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# Downregulation of miR-34a ameliorates inflammatory response and apoptosis induced by renal ischemia-reperfusion by promoting Kruppel-like factor 4 expression

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**Abstract. – OBJECTIVE: To explore the influence of micro ribonucleic acid (miR)-34a on renal ischemia-reperfusion by regulating Kruppel-like factor 4 (KLF4).**

**MATERIALS AND METHODS: A total of 36 Sprague-Dawley (SD) rats weighing 180-200 g were randomly divided into sham operation group (n=12), model group (n=12), and miR-34a inhibitor group (n=12). Renal ischemia-reperfusion modeling was performed in rats of model group and miR-34a inhibitor group. Those in the sham operation group received the same procedures without ligation. 200 μL of miR-34a inhibitor was pre-injected before modeling in rats of miR-34a inhibitor group. An automatic biochemical analyzer was used to detect serum creatinine and urea nitrogen levels in each group of rats, thus reflecting renal functions. The expressions of B-cell lymphoma 2 (Bcl-2), an apoptotic protein, and KLF4, a transcription factor, were detected via Western blotting. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) assay was conducted to measure the expression levels of miR-34a and KLF4 in rat renal tissues in each group. Immunohistochemistry was employed to determine the expressions of inflammatory factors tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10). Additionally, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining was utilized to determine the apoptotic rate in rat kidney tissues in each group.**

**RESULTS: Compared with those in the sham operation group, serum creatinine level, urea nitrogen level, expressions of miR-34a, TNF-α and Bcl-2 ($p<0.05$) increased, while the levels of KLF4 and IL-10 ($p<0.05$) decreased in the model group. Apoptosis rate was also higher in the model group than the controls. In comparison with the model group, miR-34a inhibitor group had lowered serum creatinine level, urea nitrogen level, expressions of miR-34a, TNF-α and Bcl-2, and apoptotic rate ($p<0.05$), but raised levels of KLF4 and IL-10 ($p<0.05$), showing statistically significant differences.**

**CONCLUSIONS: Downregulation of miR-34a ameliorates inflammatory response and apoptosis induced by renal ischemia-reperfusion by promoting KLF4 level, thus improving renal functions in rats.**

*Key Words:*
MiR-34a, KLF4, Inflammatory, Renal ischemia-reperfusion.

## Introduction

Renal ischemia-reperfusion injury (IRI), one of the most common problems resulting in renal insufficiency and acute kidney injury (AKI), has increasing morbidity and mortality rates and a prolonged length of stay year by year[1]. Renal IRI is mainly characterized by adenosine triphosphate (ATP) depletion, deoxyribonucleic acid (DNA) damage, lack of glycogen and oxygen supplies, immune activation, vascular leakage, endothelial cell activation, and leukocyte adhesion[2,3]. Inflammatory response and apoptosis play crucial roles in the pathogenesis of IRI. During the process of ischemia, pro-inflammatory mediators (such as cytokines) produced by renal tubular epithelial cells produce rapidly response to hypoxia, and then, mediate kidney injury after ischemia-reperfusion. Salvadori et al[4] showed that inhibiting inflammatory response can reduce renal IRI and maintain renal function. According to renal ischemia-reperfusion models, caspase activation and upregulated B-cell lym-

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phoma 2 (Bcl-2) are detected in many apoptotic cells in damaged tubules[5]. Hence, inhibiting inflammatory response and reducing apoptosis are of great significance for the treatment of renal IRI. Kruppel-like factor 4 (KLF4), an important member of the Spl-like/KLF transcription factor family, participates in various cellular functions, such as differentiation, proliferation, and inflammation by mediating transcriptional activities of multiple genes. KLF4 has anti-inflammatory and antithrombotic effects[6,7], which is changed in the early stage of renal IRI and is proven to exert a protective function by inactivating inflammatory factors or regulating the expressions of other cytokines. Micro ribonucleic acids (miRNAs) can regulate gene expressions and are involved in cellular stress responses[8]. MiR-34a is located on chromosome 1p36.23, and its ectopic expression is able to induce apoptosis. It is reported that KLF4 is the possible downstream anti-apoptotic target of miR-34a in a model of renal IRI. The overexpression of miR-34a and knockdown of KLF4 are capable of overtly promoting apoptosis[9].

Therefore, this study aims to explore whether miR-34a affects inflammatory response and apoptosis after renal IRI by regulating KLF4 level and thus improves renal function in rats.

