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Research Article

Antagonists Targeting miR-155 and miR-125a Relieved Ultrasonographic Contrast Medium SonoVue Induced Kidney Injury

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Abstract: Ultrasonographic contrast medium (UCM) enhances radiological procedures. However, it raises a serious problem that UCM induces acute kidney injury. However, the mechanism of kidney injury induced by ultrasonographic contrast medium remains elusive. This work aims to explore kidney injury from a post-transcription view. UCM was identified to induce kidney injury by activating NF-kappa B signaling. The expression of NF-kappa B was examined in mouse kidneys by western blot. The level of NF-kappa B was upregulated in the kidney with the treatment of UCM. Hereof, the expression levels of miR-155 and miR-125a, upstream of NF-kappa B, increased significantly in the UCM group, which RT-PCR detects. As a positive control, LPS was used to induce acute kidney injury. The expressions of NF-kappa B, miR-155, and miR-125a all increased in the LPS group. Further, to verify the necessity of NF-kappa B signaling in the process of UCM-induced kidney injury, the mice were treated with the antagonists of miR-155 and miR-125a, and our results showed that the antagonists of miR-155 and miR-125a repressed NF-kappa B signaling by western blot analysis. In conclusion, our results demonstrate that miR-155 and miR-125a antagonists mediate NF-kappa B signaling to relieve kidney injury, which UCM induced. The antagonists would be further tested to alleviate kidney injury clinically.

Key Words: kidney injury, ultrasonographic contrast medium, miR-155 and miR-125a antagonists, NF-kappa B signaling.

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1. INTRODUCTION

With many contrast medium-enhanced radiological procedures widely being performed in clinic[s 1, 2], it is immense to pay more attention to understanding the etiology of contrast medium-induced kidney injury. It's invaluable for formulating effective prophylactic and therapeutic regimens to reduce the incidence and associated morbidity and mortality of kidney injury. Many previous studies indicate that contrast medium-induces kidney injury [3,4]. To investigate the mechanism of kidney injury induced by contrast medium, a series of animal models were established [1]. Kidney injury occurred as we injected SonoVue [5], which was used as an ultrasonographic contrast medium by the tail vein. Increased serum creatinine and abnormal glomeruli are found in individuals with kidney injury [6]. However, the changes in molecular signaling underlying this abnormal physiological index, which is crucial for kidney injury, are still elusive. A previous study revealed that lipopolysaccharide (LPS) induces acute kidney injury. Serum creatinine and the percentage of abnormal glomeruli increased via infusing LPS [7]. More interestingly, they indicated that NF-kappa B signaling was activated by LPS treatment. NF-kappa B signaling participates in kidney injury. The expression of NF-kappa B in the UCM-treated group was significantly higher than saline control by Western blot. We suggested that NF-kappa B signaling was activated by UCM treatment. However, NF-kappa B signaling functions in each cell of the whole body. It's not specific for targets as treatment regimens. As previously indicated, miRNAs (19-22 nucleotides), which are small non-coding RNAs, are often found to be differentially regulated in different types of diseases [8-12]. Current progression also revealed targeting miRNAs might be a therapeutic strategy to suppress disease progression. MiR-125a [13] and miR-155 [9] are upstream of NF-kappa B. Overexpression of miR-125a and miR-155 activated NF-kappa B signaling [9]. In this study, we have analyzed the expression of miRNAs, miR-125a and miR-155, in the injured kidney. We found that the expression of miR-125a and miR-155 increased significantly in UCM-treated samples as detected by qRT-PCR, suggesting the increased expression of miR-125a and miR-155 leads to activation of NF-kappa B signaling. To further touch the effective regimens, we used the antagonists to inhibit miR-125a and miR155 to silence NF-kappa B signaling. The antagonists may be potential therapeutics to cure kidney injury clinically. Knockdown of miR-125a in kidney tubular cell lines resulted in the downregulation of NF-kappa B. As previously reported, miR-155 antagonist decreased NF-kappa B signaling [9]. NF-kappa B signaling was attenuated, and the serum creatinine level was reduced in antagonist-treated mice with kidney injury. It suggested that miR-155 and miR-125a antagonists mediate NF-kappa B signaling to relieve UCM-induced kidney injury. Our results demonstrate that miR-125a and miR-155 participate in kidney injury via mediating NF-kappa B signaling. Therefore, it's worth studying further to utilize the antagonists to relieve kidney injury in the clinic.

