receptor potential cation channel 6 (TRPC-6) (1:1,000) (Abcam, UK) for overnight incubation at 4°C. Following this, the samples were treated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, USA) for an additional hr. The optical density was assessed by Odyssey Infrared Imaging System (Odyssey ® Imagers, USA) and ImageJ software (NIH, USA) was employed for quantitative analysis of the blots using standard protocols.

Histopathology Study of Renal Tissues

The kidney tissues were fixed with a 10% formalin solution and then embedded in paraffin. A microtome was used to make 4-µm-thick slices, which were processed with haemotoxylin-eosin. The tissue sections were observed using a light microscope. The following scale was used to rate the level of necrosis and degeneration: no necrosis, score of 0; < 10% necrosis, score of 1; 10–25% necrosis, score of 2; 26–75% necrosis, score of 3; and > 75% necrosis, score of 4 [22].

Statistical Analysis

The data are presented as mean ± standard error (n = 10). A one-way analysis of variance was used to conduct statistical assessments. Dunnett's post-hoc test was employed for a post-hoc comparison of means. The evaluation was performed using GraphPad Prism (version 6.1; GraphPad Software Inc., USA). The statistically relevant threshold was established at $p < 0.05$.

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RESULTS AND DISCUSSION

Renal injury has a high mortality rate and conventional treatment for its management has several limitations [23]. The present investigation evaluates the nephroprotective effect of miRNA-1906 agomir on I/R-induced AKI in an animal model and also postulates the molecular mechanism of action. Renal damage may result from several pathogenic processes such as I/R. Figure 1 demonstrates that administration of the miRNA-1906 agomir reverses the changes in renal function caused by acute renal damage generated by IR. The AKI group has elevated serum BUN (167±3.1 µmol/L) and creatinine level (357±0.0 mol/L) as well as increased excretion of microalbumin (23±1.1 mg/24 hr) in the urine compared to the control group. Compared to the AKI group, the miRNA-1906 agomir group shows a decrease in serum BUN, creatinine and microalbumin levels.