## Materials and Methods

### Animal Experiments and Grouping

A total of 36 SPF male Sprague-Dawley (SD) rats weighing 180-200 g were housed in a clean and well-ventilated animal environment at 20±2°C, with a relative humidity of 60-70% and a day/night cycle of 12/12 h. They had free accesses to water and food. After adaptive feeding, 36 rats were divided into sham operation group, model group, and miR-34a inhibitor group using a random number table, with 12 rats in each group. All protocols on animals conformed to the laboratory animal guidelines. This study was approved by the Animal Ethics Committee of Shanxi Medical University Animal Center.

### Modeling

Renal IRI model in rats was established as previously described[10]. The rats were deprived of food for 12 h before surgery, with free access to water. Then, they were anesthetized with 3% pentobarbital sodium *via* intraperitoneal injection at 80 mg/kg. Next, an incision was made on the skin 0.5 cm away from the spine and 0.5 cm below the inferior border of the ribs. The muscles were separated to expose the kidneys. Bilateral renal pedicles were dissociated, and the renal artery was quickly clamped using a bulldog clamp for 45 min of ischemia. After that, the bulldog clamp was removed to restore the blood flow to create a reperfusion model. Thereafter, the incision was sutured layer by layer. After the rats sobered up, they were placed and fed in the feeding room. For the rats in miR-34a inhibitor group, 200 μL of miR-34a inhibitor was injected *via* the tail vein before the modeling of renal IRI. For those in the sham operation group, the kidneys were exposed, but the blood vessels were not ligated.

### Main Instruments and Equipment

MiR-34a inhibitor (GenePharma, Shanghai, China); tumor necrosis factor-alpha (TNF-α); interleukin-10 (IL-10), KLF4, and Bcl-2 primary antibodies (CST, Danvers, MA, USA); Leica DM4000B LED microscope (Leica, Wetzlar, Germany); terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay kit and bicinchoninic acid (BCA) protein quantification kit (Beyotime, Shanghai, China); quantitative Polymerase Chain Reaction (qRT-PCR) kit (Vazyme, Nanjing, China); immunohistochemistry kit (Maxim, Fuzhou, China); fluorescence qPCR instrument (ABI 7500, Applied Biosystems, Foster City, CA, USA), automatic biochemical analyzer (Hitachi 7080, Tokyo, Japan); Image-lab image analysis system and Motic Med 6.0 pathological graphic analysis system were prepared.

### Examination of Renal Function

Rat blood (0.5 mL) was collected *via* the abdominal aorta to separate the serum. Then, serum creatinine and blood urea nitrogen levels were detected using the automatic biochemical analyzer to evaluate renal functions.

### Detection of MiR-34a and KLF4 Expressions Through qRT-PCR Assay

Total RNAs were extracted from rat kidney tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) and lysed at room temperature for 15 min. Thereafter, chloroform was added, followed by vigorous shaking, maintained at room temperature and centrifugation. Next, the RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs), according to the instructions of an RT kit. Primer sequenc-

es: miR-34a: forward primer: 5'-CUGGCCCU-CUC-UGCCCUUCCGU-3', and reverse primer: 5'-GGGGGGCA-GGAGGGGCUCA-3'; KLF4: forward primer: 5'-CATCAGTGTTAGCAAAG-GAAGC-3', and reverse primer: 5'-GTGGCAT-GAGCTCTTGATAATG-3'; and the internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward primer: 5'-CCCAT-CACCATCTTCCAGGA-3', and reverse primer: 5'-CATCGCCCCACTTGATTTTG-3'. Amplification was carried out using the PCR instrument in a 20 μL reaction system. The reaction conditions were: 95°C for 10 min, 95°C for 15 s, and 60°C for 15 s, for a total of 40 cycles. The primers were designed and synthesized by Fuzhou Jingrui Biotechnology Co., Ltd. (Fuzhou, China). With GAPDH as the internal reference, the relative expression of the target genes was calculated by $2^{-\Delta\Delta Ct}$.

### Protein Expression Determined Through Western Blotting

Rat kidney tissues were collected, lysed in tissue lysis buffer, and homogenized using a tissue grinder. Total protein was then extracted for measuring protein concentration. Protein samples were incubated with primary antibodies at 4°C overnight and horse radish peroxidase (HRP)-labeled secondary antibody at 37°C for 1 h. Thereafter, the membrane was washed, and enhanced chemiluminescence (ECL; Millipore, Billerica, MA, USA) liquid was added, followed by band exposure using a visualizer. GAPDH was the internal reference. Image J software (Rawak Software, Inc., Hamburg, Germany) was employed to analyze the gray value of the target protein and normalized to that of GAPDH. Three effective bands were taken from each group.

