

## Original Article

# Sinomenine protects the kidney from ischemia reperfusion-induced apoptosis via up-regulation of microRNA-124 expression

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Received March 19, 2016; Accepted June 12, 2016; Epub October 15, 2016; Published October 30, 2016

**Abstract:** Sinomenine (SIN), an alkaloid from *Sinomenium acutum*, has been used in the treatment of various inflammatory diseases including rheumatism and arthritis. However, the effect and mechanisms of sinomenine on kidneys from ischemia-reperfusion (I/R) injury have not been well understood. This study aimed to test whether sinomenine attenuate renal I/R injury and reveal its underlying molecular mechanism. Renal function, oxidative stress and related parameters were studied using a kidney I/R injury model in rats, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to quantify apoptosis. MTT assay was used to detect the cell viability in HK-2 cells under hypoxia and re-oxygenation (H/R) conditions. Flow cytometry analysis was employed to measure apoptosis of H/R HK-2 cells. Expression of microRNAs was detected by real-time quantitative polymerase chain reaction (qRT-PCR) and expression of caspase 9 was analyzed by Western Blot. In vivo experimental results showed that SIN significantly decreased the renal function as evidenced by decreasing the levels of serum creatinine (Cr), blood urea nitrogen (BUN) and activities of major markers of oxidative stress, i.e. malondialdehyde (MDA), superoxide dismutase (SOD) and myeloperoxidase (MPO), and inhibited tubular epithelial apoptosis. In vitro experimental data further revealed that SIN directly guarded HK-2 cells from hypoxia-induced growth and apoptosis. In addition, RT-PCR analysis revealed that at 12 h post-injury, miR-320 and miR-378 were upregulated and miR-124, miR-21, miR-29 and miR-92a were downregulated in H/R HK-2 cells. Because miR-124 was one of the six miRNAs being most significantly downregulated, we investigated its function. Our study showed that the expression of miR-124 was significantly enhanced by SIN in H/R HK-2 cells in a dose-dependent manner and was dramatically decreased in mouse I/R injury kidney in a time-dependent manner. Furthermore, caspase 9 was identified as a direct target of miR-124 using a Luciferase reporter assay and western blot analysis. Following miR-124 silencing, SIN-induced cell viability and apoptosis were rescued in H/R HK-2 cells. Thus, SIN deserves further exploration as a novel agent to attenuate I/R injury.

**Keywords:** Sinomenine, ischemia reperfusion injury, microRNA-124, apoptosis, caspase 9

## Introduction

Renal ischemia-reperfusion (I/R) injury, frequently associated with shock or surgery, is a major cause of acute renal failure [1]. While important efforts in the advent of renal replacement therapy are ongoing, no effective therapy is currently available, with the exception of supportive treatment. Research into the pathogenesis of renal IR injury has demonstrated that tubular cell apoptosis plays a key role in the pathoetiology of renal IR injury [2, 3]. Therefore, therapeutic approaches aimed at suppressing tubular apoptosis was recognized effective ag-

ainst renal injury along with better prognosis after an episode of IR insult [4].

Increasing evidence supports that natural agents open up a novel avenue for treatment of I/R injury [5-7]. Sinomenine (SIN, 7, 8-didehydro-4-hydroxy-3, 7-dimethoxy-17-methyl- $\alpha$ , 13 $\alpha$ , 14 $\alpha$ -morphinan-6-one) is an active alkaloid originally extracted from medicinal herb *Sinomenium acutum* [8]. It has been well documented that sinomenine possesses anti-inflammatory and immune-regulatory properties [9, 10] and displays remarkable therapeutic efficacy with lower side effects in patients with mesangial

proliferative nephritis and rheumatoid arthritis (RA) [11, 12]. Recent observations in animals show that SIN pretreatment attenuates hepatic ischemia/reperfusion (I/R) injury through inducing heme oxygenase-1 (HO-1) expression, and by which it prevents IR-induced hepatocellular apoptosis [13]. Furthermore, a recent study also demonstrated that SIN could attenuate renal I/R injury by suppressing MAP kinase signaling to prevent tubular cells undergoing apoptosis [7]. Based on these discoveries, we thus examined whether SIN exerts inhibitory effects by inducing apoptosis of tubular epithelial cells, and if so, what's the underlying mechanism.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs with 18-22 nt in length. Mature miRNAs bind to the 3'-UTR of target mRNAs and repress translation of target mRNAs or induce degradation of target mRNAs [14]. Increasing evidence supports that miRNAs play indispensable roles in many biological processes including proliferation, apoptosis and so on [15-17]. Recently, several studies have suggested that miRNAs alter the response to ischemia reperfusion injury and regulate the expression of various key elements in cell survival and apoptosis [18]. And, kinds of miRNAs have been confirmed to participate in the progression of renal I/R injury [19-21]. For example, Xialian Xu et al. found miR-21 was highly upregulated in renal injury after ischemia-reperfusion and knockdown of miR-21 exacerbated renal injury after I/R. This was accompanied by upregulation of its target, programmed cell death protein 4 (PDCD4) and enhanced tubular epithelial apoptosis [22]. Simin Liang et al. provided strong evidence that miR-26a was involved directly in renal innate immune response against renal I/R injury by modulating tregs expansion [23]. Recent study indicated miR-124 was an endogenous regulator of Ku70 that improves ischemia/reperfusion (I/R)-induced brain injury and dysfunction [24]. However, whether microRNAs participate in the anti-apoptotic effects of SIN was unknown.