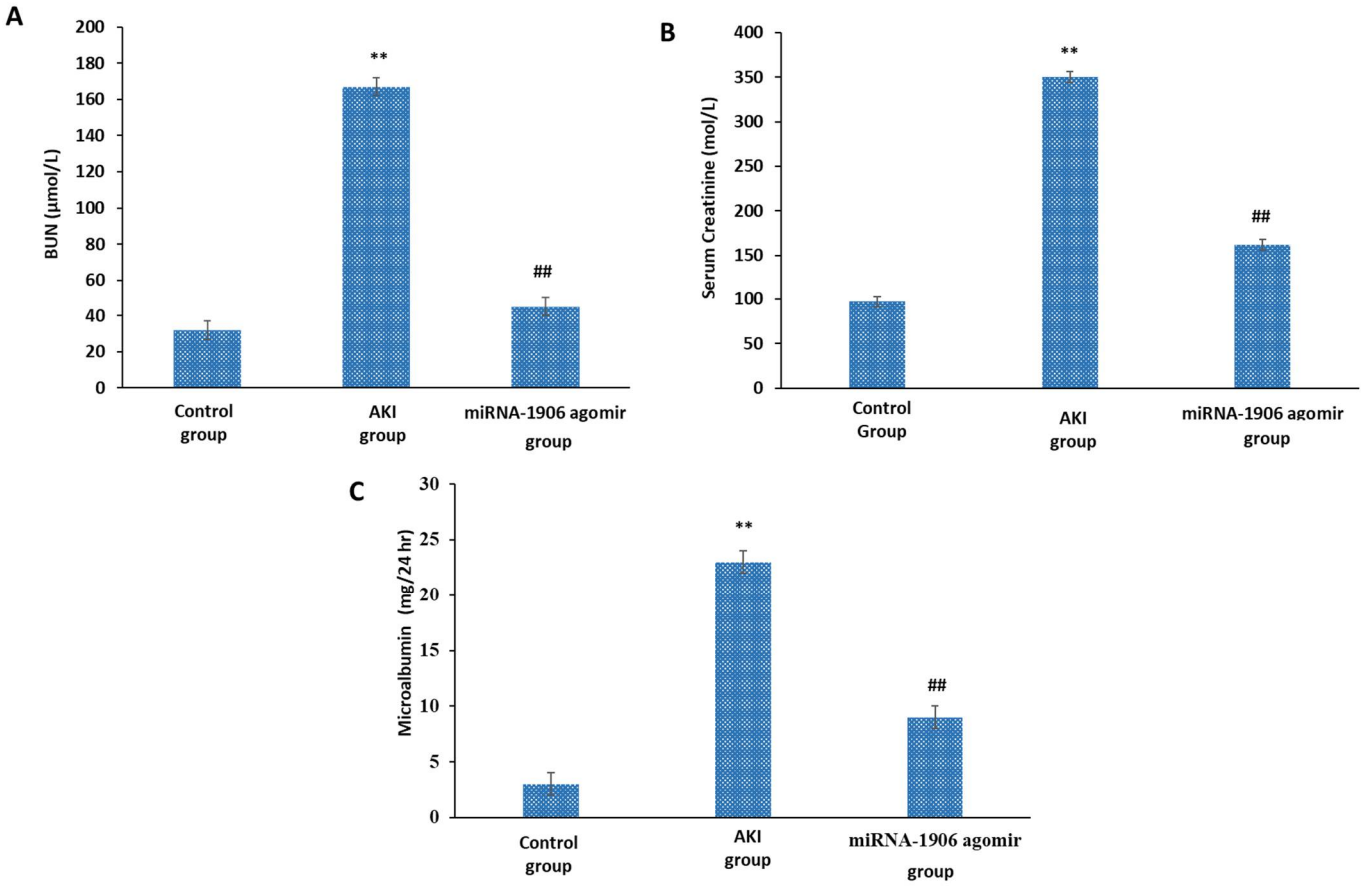

Figure 1. miRNA-1906 agomir restores altered biochemical markers of renal injury in mice with I/R-induced AKI: A) BUN level; B) creatinine level; C) microalbumin level.
Values are mean ± standard error (n = 10). ** $p < 0.01$ compared to control group, $^{\#\#}$ $p < 0.01$ compared to AKI group

Figure 2 demonstrates the effects of miRNA-1906 agomir on the level of malondialdehyde, glutathione and the functioning of SOD in the renal tissues of mice with I/R-induced AKI. The level of malondialdehyde was higher (7.2±1.23 nmol/mg) and the level of glutathione was lower (3.8 nmol/mg) in the kidney tissue of the AKI group compared to the control group. Additionally, the AKI group shows a lower level (0.8±0.1 U/mg) of SOD activity in the tissue compared to the control group. Administration of the miRNA-1906 agomir reduces the changes in the concentrations of malondialdehyde and glutathione, as well as the activity of SOD.