2. MATERIALS & METHODS

2.1. Animal

Male C57BL/6 mice, 8 weeks of age, were housed in cages at 20-25 ℃ with water and food available *ad libitum*. All animal experiments were conducted by our Institutional Animal Ethics Committee and Animal Care Guidelines for the Care, and this experiment was approved by our Animal Ethics Committee and Animal Care with the number 2021-06.

2.2. Serum creatinine and abnormal glomeruli analysis

Serum creatinine (enzymatic method) concentration was measured with an autoanalyzer (Accute TBA-40FR, Toshiba Medical Systems, Tochigi, Japan)[14]. The kidney from mice treated with ultrasonographic contrast medium (SonoVue [5], 200 ug in 200 ul), 20 mg/kg LPS [7], or saline-infused via tail veins were histopathologically examined. Mice were treated with 0.12 nmol miR-155/125a antagonist (2 ul). The histological evaluation was conducted in a blinded manner. Mice were treated with 0.12 nmol miR-155/125a antagonist (2ul) via tail veins to measure creatinine and analyze abnormal glomeruli. The antagonists were purchased from GenePharma, Co.Ltd. (Shanghai, China).

2.3. RNA isolation and cDNA preparation

Total RNA was extracted from kidney samples (~100-180 mg) using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. RNA concentration was measured using Nanodrop 1000 (Thermo Scientific). 300ng total RNA was reverse transcribed using an oligo-d(T) primer and RNase H-MMLV reverse transcriptase according to the protocol of the manufacturer (Promega).

2.4. qRT-PCR

The cDNA was diluted to give a total volume of 200 ul, and 5 ul of this dilution was used for each PCR reaction. The quality of the cDNA was confirmed by the amplification of glyceraldehyde-3-phosphatedehydrogenase (GAPDH, cytosolic protein), and only samples with consistent and strong amplification were included in the final analyses. The PCR primer sequences used for quantitative real-time PCR were: NF-kappa B, forward primer: GAGACATGGAGAGTTGCTAC, reverse primer: GCCTTCACAGCCATATCGAA; miR-155, forward primer: CAGCCTACACGGTGGGAGC, reverse primer: CTGCTCTGAGTCATTGTGCTGG; miR-125a, forward primer: GTCCTCACAACGATTCCACAAG, reverse primer: GTGCAGGGTCCGAGGT; GAPDH, forward primer: TCATGACCACAGTGGATGCC, reverse primer: GGAGTTGCTGTTGAAGTCGC.

2.5. Western blotting

Western blotting was performed as previously described [7]. Protein was extracted from frozen kidneys using radio-immunoprecipitation-assay buffer (RIPA) and sonication. An anti-GAPDH antibody was used to control for equal protein loading. Membranes were washed with 0.1% TBS-T and incubated with a secondary antibody (ECL Rabbit or Mouse IgG, HRP Linked whole antibody) for 1h at room temperature. Membranes were washed with TBS-T, and chemiluminescent detection was performed using ECL western blot substrate or Super Signal West Pico substrate (Thermo Scientific).

2.6. Statistical analysis

Data were expressed as mean $\pm$ SEM. Student's t-test and one-way ANOVA evaluated statistical differences between more than two test groups. Data were considered statistically significant when $P<0.05$. Data were considered statistically highly significant when $P<0.01$. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA).

3. RESULTS

3.1. Ultrasonographic contrast medium-induced acute kidney injury

The mice were treated with ultrasonographic contrast medium SonoVue to increase serum creatinine (Fig.1). As the positive control, LPS also increased serum creatinine levels. The enzymatic method was used to analyze serum creatinine in saline, SonoVue, and LPS groups. The serum creatinine level increased in both SonoVue and LPS groups (Fig.1A). Simultaneously, the abnormal glomeruli number in the kidney with SonoVue and LPS treatment was measured. The percentage of abnormal glomeruli increased in SonoVue and LPS groups, compared to that of saline (Fig.1B).