The primary objective of the present study was to elucidate the effects and underlying molecular mechanisms of the anti-apoptotic action of SIN. Considering the important roles of miR-124 and apoptosis in I/R injury, we investigated whether miR-124 contributes to the anti-apoptotic effect of SIN.

## Materials and methods

### *Animals and drugs*

Male C57BL/6 mice (H-2b, 22-25 g, Joint Venture SIPPR BK Experimental Animal Co., Shanghai, China) were housed in a specific pathogen-free environment and fed with laboratory chow and ad libitum. All studies were approved by the Animal Care Committee of The First Hospital of Lanzhou University, and were conducted in accordance with our institutional guidelines. SIN (98% purity verified by HPLC) was purchased from Sigma Co., Ltd (USA), and was freshly dissolved in normal saline (NS) for intravenous (i.v.) injection.

After 7 days of acclimation, 18 rats were randomly divided equally into 3 groups: Sham-operated (SO), I/R and SIN+I/R groups. The Sham and I/R groups were treated with saline solution, and the SIN+I/R groups were treated with SIN (200 mg/kg) by intraperitoneal injection 1 hour before surgery [7, 13, 25]. The rats in the sham group underwent the same surgical procedure but without occlusion of the renal pedicles. The rats in the SIN+I/R and I/R groups were subjected to renal I/R injury by methods previously described [26]. Mice from each group (6 mice/group) were sacrificed 8 h and 24 h after reperfusion to obtain blood and kidney samples, respectively.

### *Cell culture and in vitro I/R injury*

Human renal proximal tubular epithelial cells (human kidney-2, HK-2; ATCC, Manassas, VA) were cultured in DMEM (Invitrogen Corp., Carlsbad, CA) with 10% fetal bovine serum under a 5% CO<sub>2</sub> and 95% air atmosphere at 37°C. H/R model was established using the mineral oil coverage [27, 28]. In general, HK-2 cells were seeded in the 6-well plates at 5×10<sup>5</sup>/ml and incubated for 24 h to reach a confluence of 70-80%. Then the medium was removed and washed with PBS for twice. The mineral oil (Sigma-Aldrich, St. Louis, USA) was added into the plates to fully cover the cells and the cells were cultured in the incubator. One hour later, the oil was removed and the plates were washed with PBS for three times. The cells were then incubated with DMEM medium without FBS to mimic the reperfusion process and harvested at the appropriate time points.

## Measurement of renal function

Renal function was monitored by measuring BUN and serum creatinine using analytical kits from Biotron Diagnostics (Hemet, CA) and Stanbio Laboratory (Boerne, TX), respectively.

## Detection of SOD activity

Superoxide dismutase (SOD) activity was evaluated by an NBT (nitroblue tetrazolium) method. The inhibition of NBT reduction caused by the xanthine-XO system, which was a superoxide generator, was used to represent the SOD activity in renal homogenates. After mixed with 1.0 ml ethanol/chloroform mixture (5/3, v/v), the samples were centrifuged, the activity was assessed in the ethanol phase of the lysate. One unit of SOD meant the enzyme amount when 50% inhibition occurred in the NBT reduction rate. The results were expressed in units/mg protein.

## Detection of MDA activity

The concentration of malondialdehyde (MDA) was calculated by the thiobarbituric acid (TBA) method using assay kit from Jiancheng Co. (Nanjing, China). A spectrometer was used to measure the absorbance at 450 nm.

## Detection of MPO activity

Myeloperoxidase (MPO) activity in the renal samples was measured with a commercial kit (NJJC Bio Inc., Nanjing) according to the manufacturer's instructions.

## TUNEL assay

Apoptotic cell death of kidney was detected by the nuclear changes showed by widespread terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. Firstly, tissue sections were heated at 65°C to be rehydrated and then washed in xylene and rehydrated through a graded series of ethanol. Sections were incubated with 10 µg/ml protease K (Sigma, St Louis, MO) for 30 min at 37°C, and then with 0.5% Triton X-100 for 10 min. Slides was rinsed twice with PBS, and then incubated with 50 µl of TUNEL reaction mixture for 60 min at 37°C in a humidified atmosphere in the absence of light. The amount of apoptosis cells was examined at ×400 magnification and 20 cortical fields for each animal examined were randomly chosen.