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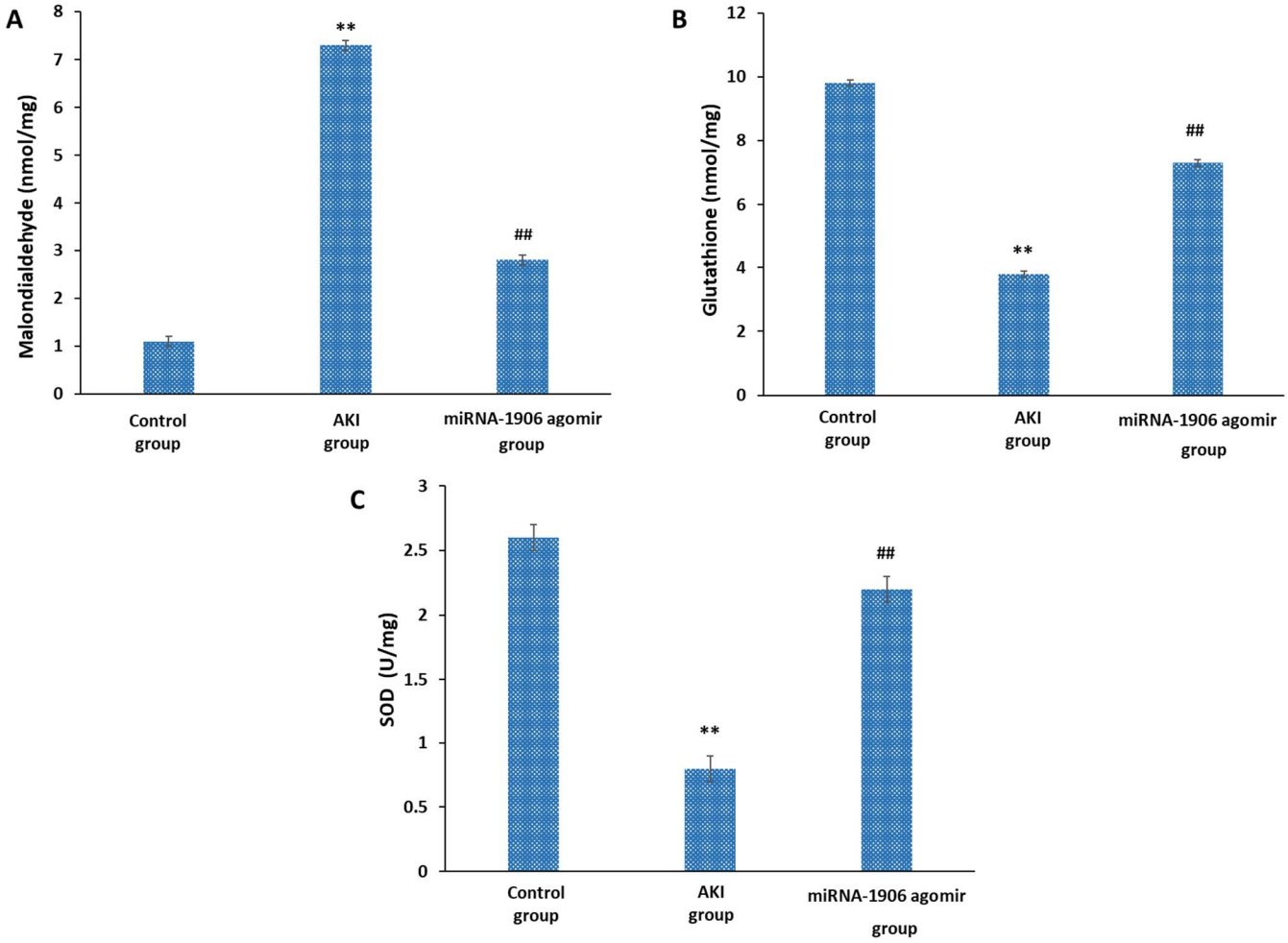

Figure 2. miRNA-1906 agomir restores altered markers of oxidative stress in the renal tissues of mice with I/R-induced AKI: A) malondialdehyde level; B) glutathione level; C) SOD level. Values are mean ± standard error (n = 10). ** $p < 0.01$ compared to control group, $^{\#\#}$ $p < 0.01$ compared to AKI group

The AKI group shows elevated levels of PGE-2 (296±0.63 pg/mg), TNF-α (276±0.33 pg/mg) and IL-1 (152±0.09 pg/mg) in homogenised renal tissues compared to the control group. However, miRNA-1906 agomir treatment reduces the levels of PGE-2 (106±0.39 pg/mg), TNF-α (174±0.11 pg/mg) and IL-1β (63±0.33 pg/mg) in the tissue homogenate compared to the AKI group (Figure 3).