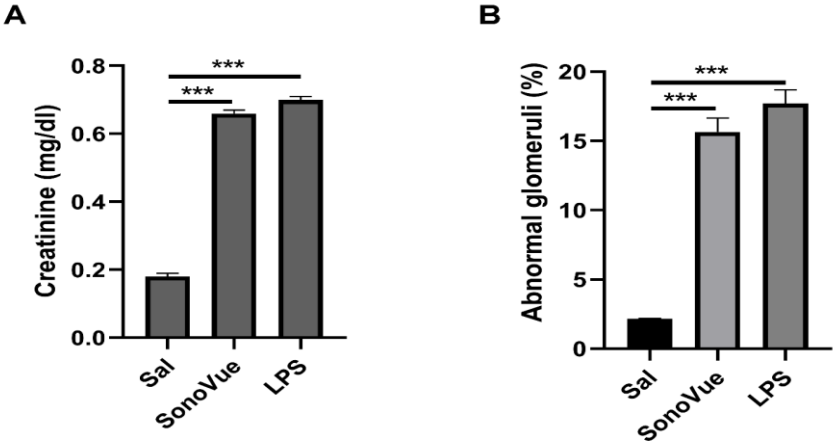

Fig.1 Ultrasonographic contrast medium increased serum creatinine level and percentage of abnormal glomeruli.

(A) Serum creatinine level in ultrasonographic contrast medium treatment (n=6) and in corresponding normal control with saline (n=6). As the positive control, 20mg/kg LPS induced kidney injury (n=6). The levels of serum creatinine in the Ultrasonographic contrast medium group (P=0.0003) and LPS group (P=0.0002) were significantly higher than those in the controls. (B) The percentage of abnormal glomeruli was analyzed from the kidney with SonoVue treated. The analysis of the percentage of abnormal glomeruli (SonoVue, P=0.014, LPS, P=0.024) significantly up-regulated compared to normal saline control.

3.2. NF-kappa B signaling activity increased via the treatment of UCM.

As previously revealed, LPS mediates NF-kappa B signaling activation and results in kidney injury. Next, NF-kappa B signaling activity was investigated after the treatment of UCM (Fig.2). The mRNA expression level of NF-kappa B increased in the kidney of SonoVue and LPS groups (Fig.2A). Then, the protein level of NF-kappa B which were treated with SonoVue and LPS was measured. It was identified that the expression of NF-kappa B was increased in SonoVue and LPS groups (Fig.2B, C). The UCM group can significantly increase p65 phosphorylation levels compared to controls (Fig. 2D), and the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) was significantly increased under UCM treatment (Fig. 2E). This result suggested that UCM induced kidney, activated NF-kappa B signaling to result in kidney injury as LPS treatment. However, what is the abnormal upstream of NF-kappa B signaling leads to kidney injury?