## RNA extraction and real-time PCR

Total RNA was isolated using TRIzol (Invitrogen, CA) and miRNeasy mini kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. Total RNA from each sample was reverse-transcribed to cDNA using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan) and qRT-PCR was performed using the SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan). The forward primers used for the miRNA quantification were the same exact sequences of the mature miRNA genes. A universal reverse primer was provided in the NCode SYBR miRNA qRT-PCR kit. The relative microRNA levels were normalized to U6 expression for each sample. Analyses of gene expression were performed by the  $2^{-\Delta\Delta Ct}$  method.

## Transfection

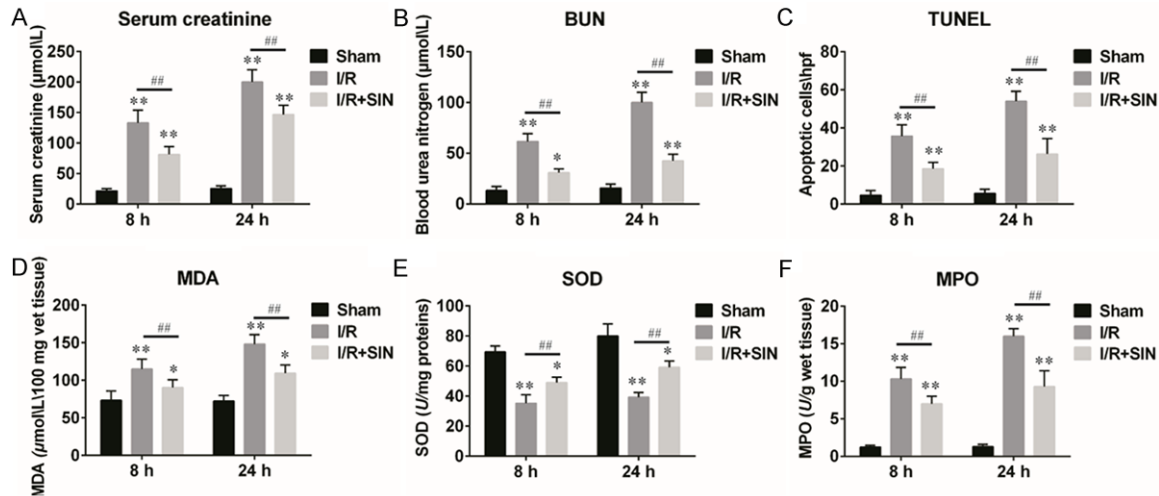
The HK-2 cells were plated into six-well plates and grown to 30-50% confluence after 24 hours of incubation and were then transfected with miRNA mimics, miRNA inhibitor or the non-sense controls for each of them at a final multiplicity of infection of 10 using siLent Fect™ Lipid reagent (Life Science Research). The cells were then diluted in DMEM/F12 without serum (GeneChem, Shanghai, China). After 4 h of incubation in a CO<sub>2</sub> incubator at 37°C, the medium was changed to 10% FBS containing DMEM. The efficiencies of miRNA mimics, miRNA inhibitor and negative control were tested by quantitative real time polymerase chain reaction (qRT-PCR). 24 h later, the cells were subjected to the H/R procedures.

## MTT cell viability assay

HK-2 cells were seeded in 96-well culture plates with  $1 \times 10^4$  cells/well, and incubated at 37°C with 5% CO<sub>2</sub>. After treating with 100 µM of H<sub>2</sub>O<sub>2</sub>, MTT assay (Amresco, Solon, USA) was performed. Briefly, 20 µL of MTT solution (5 mg/ml) was added to each well, and the cells were continuously incubated for 4 h. Formazan crystals were then dissolved in 150 µL DMSO. The optical density (OD) of the wells was measured with a microplate reader (BioTek, Richmond, USA) at 490 nm.

## Cell apoptosis detection by flow cytometry

HK-2 cells were collected after treatment, and then washed twice with PBS. The cells were



**Figure 1.** SIN provides protection for mice against I/R-induced renal injury. The mice were first subjected to 30 min of renal ischemia followed by reperfusion to induce I/R injury. Blood samples were next collected 8 h and 24 h after reperfusion to determine serum levels for creatinine (A) and blood urea nitrogen (B), respectively. Mice treated with SIN showed significantly lower levels of serum creatinine and blood urea nitrogen. (C-E) SIN exerted anti-oxidative effects against renal ischemia reperfusion injury. The levels of MDA (C), SOD (D) and MPO (E) were determined in individual group. (F) Renal cellular apoptosis was determined by TUNEL assay. TUNEL positive cell number is counted at  $\times 400$  magnification. ## $P < 0.01$  (SIN+I/R vs. Sham group); \* $P < 0.05$ , \*\* $P < 0.01$  (SIN +I/R vs. I/R group). Six mice were included in each study group.