### Determination of Expression of Inflammatory Factors by Immunohistochemistry

After reperfusion, the kidneys were removed from the rats in each group to prepare into coronal sections (5 μm in thickness). Then, the sections were deparaffinized with xylene, dehydrated with graded ethanol, and incubated in citric acid for antigen retrieval. Sections were added dropwise with 3% $H_2O_2$ to inactivate endogenous peroxidase at room temperature for 10 min, washed with phosphate-buffered saline (PBS) for three times (5 min/time), and blocked with 5% goat serum at room temperature for 10 min. After washing with PBS for three times (5 min/time), the sections reacted with TNF-α and IL-10 primary antibodies in a wet box at 4°C overnight. After that, they were rewarmed in an oven at 37°C for 30 min, washed with PBS for three times (5 min/time), and incubated with the secondary antibody at room temperature 10 min. Following PBS washing for three times (5 min/time), streptavidin-peroxidase was added. 10 min later, the sections were rinsed with PBS for three times (5 min/time), and counterstained with diaminobenzidine (DAB) solution (Solarbio, Beijing, China) and hematoxylin. Finally, the sections were washed, sealed, and analyzed using the Motic Med 6.0 pathological graphic analysis system.

### Detection of Apoptosis via TUNEL

TUNEL-4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining assay was performed in accordance with the instructions of the TUNEL kit. The paraffin-embedded sections of kidney tissues were deparaffinized with xylene, dehydrated with graded ethanol, and incubated in citric acid for antigen retrieval. After 8-min permeabilization, 500 μL of TUNEL reaction mixture was applied. Next, the sections were stained with DAPI, mounted with anti-fluorescence quenching medium and observed using a fluorescence microscope. Apoptotic cells were counted for calculating apoptosis rate. Apoptosis rate = number of positive cells/total number of cells ×100%.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 24.0 software (IBM Corp., Armonk, NY, USA) was used for statistical processing of data. Data in each group were expressed as mean ± standard deviation (χ±s). The differences between the groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test, followed by post-hoc test (Least Significant Difference). $p<0.05$ indicated that the difference was statistically significant.

## Results

### Comparisons of Renal Function Indicators

The serum creatinine and urea nitrogen levels were higher in the model group compared with those in the sham operation group ($p<0.05$), and

**Table I.** Comparison of renal function among each group of rats ($\bar{x} \pm s$).

| Group | No. | Serum creatinine (μmol/L) | Urea nitrogen (mmol/L) |
|---|---|---|---|
| Sham operation group | 6 | 6.78 ± 0.65 | 57.49 ± 7.65 |
| Model group | 6 | 47.23 ± 6.77* | 216.52 ± 29.75* |
| MiR-34a inhibitor group | 6 | 25.84 ± 4.32# | 154.29 ± 18.64* |

**Note:** *$p < 0.05$ *vs*. sham operation group, #$p < 0.05$ *vs*. model group.

they were lower in miR-34a inhibitor group than those in the model group ($p<0.05$), showing statistically significant differences (Table I).

### Expression Levels of MiR-34a and KLF4 in Rats

Compared with those in the sham operation group, higher level of miR-34a and lower level of KLF4 were found in the kidney tissues in the model group ($p<0.05$). In comparison with the model group, miR-34a inhibitor group exhibited reduced level of miR-34a ($p<0.05$) and upregulated KLF4 ($p<0.05$). The differences were statistically significant (Figure 1).

### Expressions of KLF4 and Bcl-2 Proteins in Rats

Compared with those in the sham operation group, the level of the apoptotic protein Bcl-2 rose ($p<0.05$), and that of KLF4 protein was reduced ($p<0.05$) in the model group. In comparison with model group, miR-34a inhibitor group had decreased level of Bcl-2 ($p<0.05$) and raised level of KLF4 ($p<0.05$). The differences were of statistical significance (Figures 2 and 3).