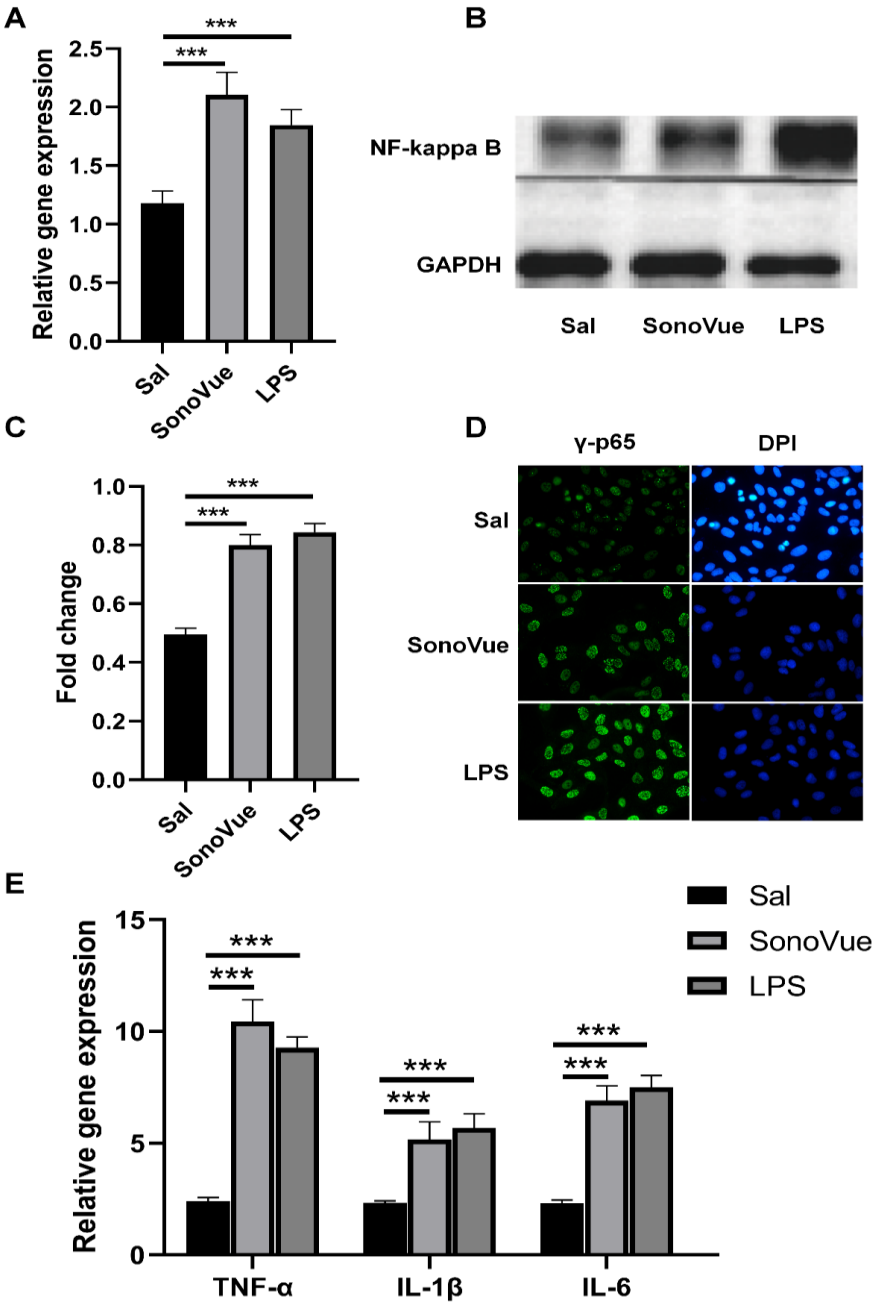

Fig.2 The expression of NF-kappa B was up-regulated by ultrasonographic contrast medium.

(A) Expression of mRNA of NF-kappa B in three groups (n=6). Expression levels were standardized for GAPDH expression. Expression of NF-kappa B (SonoVue, P=0.032, LPS, P=0.013) was significantly increased in SonoVue and LPS than in saline.

(B) Western blot analysis analyzed 30 ug of protein lysates from the kidney for NF-kappa B protein expression. The protein expression level of NF-kappa B (SonoVue, P=0.0095, LPS, P=0.024, n=6) was significantly increased in SonoVue and LPS than in saline.

(C) The statistics for NF-kappa B protein expression by Western blot analysis.

(D) Immunofluorescence for detection of p-p65. Scale bar = 50 μm (400×). Data are presented as mean ± SEM of three independent experiments. *p < 0.05.

(E) Expression of pro-inflammatory cytokines mRNA (IL-1β, IL-6, and TNF-α) in three groups (n=6). Expression levels were standardized for GAPDH expression. Expression of pro-inflammatory cytokines (SonoVue, P=0.032, LPS, P=0.013) was significantly increased in SonoVue and LPS than in saline.

3.3. MiR-125a and miR-155 increased during kidney injury.

MiR-125a [13] and miR-155 [9] are upstream of NF-kappa B. Therefore, the expression of miR-125a and miR-155 in the kidney with SonoVue treatment was measured. (Fig. 3). It was found that the expression of miR-125a (Fig. 3A) and miR-155 (Fig. 3B) was significantly increased in samples with UCM as detected by qRT-PCR, suggesting that the increased expression of miR-125a and miR-155 lead to activation of NF-kappa B signaling. The induction of pro-inflammatory cytokines could be enhanced by transfection of miR-125a-5p mimics or miR-155 mimics (Fig. 3C, D). Alternatively, it can be attenuated by transfection with miR-125a or miR-155 antagonists (Fig. 3E, F).