resuspended in 500  $\mu$ L binding buffer at a concentration of 106/ml and then mixed with 10  $\mu$ L Annexin V (Bio-Science, Co. Ltd, Shanghai, China) for 10 min in the dark at room temperature (RT), followed by the addition of 5  $\mu$ L PI (Bio-Science, Co. Ltd., Shanghai, China). After incubation at RT in the dark for 5 min, samples were analyzed by a FACS Aria flow cytometry (BD Biosciences, San Jose, CA, USA).

#### Luciferase reporter assay

Dual luciferase assays were conducted in a 24 well plate format. pGL3-caspase 9 3'UTR report/pGL3-caspase 9 3'UTR Mutant report + TK100 Renilla report were transfected into 70% confluent HEK293 cells, along with miR-124 mimic, miR-124 inhibitor or each control. After 48-h transfection, firefly and renilla luciferase were quantified sequentially using the Dual Luciferase Assay kit (Promega, USA) following the manufacturer's recommendations.

#### Western blot analysis

Protein extracts from HK-2 cells were subjected to 10% SDS-PAGE and subsequently transferred to a PVDF membrane. The membrane was blocked with 5% (w/v) nonfat milk and incubated sequentially with the primary antibody

against caspase 9 (rabbit, 1:5000, Abcam, Cambridge, UK) in TBST containing 5% bovine serum albumin overnight at 4°C. Anti- $\beta$ -actin antibody was used as an internal control. After washing three times with TBST, the membrane was incubated at room temperature for 2 hours with horseradish peroxidase-conjugated secondary antibody (anti-rabbit, 1:2000, Cell Signaling Technology) diluted with TBST. The detected protein signals were visualized using an enhanced chemiluminescence (ECL) system western blot kit (Thermo Fisher Scientific, Waltham, MA, USA).

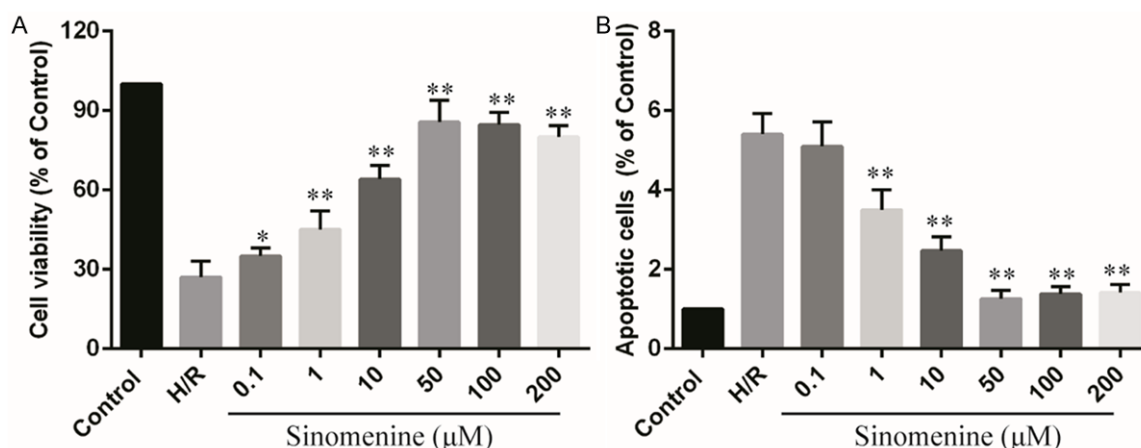
#### Statistical analysis

All data were expressed as mean  $\pm$  SD. Results were analyzed by one-way ANOVA, and Student's t test was employed for assessing statistical significance if differences were established. In all cases,  $P$  value  $< 0.05$  was considered with statistical significance.