The impact of miRNA-1906 agomir on proteins involved in podocyte damage was assessed by measuring the expression levels of desmin, caspase-3, TRPC-6 and nephrin in the kidney tissue homogenate (Figure 4). The AKI group exhibits a decrease in the expression levels of desmin (2.45 ± 0.01), caspase-3 (2.62 ± 0.03), TRPC-6 (0.1 ± 0.01) and nephrin (0.25 ± 0.06) compared to the control group. Administration of the miRNA-1906 agomir results in an elevation of the expression of all four proteins, in contrast to the AKI group.

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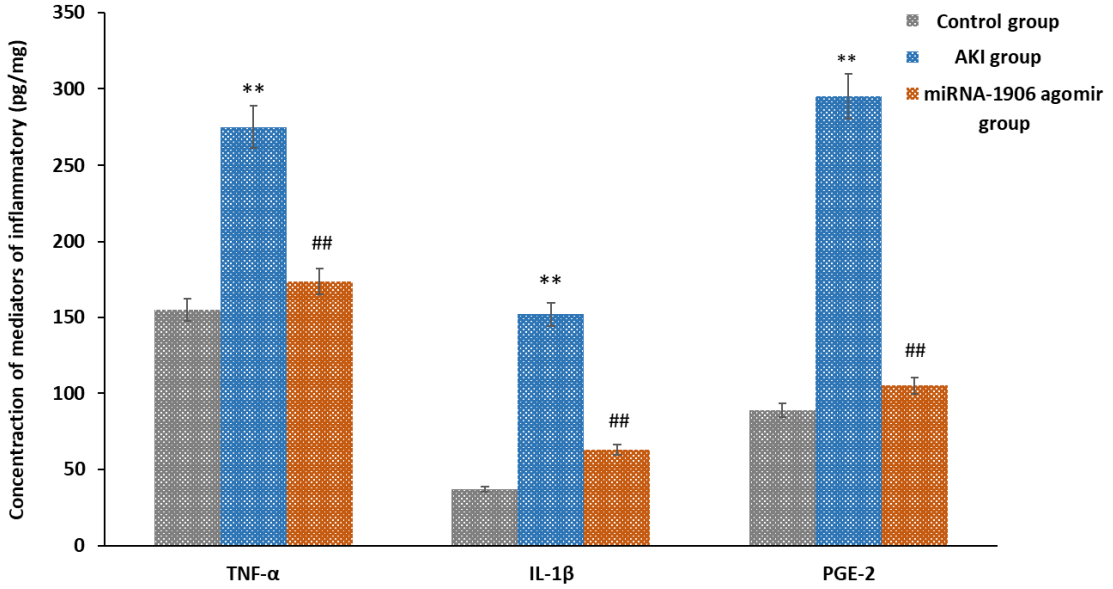

Figure 3. miRNA-1906 agomir restores level of inflammatory mediators PGE-2, TNF-α and IL-1β in renal tissues of mice with I/R-induced AKI.
Values are mean ± standard error (n = 10). ** $p < 0.01$ compared to control group, ## $p < 0.01$ compared to AKI group