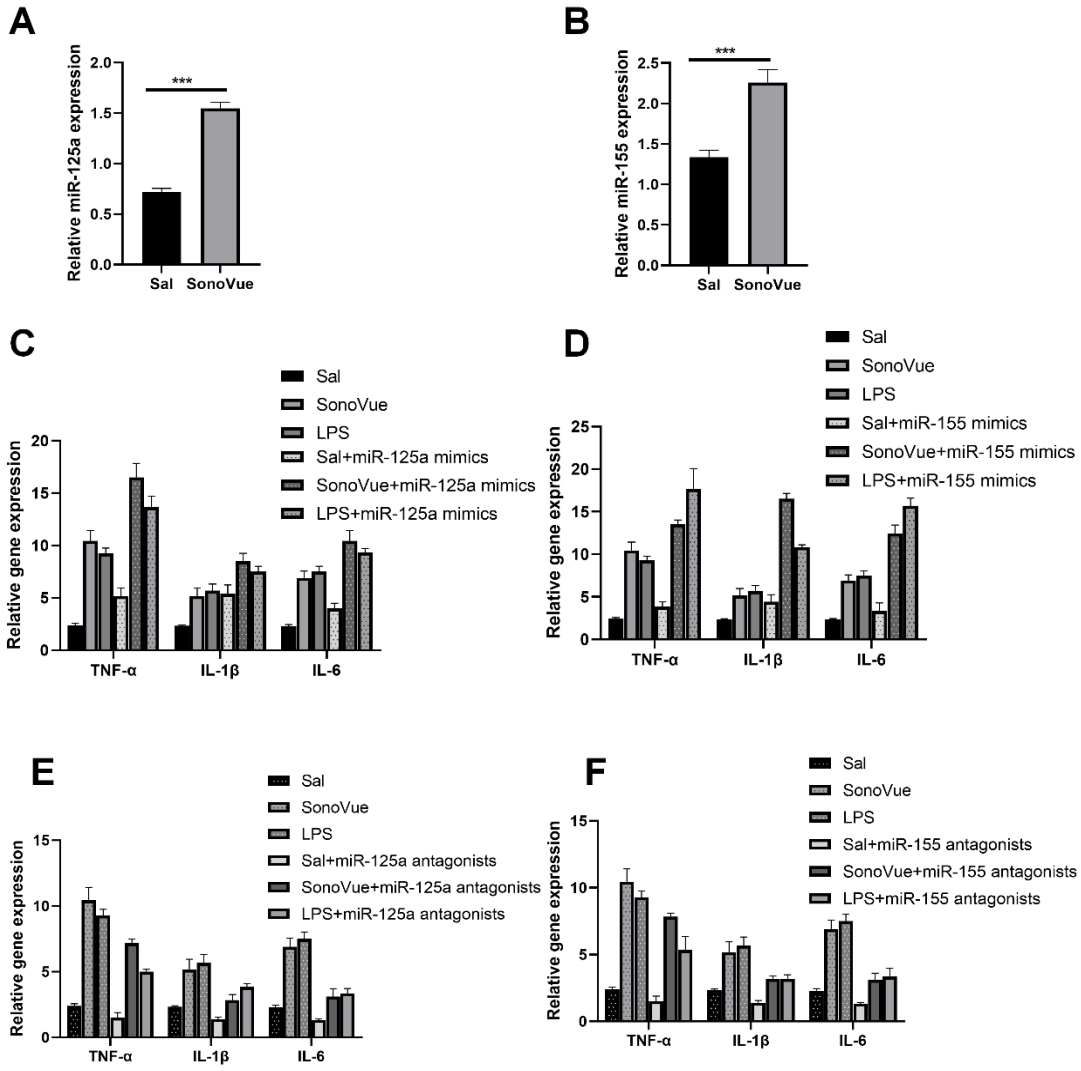

Fig.3 miR-125a and miR-155 increased by ultrasonographic contrast medium.

(A) Expression of mRNA of miR-125a in ultrasonographic contrast medium treatment(n=6). Expression levels were standardized with GAPDH expression. Expression of miR-125a was significantly increased in SonoVue than in saline (P=0.0002).

(B) Expression of mRNA of miR-125a in a panel of ultrasonographic contrast medium treatment(n=6). Expression levels were standardized for GAPDH expression. Expression of miR-125a was significantly increased in SonoVue than in saline (P=0.03).

(C) Expression of pro-inflammatory cytokines mRNA (IL-1β, IL-6, and TNF-α) in six groups (Sal, SonoVue, LPS, Sal+ miR-125a mimics, SonoVue + miR-125a mimics, and LPS + miR-125a mimics) (n=6). Expression levels were standardized for GAPDH expression.

(D) Expression of pro-inflammatory cytokines mRNA (IL-1β, IL-6, and TNF-α) in six groups (Sal, SonoVue, LPS, Sal+ miR-155 mimics, SonoVue + miR- miR-155 mimics, and LPS + miR- miR-155 mimics) (n=6). Expression levels were standardized for GAPDH expression.