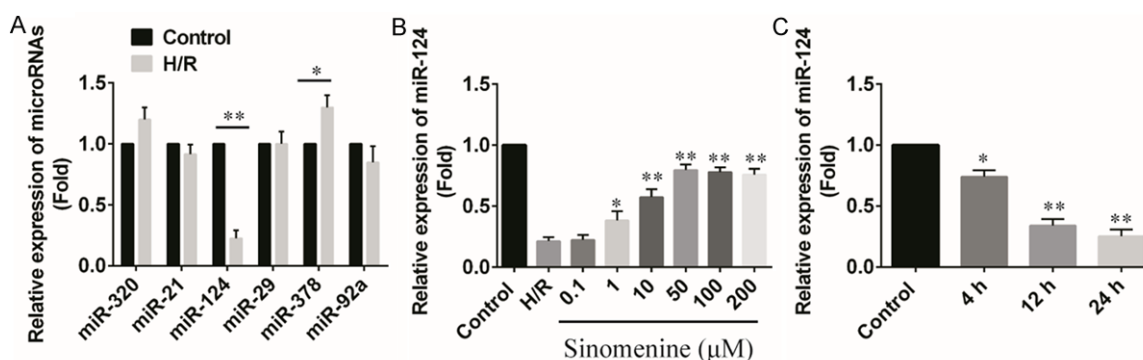
#### Results

##### Administration of SIN protects mice from I/R-induced renal injury

To investigate the effect of SIN on I/R-induced renal injury, and for this purpose, we checked



**Figure 2.** The protective effect of SIN in H/R-induced HK-2 injury. HK-2 cells were submitted to hypoxia/reoxygenation (H/R) protocol. A. Cells were incubated in medium containing various concentrations of SIN for 24 h. Cell viability was all examined by MTT assay and the data were shown as means  $\pm$  SD of three independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01 relative to H/R treated cells. B. Cell apoptosis was all examined by Flow cytometry assay and the data were shown as means  $\pm$  SD of three independent experiments. \*\* $P$  < 0.01 relative to H/R treated cells.



**Figure 3.** miR-124 expression in ischemic kidney and H/R HK-2 cells. A. Expression level of six miRNAs in kidneys with ischemia reperfusion injury (n = 6). B. The expression of miR-124 was detected by real-time RT-PCR in H/R HK-2 cells after various concentrations of SIN treatment for 72 h. \* $P$  < 0.05, \*\* $P$  < 0.01 compared to H/R treated cells. C. Expression level of miR-124 was validated by RT-PCR in kidneys with ischemia reperfusion injury at different time points (n = 6). \* $P$  < 0.05, \*\* $P$  < 0.01 vs. Control.

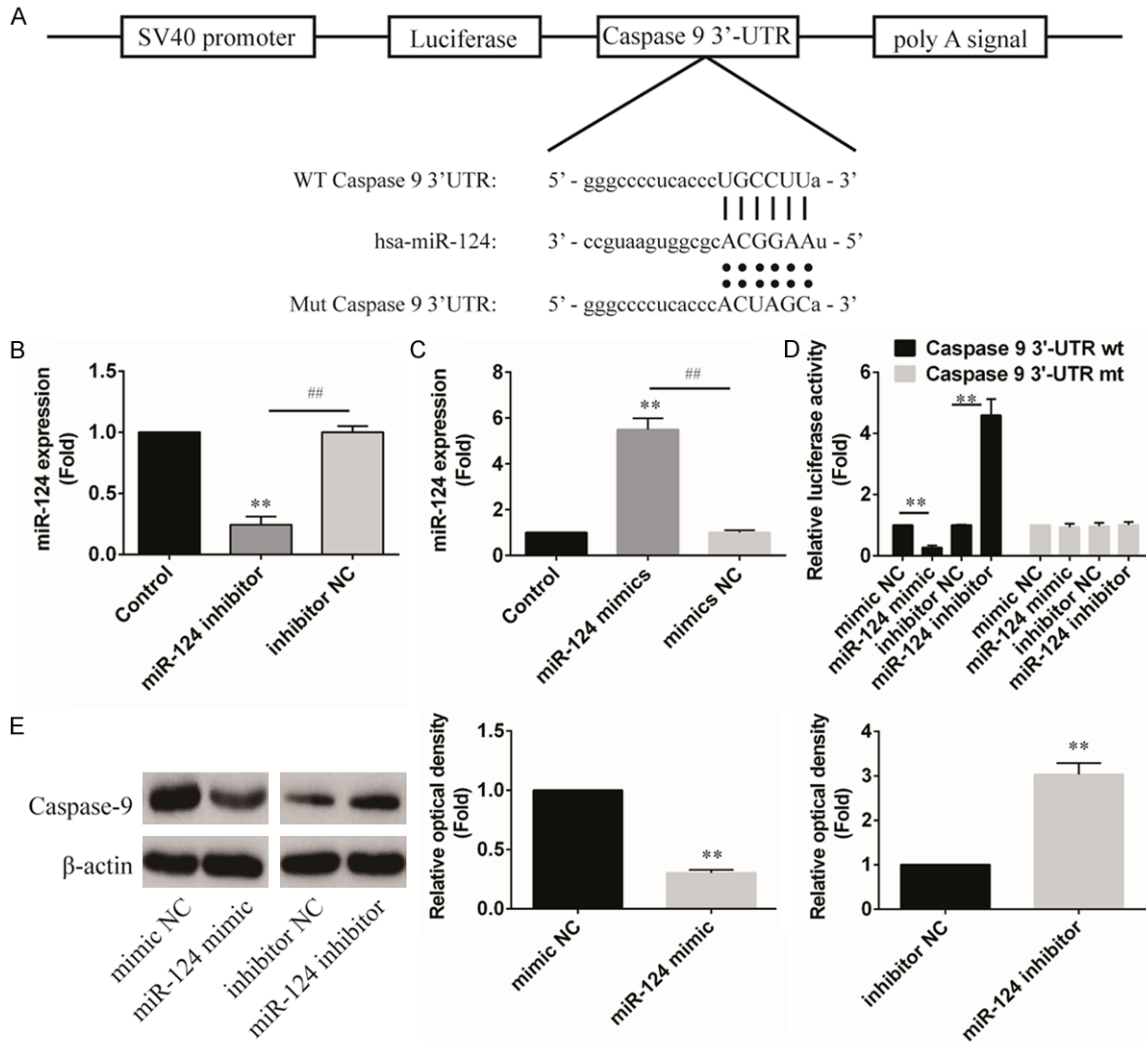
the renal function; it showed that SCR and BUN levels were higher in I/R groups as compared to the Sham group, indicating that I/R insult induced severe renal damage. Intraperitoneal injection of SIN (200 mg/kg) reduced BUN and SCR levels as compared with I/R group (Figure 1A and 1B).