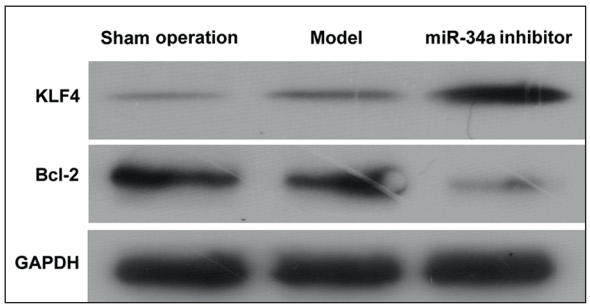


**Figure 2.** Comparisons of protein levels of KLF4 and Bcl-2 in rat renal tissues in each group.

### Expressions of Inflammatory Factors TNF-α and IL-10

The results of immunocytochemistry showed that the expression level of the proinflammatory

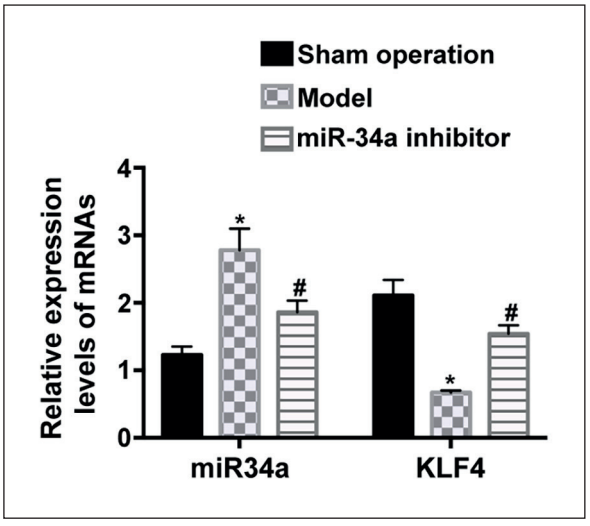


**Figure 1.** Relative expression levels of miR34a and KLF4 in each group of rats. Note: *$p<0.05$ *vs.* sham operation group, #$p<0.05$ *vs.* model group.

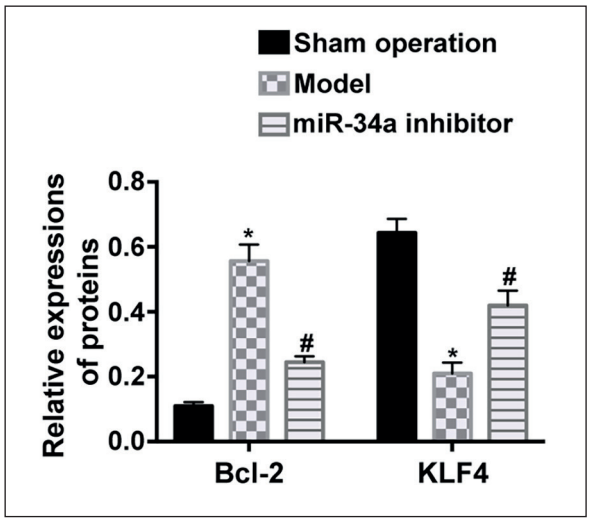


**Figure 3.** Comparisons of positive expressions of KLF4 and Bcl-2 in rat renal tissues in each group. Note: *$p<0.05$ *vs.* sham operation group, #$p<0.05$ *vs.* model group.

**Table II.** Expression of inflammatory factors in rats in each group ($\bar{x} \pm s$).

| Group | No. | TNF-α | IL-10 |
|---|---|---|---|
| Sham operation group | 6 | 0.021 ± 0.0.004 | 0.018 ± 0.003 |
| Model group | 6 | 0.431 ± 0.032* | 0.357 ± 0.025* |
| MiR-34a inhibitor group | 6 | 0.027 ± 0.016# | 0.579 ± 0.048# |

**Note:** *$p < 0.05$ *vs*. sham operation group, #$p < 0.05$ *vs*. model group.

factor TNF-α was higher in the model group than that in the sham operation group ($p<0.05$), while it was lower in miR-34a inhibitor group than that in the model group ($p<0.05$). Expression level of the anti-inflammatory factor IL-10 was lower in the model group than that in the sham operation group ($p<0.05$), while it was higher in miR-34a inhibitor group than that in the model group ($p<0.05$), displaying statistically significant differences (Table II and Figure 4).

### Apoptosis in Rat Renal Tissues

According to TUNEL staining results, the apoptosis rate was raised in the model group and miR-34a inhibitor group compared with that in the sham operation group ($p<0.05$), while it was lower in miR-34a inhibitor group than that in the model group ($p<0.05$), and the difference was statistically significant (Figures 5 and 6).