(E) Expression of pro-inflammatory cytokines mRNA (IL-1β, IL-6, and TNF-α) in six groups (Sal, SonoVue, LPS, Sal+ miR-125a antagonists, SonoVue + miR-125a antagonists, and LPS + miR-125a antagonists) (n=6). Expression levels were standardized for GAPDH expression.

(F) Expression of pro-inflammatory cytokines mRNA (IL-1β, IL-6, and TNF-α) in six groups (Sal, SonoVue, LPS, Sal+ miR-miR-155 antagonists, SonoVue + miR- miR-155 antagonists, and LPS + miR- miR-155 antagonists) (n=6). Expression levels were standardized for GAPDH expression.

3.4. Antagonists of miR-125a and miR-155 mediate NF-kappa B signaling to reverse kidney injury.

To verify that, miR-125a and miR-155 participate in kidney injury, the antagonists of miR-125a and miR-155 were used to treat mice with UCM-induced kidney injury. The expression levels of NF-kappa B decreased in kidneys treated with anti-miR-125a (Fig.4A). The expression levels of NF-kappa B also decreased in kidneys with antagonists of miR-155. (Fig. 4B) More interestingly, the antagonist of miR-155 could reverse the level of serum creatinine, which was increased with UCM treatment. (Fig. 4C) The level of serum creatinine in anti-miR-155 group has no significance, compared with saline (Fig.4C). Furthermore, immunofluorescence analysis showed that miR-125a and miR-155 antagonists significantly reduced UCM-stimulated NF-κB p65 phosphorylation. (Fig.4D) Together, these data indicated that miR-155 and miR-125a antagonists mediated NF-kappa B signaling to relieve kidney injury induced by UCM. The antagonists might be further tested to alleviate kidney injury in the clinic.

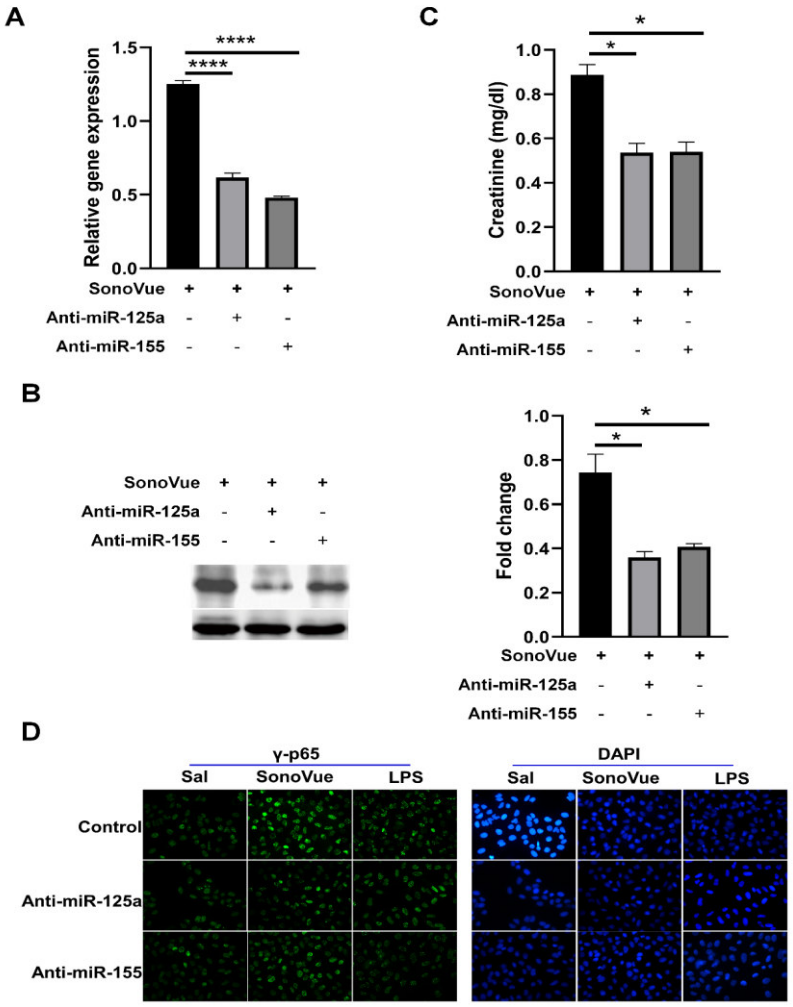

Fig.4 Antagonist of miR-125a and miR-155 reduced NF-kappa B to reverse kidney injury.