To uncover the protective mechanisms of SIN, we evaluated the presence of oxidative stress in I/R-damaged rat kidney. The results showed that the activities of SOD were significantly decreased in I/R group; pretreatment with SIN increased the SOD levels in I/R rats (Figure 1C). In contrast, MDA level was higher in I/R

rats than in the sham control rats, which was also decreased with the pretreatment of SIN (Figure 1D). MPO was assayed in the present study to assess leukocyte infiltration. MPO activity was significantly increased in I/R group, which was inhibited by SIN pretreatment, indicating that SIN may decrease I/R induced inflammatory infiltration (Figure 1E).

Considering the important roles of tubular cell apoptosis in I/R injury, we performed TUNEL staining to investigate the apoptosis of renal tissues. The results showed that TUNEL positive cells appeared occasionally in the sham operated group, but were greatly increased in





**Figure 4.** miR-124 directly binds and downregulates caspase 9. **A.** Schema of the firefly luciferase reporter constructs for the caspase 9, indicating the interaction sites between miR-124 and the 3'-UTRs of the caspase 9. **B** and **C.** The expression of miR-124 after treatment with miRNA mimics, miRNA inhibitor or the nonsense controls for each of them (n = 6). **D.** Luciferase activities. HK-2 cells were co-transfected with firefly luciferase constructs containing the caspase 9 wild-type or mutated 3'-UTRs and miR-124 mimic, mimic NC, miR-124 inhibitor or inhibitor NC, as indicated (n = 6). **E.** Protein expression of caspase 9 after treatment with miR-124 mimic or miR-124 inhibitor (n = 6). All data represent the mean ± SD results of three independent experiments. \*\*P < 0.01.

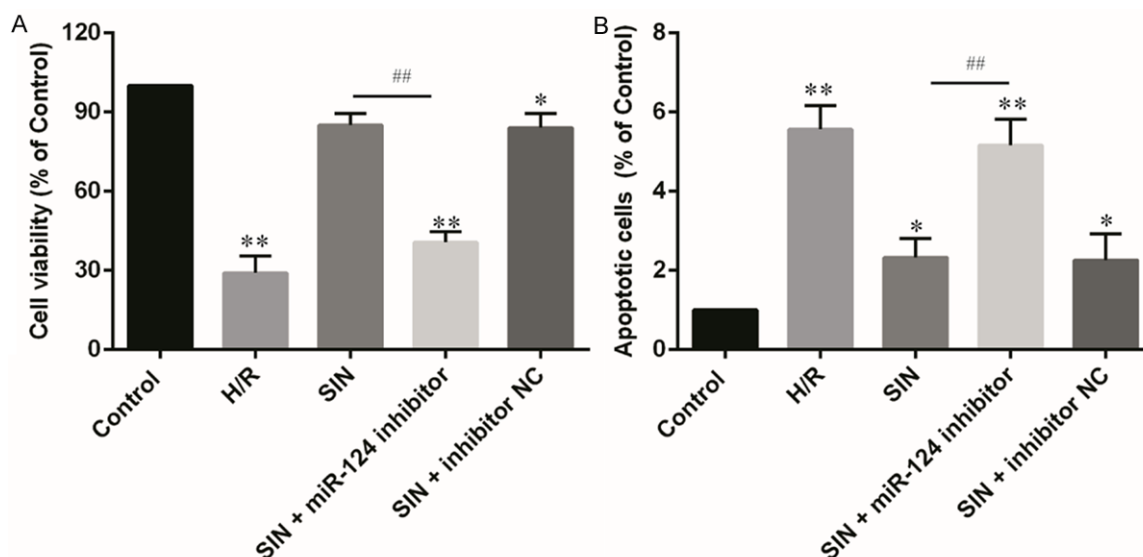
I/R treatment group. Pretreatment with SIN significantly reduced the renal apoptosis in I/R rats (**Figure 1F**). Together, our data support that SIN protects mice against I/R-induced renal injury at least in part by inhibiting tubular cell apoptosis.