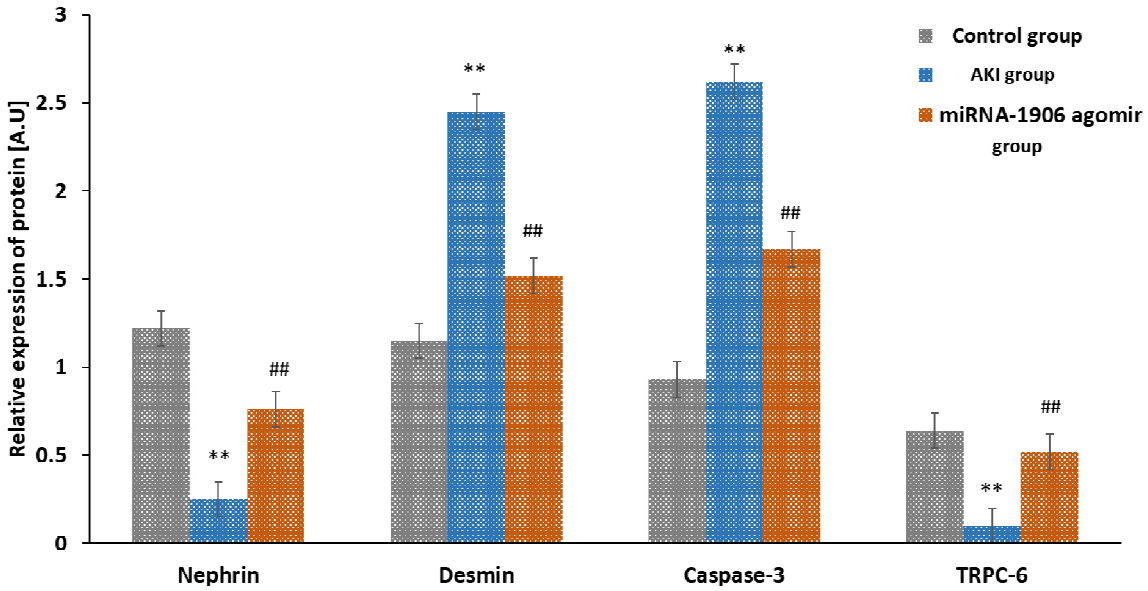

Figure 4. miRNA-1906 agomir restores expression of proteins that contribute to development of podocyte injury in renal tissues of mice with I/R-induced AKI.
Values are mean ± standard error (n = 10). ** $p < 0.01$ compared to control group, ## $p < 0.01$ compared to AKI group

Figure 5 depicts the impact of miRNA-1906 agomir on the SLIT2/ROBO1 pathway in mice suffering from acute renal damage generated by I/R. The renal tissues of the AKI group exhibit a reduction in the expression levels of Akt (0.3 ± 0.01), ROBO1 (0.25 ± 0.05), and SLIT2 (0.32 ± 0.01) compared to the control group. However, the level of Akt (0.8±0.02), ROBO1 (0.68±0.05) and SLIT2 (0.76±0.03) expression is notably increased in the tissue homogenate of the miRNA-1906 agomir group in comparison to the untreated AKI group.

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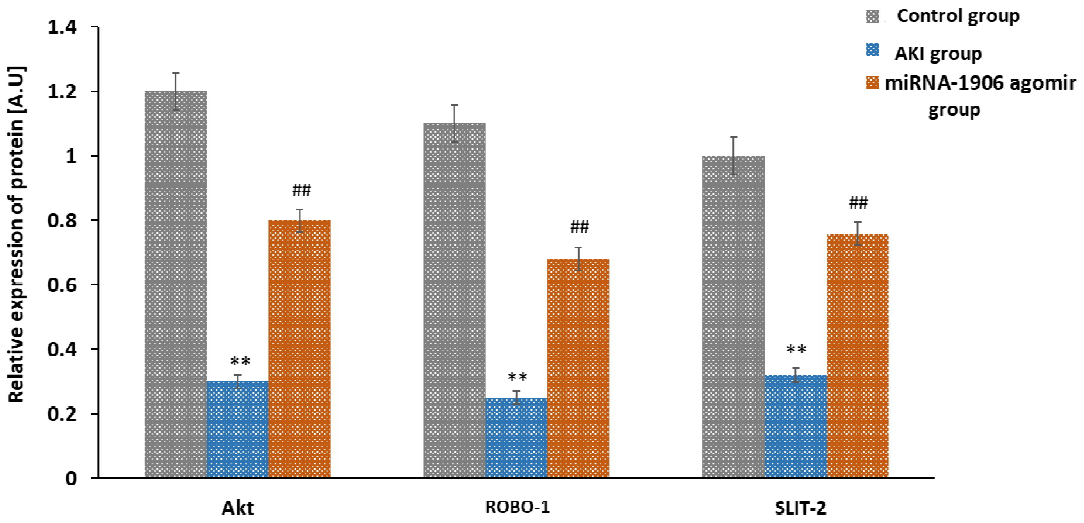