## Discussion

IRI, an injury due to sudden temporary interruption or sharp decrease of blood flow in a specific organ, is usually correlated with intense inflammation and oxidative stress. It eventually damages organ functions. AKI caused by renal IRI is featured by high morbidity and mortality rates in various injuries. The pathological progress of renal IRI is complicated, involving activation of neutrophils, and release of reactive oxygen species (ROS) and inflammatory mediators. Inflammation is a common pathological feature of renal IRI that triggers the inflammatory cascade. Hence, inhibiting inflammatory response can effectively protect kidney tissues[11,12]. Chemokines are the major mediators regulating leukocyte infiltration and inflammation by mediating inflammatory cytokines and adhesion molecules[13].

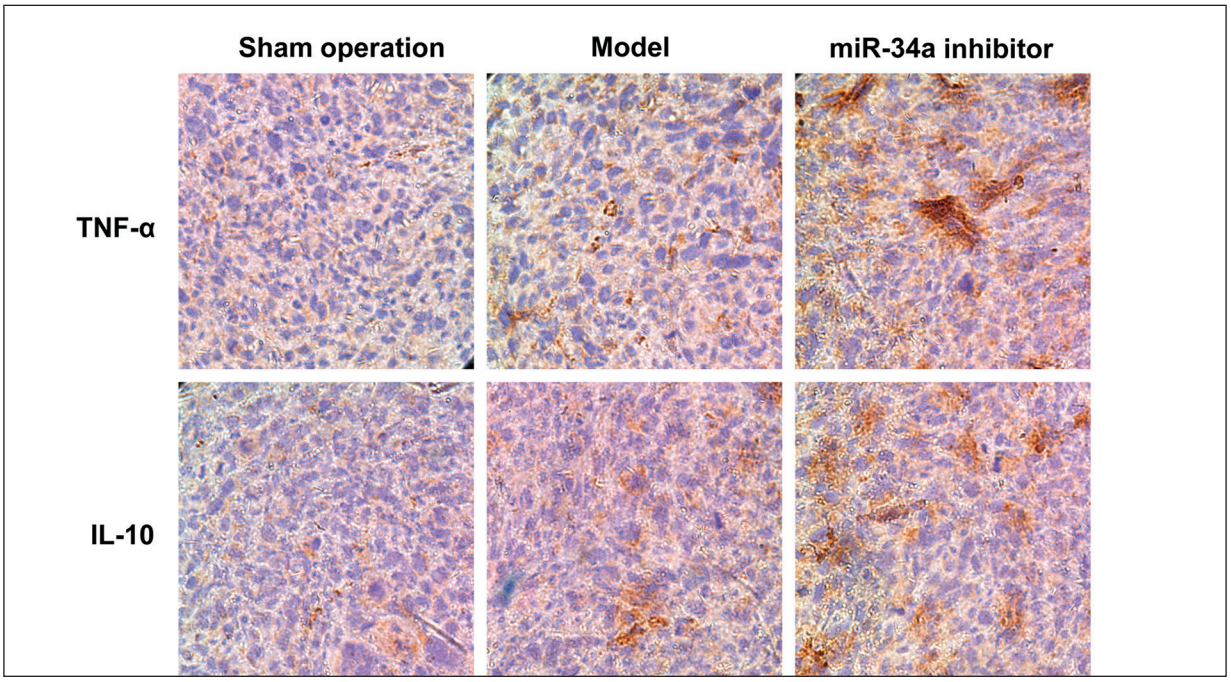


**Figure 4.** Expression of inflammatory factors in rats in each group (magnification: 200×).

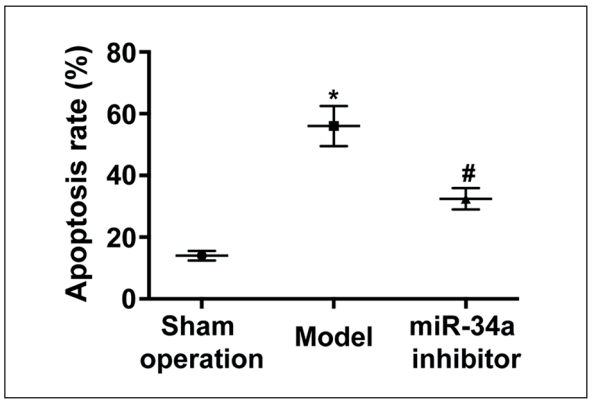


**Figure 5.** Comparison of apoptosis rate in rat renal tissues in each group. Note: $^{*}p<0.05$ *vs.* sham operation group, $^{\#}p<0.05$ *vs.* model group.