#### Effect of SIN from H/R in in-vitro experiment

To further show the role of SIN in the I/R injury, we used an H/R model in a renal epithelial cell line (HK-2). HK-2 cells were treated with SIN at different concentration (0.1-200 µM), and then

the cells were analyzed by MTT Assay. As shown in the **Figure 2A**, SIN slightly increased the cell viability of HK-2 cells at the dose of 0.1 µM, and significantly increased the cell viability of HK-2 cells at the dose of 1, 10 and 50 µM. In addition, SIN increased the cell viability of HK-2 cells in a dose dependent manner at 0.1-50 µM.

To detect whether the promotion of cell viability was associated with cell apoptosis, flow cytometry assay was applied to detect apoptotic rate of HK-2 cells treated with SIN. As shown in



**Figure 5.** Knock down of miR-124 alleviates the anti-apoptotic effects of SIN in H/R HK-2 cells. A. Cell viability was all examined by MTT assay after SIN treatment or miR-124 inhibitor or the combination. Knock down of miR-124 decreased the cell viability induced by SIN in H/R HK-2 cells. B. Cell apoptosis was all examined by Flow cytometry assay after SIN treatment or miR-124 inhibitor or the combination in H/R HK-2 cells. Knock down of miR-124 alleviated the reduction of the apoptosis induced by SIN in H/R HK-2 cells.  $n = 5$ ,  $##P < 0.01$  vs. Control;  $*P < 0.05$ ,  $**P < 0.05$  vs. SIN.

**Figure 2B**, compared with the H/R group, apoptosis was markedly decreased in HK-2 cells after treating with SIN in a dose dependent manner at 0.1-50  $\mu$ M. The results indicated that SIN had a potent protective effect against HK-2 cells under H/R condition in a dose-dependent manner and that the best dose for protective effect was 50  $\mu$ M.

#### *Sinomenine promotes expression of miR-124*

Multiple miRNAs, such as miR-21 [22], miR-320 [29], miR-124 [24], miR-92a [30], miR-29 [31] and miR-378 [23] were shown to be involved in renal ischemia-reperfusion injury by altering the expression of key genes associated with cell survival and apoptosis. Thus, we assumed that SIN induces the apoptosis of renal proximal tubular epithelial cells by upregulating expression of miRNAs. The level of miRs was determined by real-time RT-PCR after treating with SIN in H/R HK-2 cells for 48 h. **Figure 3A** showed that SIN increased the levels of miR-320, miR-378 and miR-29 in H/R HK-2 cells, while decreased the levels of miR-21, miR-124 and miR-92a. Thus, our data indicate that a set of microRNAs is frequently aberrantly expressed in H/R HK-2 cells. We then focused on miR-124, because it was significantly down-

regulated in H/R HK-2 cells treated with SIN. Therefore, miR-124 was selected for further study.

To investigate whether SIN treatment could regulate the expression of miR-124 in H/R HK-2 cells, we performed miRNA RT-PCR assay. As shown in **Figure 3B**, miR-124 expression was significantly increased after SIN treatment in H/R HK-2 cells in a dose-dependent manner and that the best dose was 50  $\mu$ M. Further experiments confirmed that miR-124 was decreased in I/R-damaged rat kidney (**Figure 3C**). These results suggest that the SIN might exert its protection by regulating the expression of miR-124.

#### *miR-124 directly targets at the 3' UTR of caspase 9*

To test whether caspase 9 is a direct target of miR-124, we constructed a firefly luciferase reporter containing the potential binding site for miR-124 in caspase 9 3'-UTR (**Figure 4A**). The constructs were then co-transfected into HEK-293 cells together with the miR-124 mimic, its inhibitor or negative control, and luciferase activity was measured 48 h after transfection. Along with significant changes in

the expression level of miR-124 (**Figure 4B** and **4C**), we found that miR-124 significantly repressed the activity of reporter vectors harboring wt 3'-UTRs of caspase 9, whereas the mutations of potential miR-124 binding sites in these 3'-UTR regions largely abrogated the inhibitory effects of miR-124 (**Figure 4D**).