(A) Expression of mRNA of NF-kappa B in three groups (n=6). Expression levels were standardized for GAPDH expression. Expression of NF-kappa B (anti-miR125a, P<0.0001, anti-miR155, P<0.0001) decreased significantly than of control.

(B) Western blot analysis analyzed 30 ug of protein lysates from the kidney for NF-kappa B protein expression. The expression level of NF-kappa B (anti-miR125a, P=0.018, anti-miR155, P=0.033, n=4) was significantly reduced in the antagonists treated group than in control.

(C) The levels of serum creatinine in a panel of anti-miR125a (n=6), anti-miR155 (n=6), and in corresponding normal control (n=6). The serum creatinine levels in the anti-miR125a group (P=0.028) and anti-miR155 group (P=0.028) decreased significantly than in the control group.

(D) Immunofluorescence for detection of p-p65. Scale bar = 50 μm (400×). Data are presented as mean ± SEM of three independent experiments. *p < 0.05.

4. DISCUSSION

Our study provides strong evidence that UCM induced kidney injury by activating NF-kappa B signaling. The upstream of NF-kappa B signaling, miR-125a and miR-155, was also activated by UCM. Antagonists of miR-125a and miR-155 could reverse kidney injury by suppressing the NF-kappa B signaling pathway. This is the first time to demonstrate that antagonists of miR-125a and miR-155 could relieve kidney injury induced by UCM. Many studies demonstrated contrast medium-mediated kidney injury [1-4, 6, 15-18]. Therefore, UCM was used to induce kidney injury. Serum creatinine was considered a powerful index to justify kidney injury [4, 19]. In our study, it is found that the level of serum creatinine is dramatically increased by UCM treatment. This finding strongly argued against previous reports that UCM didn't induce acute kidney injury [17]. Kidney injury is dosage-dependent on UCM. This data should have been shown. Another critical index of kidney injury is the percentage of abnormal glomeruli [20]. Similar findings have previously been reported that UCM increased the percentage of abnormal glomeruli [16]. These findings indicate that a high dosage of UCM leads to kidney injury. The mechanism of kidney injury is reported by NF-kappa B signaling activation. In this case, the expression of NF-kappa B was measured. The expression of NF-kappa B increased significantly by UCM treatment. As the positive control, LPS also increased the expression of NF-kappa B. It's consistent with previous studies [5]. Current progression also revealed targeting miRNAs would be a therapeutic strategy to suppress diseases. miRNAs function by regulating the expression of target genes by either inducing mRNA degradation or inhibiting mRNA translation [11, 12]. MiR-125a [13] and miR-155 [9] are upstream of NF-kappa B. Our analyses showed that UCM upregulated miR-125a and miR-155 targeting NF-kappa B. Given the function of miR-221 targeting NF-kappa B [9, 13, 21], miR-125a/155 were supposed to activate the NF-kappa B signaling pathway. Further, the antagonist of miR-125a/155 could reverse kidney injury. The antagonists were used to treat UCM-treated mice. First of all, NF-kappa B signaling was suppressed. Western blot analyses of NF-kappa B in the antagonists' group showed significantly lower expression than that of UCM treated group. The

expression level of mRNA of NF-kappa B was also reduced by antagonist treatment. It is suggested that antagonists mediated NF-kappa B by suppressing the expression. These phenomena were consistent with previous studies. Further, antagonists of miR-125a/155 reversed the increased level of serum creatinine and decreased the percentage of abnormal glomeruli by UCM. This result suggests that the antagonist of miR-125a/155 may be the potential regimen for kidney injury. However, the inflammation regulation pathways form a large network[22], and the NF-kappa B pathway in UCM-induced Kidney injury may be regulated by other genes, which made this research very preliminary.

5. CONCLUSION

Our results demonstrate that miR-125a and miR-155 participate in kidney injury via mediating NF-kappa B signaling. And antagonists of miR-125a and miR-155 reduced NF-kappa B signaling and alleviated kidney injury. Therefore, it's worth studying further to utilize the antagonists to relieve kidney injury in the clinic eventually.

6. FUNDINGS

Baiyin Integrated Traditional Chinese and Western Medicine Hospital supported this work.

7. AUTHORS CONTRIBUTION STATEMENT

Zhenqiang Yao performed all the experiments. Zhengshun Zhang conceived the project and wrote the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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