During the process of ischemia, hypoxia-induced suppression in oxidative phosphorylation of mitochondria, damaged ATP synthesis and inactivated cellular energy-dependent processes to trigger cell death[14,15].

KLFs are transcription factors. As a member of the KLFs, KLF4 in endothelial cells protects kidneys in case of AKI[16]. Conditional knockout of KLF4 in mice regulates the expressions of cell adhesion molecules and infiltration of neutrophils and lymphocytes to promote ischemic AKI[7]. Specific KLF proteins can serve as therapeutic targets for kidney injury. Besides, the protective effects of statins relies on upregulation of KLF4 expression.

MiRNAs play a crucial role in post-transcriptional regulation of gene expressions by inhibiting the translation of target mRNAs. Since they are small-sized and stably expressed, they can also be inactivated with RNA antagonists or used as therapeutic agents. MiR-21 is upregulated after renal ischemia preconditioning, and the knockdown of miR-21 impairs renal function[17]. Therefore, miR-21 contributes to the protective effect of renal ischemia preconditioning against renal IRI. MiR-21 is expressed in renal tubular epithelial cells and leads to increased cell death. However, the overexpression of miR-21 does not result in pronounced cell survival after oxidative stress. Besides, the knockdown of miR-21 is effective only in conjunction with renal ischemia preconditioning. Therefore, miR-21 participates in the protection against renal IRI, but it has a more complex mechanism[18]. However, Akbari et al[19] have demonstrated that the induction of local ischemia and reperfusion increases the concentration of serum miR-34a, and such an increase may be induced by ROS. In the renal ischemia-reperfusion model, IRI causes autophagy inactivation and downregulation of miR-34a in renal tissues. *In vitro* data have shown that upregulating miR-34a leads to increased apoptosis, while repressing miR-34a facilitates self-reactivity of endothelial cells, thus exerting the protective mechanism[20]. In the model of sepsis-associated renal injury, the mRNA expression of miR-34a notably increases in blood and kidney tissues, while the expression of KLF4 decreases. Moreover, miR-34a expression in blood is positively correlated with serum creatinine and urea nitrogen level. KLF4 may be the target gene of miR-34a[21].

In this study, it was found that in rat models of renal IRI, miR-34a expression was elevated, while KLF4 expression was downregulated, and they were responsible for inflammatory response and apoptosis. The knockdown of miR-34a upregulated KLF4, and further protected the inflammatory response and apoptosis in rats with renal IRI.

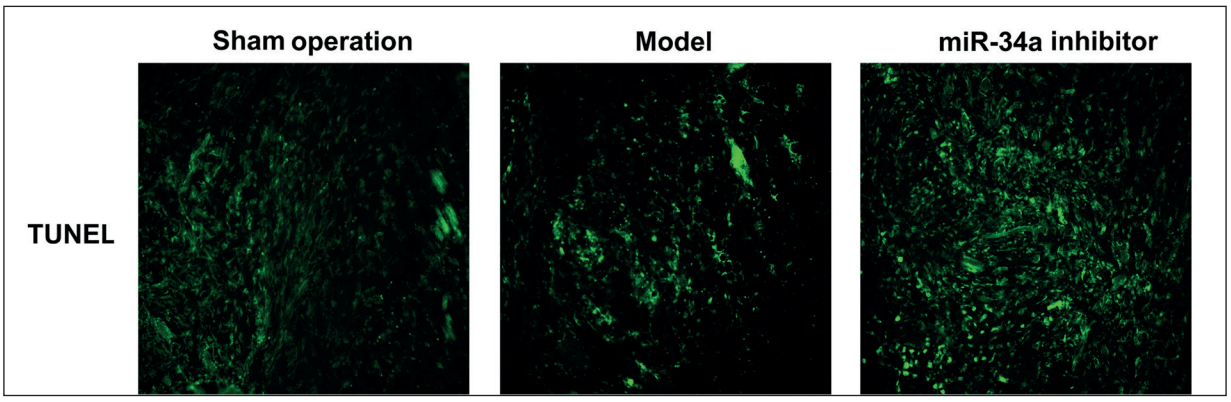


**Figure 6.** Apoptosis in rats in each group (magnification: 400×).

## Conclusions

In summary, miR-34a modulates KLF4 to participate in the inflammation and apoptosis induced by renal ischemia-reperfusion, and it may become a new target for the treatment of renal ischemia-reperfusion.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

### Funding Acknowledgements

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