To evaluate whether miR-124 regulated caspase 9 expression, we detected the protein expression level of caspase 9 in miR-124 mimic or miR-218 inhibitor infected cells. Western blot analysis showed that miR-124 overexpression markedly decreased the protein level of caspase 9, whereas miR-124 inhibition increased the protein expression of caspase 9 (**Figure 4E**). Together, these results indicated that caspase 9 is a direct target of miR-124, which suggested that miR-124 may regulate cell apoptosis by targeting caspase 9.

## *Role of miR-124 in the protection of SIN from H/R in in-vitro experiment*

To examine whether the up-regulation of miR-124 contributes to the anti-apoptotic effects of sinomenine in H/R HK-2 cells, we knocked down the expression of miR-124 by transfection with miR-124 inhibitor, and then observed the alteration of SIN-induced apoptosis in H/R HK-2 cells. As expected, we found that knock-down of miR-124 decreased the cell viability induced by SIN (**Figure 5A**) and alleviated the reduction of the apoptosis induced by SIN (**Figure 5B**), indicating that the elevation of miR-124 is involved in the anti-apoptotic effects of sinomenine in H/R HK-2 cells.

## Discussion

In the present study, we demonstrated that SIN effectively protected I/R-induced renal injury both in a mouse model and in H/R model in a renal epithelial cell line (HK-2). The protective effect of SIN was at least in part, mediated by miR-124, as proved by the fact miR-124 alleviated the reduction of the apoptosis induced by SIN through regulating the expression of caspase 9. Taken together, our work revealed the anti-apoptotic effects of SIN in HK-2 cells and uncovered the participation of miR-124 in this process. And, our studies indicate that SIN could be a novel agent for attenuating I/R injury.

Sinomenine, which is a purified alkaloid extracted from the Chinese herb *Sinomenium acutum*, possesses multiple biochemical characteristics, such as anti-inflammatory and anti-apoptotic properties [7]. And, a study from Song S et al. further demonstrated that SIN could induce heme oxygenase-1 expression, and by which it prevents IR-induced hepatocellular apoptosis, which indicate the potential therapeutic effects of SIN on Hepatic I/R injury [13]. In addition, SIN protects against UUO-associated kidney injury essentially through Nrf2 signaling activation since lack of Nrf2 significantly eliminates its renoprotective functions. These studies imply that SIN may have a potential protective action against renal I/R injury. Our data reinforce this protection of SIN by showing that SIN treatment ameliorated renal dysfunction, and inhibited tubular apoptosis. On the other hand, we determined the oxidative stress in renal I/R injured rats with or without SIN pretreatment. It showed that SIN reversed the elevated MPO (representing the degree of neutrophil accumulation) and MDA (indicating of lipid peroxidation), and recovered the deceased SOD induced by renal I/R injury. These data suggested that SIN could be used for the treatment of renal I/R injury.

Recent experimental studies have demonstrated the role of microRNAs in renal I/R injury, such as miR-210, miR-21 and so on [32, 33]. Xiujuan Liu et al. found that the miR-21 level was increased during renal I/R injury, which inhibited autophagy activity and aggravated I/R injury by targeting Rab11a [34]. A study performed by Lorenzen JM et al. showed that miR-126 and miR-24, which have been demonstrated to be highly enriched in endothelial cells, were therapeutically modulated and shown to ameliorate renal I/R injury in mice [35]. Increasing evidence supports that miRNAs are involved in the anti-apoptotic action of various agents, such as curcumin, notoginsenoside R1 and so on [6, 36]. However, it is unknown whether miRNAs mediated the anti-apoptotic effect of SIN against renal I/R injury.

A variety of studies have demonstrated that miR-124 participated in the progression of I/R injury [24, 37, 38]. For example, miR-124 is down-regulated during hepatic IRI and protect human hepatic LO2 cells from H<sub>2</sub>O<sub>2</sub>-induced injury through the AKT pathway by targeting



Rab38 [37]. Doeppner TR et al. showed that viral vector-mediated miR-124 delivery increased the resistance of cultured oxygen-glucose-deprived cortical neurons in vitro and reduced brain injury as well as functional impairment in mice submitted to middle cerebral artery occlusion. found that miR-124 promoted neuronal survival under ischemic conditions via Usp14-dependent REST degradation [38]. Our data showed that SIN promoted the expression to miR-124, which mediated the anti-apoptotic effect of SIN in renal I/R injury and HK-2 cells from H/R induced injury.

Interestingly, our work revealed that miR-124 is a novel mechanism by which SIN produces anti-apoptotic effects. However, emerging reports showed that other miRNAs also contribute to the protection against renal I/R injury. Maybe this is the reason why the dysregulation of caspase 9 were not fully recovered when we knocked down of miR-124 before treating with SIN in H/R HK-2 cells. Examining the roles of other miRNAs in SIN-induced protection against renal I/R injury warrants our future research.

#### Disclosure of conflict of interest

None.

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