Figure 5. miRNA-1906 agomir ameliorates expression of Ak strain transforming protein (Akt), ROBO1 and SLIT2 in renal tissues of mice with I/R-induced AKI.
Values are mean ± standard error (n = 10). ** $p < 0.01$ compared to control group, $^{\#\#}$ $p < 0.01$ compared to AKI group

The histological changes in the renal tissues of mice with kidney damage induced by I/R were assessed using hematoxylin and eosin staining. In the control group the kidney tissue is morphologically normal (Figure 6). The AKI group shows leukocyte infiltration, swelling, congestion, hemorrhage and tubular damage in the glomeruli. In contrast, the tissues of mice treated with miRNA-1906 agomir appear to be morphologically normal (Figure 6A). Necrosis and degeneration scores are shown in Figure 6B. There is more necrosis and degeneration in the AKI group than in the control group. However, there is less necrosis and degeneration in the miRNA-1906 agomir group than in the AKI group.

Kidney tissues are affected by oxidative stress, which arises from the generation of reactive oxygen species, resulting in a disturbance in the renal system [24]. Oxidative stress triggers cell death by necrosis or apoptosis, impacts the levels of malondialdehyde, glutathione and modifies the functioning of catalase, SOD, xanthine oxidase and inducible nitric oxide synthase [25]. Drugs used for the management of kidney disease rely on vigorous antioxidant activity to treat kidney injury [26]. Inflammation is a significant contributor to kidney damage and studies have shown that TNF-α and IL-1β are involved in the development of kidney injury [27]. There is sufficient evidence to support the notion that oxidative stress leads to increased activity of TNF-α and IL-1β [28]. The study's findings demonstrate that the activities of TNF-α and IL-1β increase in the kidney tissue homogenate after I/R injury. However, the administration of the miRNA-1906 agomir effectively decreases the activity of these cytokines to normal levels. Research has reported a reduction in podocyte count in renal injury [29]. Podocyte injury increases the excretion of albumin in urine, which is used as a marker of renal injury. Desmin, nephrin and TRPC-6 help maintain the structure and function of podocytes [30]. In renal injury, a high level of inflammatory mediators alter the expression of proteins secreted by podocytes [31]; miRNA-1906 agomir has been reported to prevent this phenomenon [32, 33]. Moreover, studies have demonstrated that the SLIT2/ROBO1 pathway plays a role in the progression of podocyte injury. As a result, this pathway is a prospective target for the progression of novel medications that can treat kidney injury [33]. The outcomes

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reveal that the miRNA-1906 agomir attenuates alterations in the expression levels of desmin, nephrin and TRPC-6 in the kidney tissues of AKI mice [34].

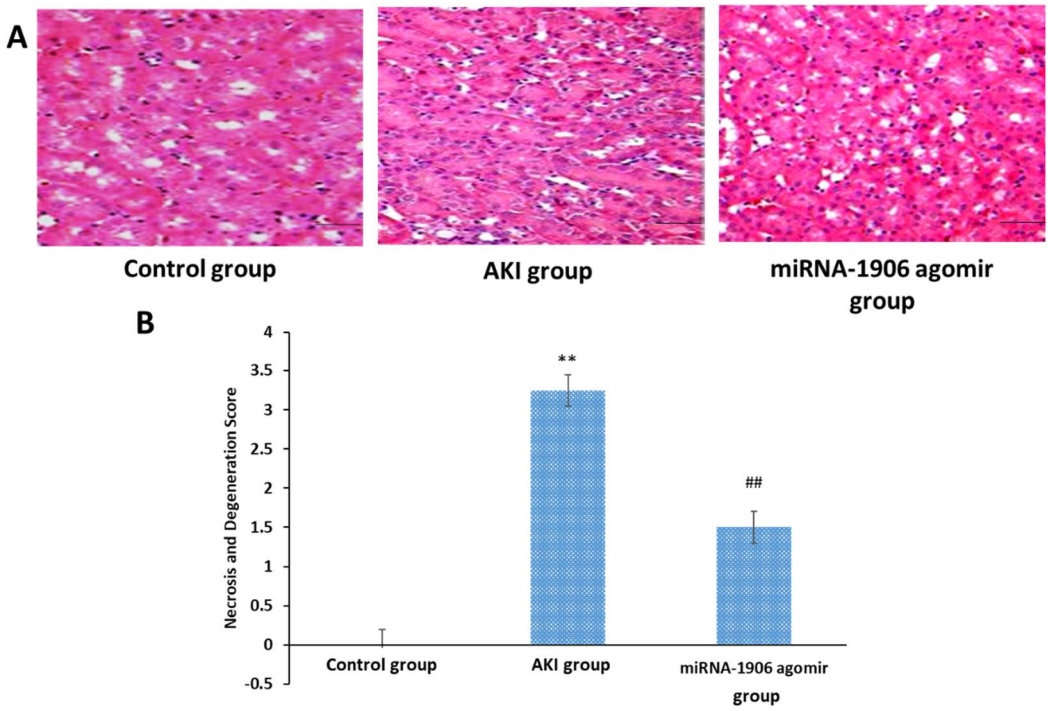

Figure 6. Effects of miRNA-1906 agomir on histopathology of renal tissues and necrosis and degeneration scores in mice with I/R-induced AKI: (A) histopathology of renal tissues shown by haemotoxylin-eosin staining (200×); B) necrosis and degeneration scores.
Values are mean ± standard error (n = 10). **$p < 0.01$ compared to control group, $^{\#\#}$ $p < 0.01$ compared to AKI group

The present study has elucidated the nephroprotective impact and molecular function of miRNA-1906 activation in I/R-induced AKI in mice. The present data show that the miRNA-1906 agomir protects against podocyte injury by attenuating alterations in the expression level of SLIT2 and ROBO1 proteins. The research reveals that the levels of creatinine, BUN and microalbumin decrease after treatment with miRNA-1906 agomir, in contrast to AKI. In mice with acute kidney damage from I/R, miRNA-1906 agomir reduces the accumulation of harmful substances and inflammation inside the renal tissue. Administration of the miRNA-1906 agomir significantly increases the concentrations of desmin, caspase-3 and TRPC-6 while concurrently decreasing nephrin levels relative to AKI. The miRNA-1906 agomir mitigates disruptions in the SLIT2/ROBO1 pathway, hence reinstating podocyte functioning.

This study is primarily a cellular, animal-based study, which may have inherent limitations in data quality and representativeness. The findings lack experimental validation through clinical studies, which would be necessary to confirm the biological relevance of the identified hub genes, their nephroprotective effect via the SLIT2/ROBO1 signalling pathway and the molecular function of miRNA-1906 stimulation on I/R-induced AKI. Future research should focus on conducting clinical trials to assess the translational potential of these findings in human subjects. Such studies would help elucidate the mechanisms at play and potentially lead to novel therapeutic strategies for managing AKI.

CONCLUSIONS

The SLIT-ROBO signalling pathway plays a significant role in several renal diseases. The present investigation has examined the nephroprotective effects of the SLIT2/ROBO1 signalling pathway and the molecular function of miRNA-1906 stimulation on I/R-induced AKI in animals. The findings have indicated that miRNA-1906 agomir decreases oxidative stress, inflammation and cellular apoptosis-promoting elements in renal tissues. It also diminishes alterations in the SLIT2/ROBO1 pathway, thus restoring podocyte functionality.

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