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Experimental study on the regulation of the cholinergic pathway in renal macrophages by microRNA-132 to alleviate inflammatory response

The regulation of the cholinergic pathway by microRNA-132

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Abstract: MicroRNA-132 (miR-132) is correlated with inflammatory response regulation, although its effect on acute kidney injury to provide protection against hemorrhagic shock remains currently unknown. AChE in macrophages of the kidney subjected under hemorrhagic shock is presumed to be regulated by miR-132 after its transcription to alleviate the inflammatory response accordingly. Antagonists such as acetylcholine (Ach) (concentration 10^{-4}mol/L) and galanthamine (Gal) (concentration 10μmol/L) were added into separate groups 1 hour after the macrophages in the kidney were isolated and cultured to induce injury under oxygen and glucose deprivation (OGD) and then cultured for 24 hours. To analyze the effect of miR-132, we placed the renal epithelial cells transfected with miR-132 plasmids with stable expression over the renal macrophages to create a double cell culture system. The expression levels of inflammatory factors and apoptosis under OGD were significantly higher in renal macrophages than in other experimental groups. Moreover, the expression of miR-132 in macrophages of the double cell culture system showing stable expression of miR-132 increased, whereas that of several inflammatory factors was significantly inhibited. The expression levels of AChE mRNA and protein in the macrophages significantly decreased. The cholinergic anti-inflammatory pathway in renal macrophages is regulated by miR-132 via inhibition of the hydrolytic activity of cholinesterase to alleviate inflammatory response, which may play a role in the prevention and treatment of kidney injury caused by hemorrhagic shock.

Keywords: cholinergic anti-inflammatory pathway; microRNA-132; renal macrophages.

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1 Introduction

Hemorrhagic shock is a common complication following trauma and surgery, as well as one of the common issues in emergency cases and critical illnesses [1,2]. Hemorrhagic shock can cause extensive organ dysfunction and metabolic disorders. The death rate yielded by trauma-associated hemorrhagic shock is relatively high, and the primary cause of death in this condition is multiple organ dysfunction syndrome (MODS) induced by sharp reduction in the effective circulating volume, tissue hypoperfusion, reperfusion injury, and endotoxin translocation, among others [3]. Additionally, acute kidney injury (AKI) is one of the primary causes of death among patients with MODS.

AKI incidence constantly increases, and irreversible reduction in renal function may be subsequently found in some patients who have suffered from AKI, potentially resulting in chronic kidney disease (CKD) or end-stage renal disease (ESRD) and ultimately in reduced survival of patients [4]. AKI is an independent risk factor for CKD and ESRD, as well as death and other diseases related to kidney disorders.

The microenvironment of macrophages in the kidney changes resulting from the release of large amount of inflammatory mediators when ischemic injury occurs in the kidney [5]. Therefore, in addition to active fluid resuscitation therapies at the early stage of hemorrhagic shock, anti-inflammatory treatment measures that block or reduce the production and release of inflammatory mediators can significantly prevent the progress of hemorrhagic shock [6]. The cholinergic anti-inflammatory pathway is a vagal-mediated pathway for real-time regulation of inflammatory response. When an inflammatory response occurs in the human body, vagal afferent neurofibers are stimulated by local inflammatory factors to transfer the inflammatory signals to the hypothalamus [7, 8]. Acetylcholine is then released into the inflammatory site through vagal efferent neurofibers to activate nicotinic ACh receptor 7 (nAChRα7), thereby inhibiting the activation of monocyte macrophages and reducing the release of proinflammatory factors, such as TNF-α, and ultimately regulating the inflammatory response [9-11]. This study performed isolation and culture of macrophages in the kidney to explore the regulatory function of miR-132 in the cholinergic pathway and its effect on the macrophages.

2 Materials and methods

2.1 Culture of macrophages in the kidney

All procedures in the animal experiments were conducted in accordance with the guidelines developed by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center (Permit SYXK 2015-2016). 10 male eight-week-old SD rats (220g to 250g) were sacrificed by cervical dislocation and disinfected by soaking in 75% alcohol for 5 minutes. The rats were retrieved and positioned upside down, and the bilateral kidneys were surgically removed under sterile conditions. The kidneys were placed in a flat plate filled with precooled RPMI-1640 (Gibco, NY, USA) culture solution (containing 1% calf serum), and the connective tissues and fats were removed. The kidney tissue was squeezed through steel wire gauze (200 mesh) to obtain single cells, which were centrifuged at 200 ×g for 10 minutes and the supernatant was then extracted. A cell solution containing approximately 1 × 108 cells/ml was prepared using RPMI-1640 culture solution containing 1% calf serum. The single cells obtained were inoculated with DMEM culture solution containing 10% FBS (Gibco, NY, USA) in a cell culture flask and placed in CO2 incubator with 5% volume fraction at 37°C under saturated humidity. The macrophages were purified using an adherent method.

2.1.1 Construction of oxygen–glucose deprivation (OGD) model of renal macrophages

The renal macrophages were inoculated on a 24-well plate at a density of 3.0×10^4 cells/ml with 1 ml of culture medium per well. After 24 hours, the culture solution was replaced with serum-free medium and then cultured for another 24 hours; the original culture solution was discarded. For cells in the normal group, Earle's fluid was added; for cells in the hypoglycemia and hypoxia groups (10%XB+OGD), Na2S2O4 sugar-free Earle's fluid (4 mmol/ml) was added after the cells were washed once with sugar-free Earle's fluid. The duration for induction of injury under hypoglycemia and hypoxia was 1 hour. Earle's fluid was used to wash the cells once after 1 hour. Complete culture solution (10% FBS+1640) was added into the culture, and then the cells under hypoglycemia and hypoxia were divided into two groups; ACh (10×10^{-5} mol/L, 10%XB+OGD+ACh) was added in one group, whereas the mixture of ACh (10×10^{-5} mol/l) and galanthamine (Gal) (10 μmol/l) was added into the other group and then cultured for 24 hours (10%XB+OGD+ACh+Gal). All of the s were repeated three times.

2.2 Cell transfection

Renal epithelial cells were inoculated in a 12-well culture plate, resulting in 50–70% cell confluency when transfection was performed. miRNA-132 (RiboBio Co.,LTD, Guangzhou, China) was overexpressed, and the control vector (6 μg) was transfected into the cells using Lipofectamine 2000 (Invitrogen, USA). The mixed liquor was removed after 8 hours, and then 2 ml of DMEM was added into the complete culture solution. The cells were observed under a fluorescent microscope at 3–5 days after

transfection. After miRNA-132 was stably expressed in renal epithelial cells, the abovementioned renal macrophages under hypoglycemia and hypoxia were inoculated in a 96-well culture plate at a density of 5×10^3 cells/cm^2. When the cells were overgrown by 50–60%, the DMEM culture solution containing 10% FBS was placed in a double cell culture system containing renal tubular epithelial cells; this culture system was used for transfection and stable expression of miRNA-132 for 24 hours.

2.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The expression levels of TNF-α, IL-1β, IL-10, TGF-β, IL-4 and IL-6 in cultured cell supernatant were determined using ELISA kit (Senxiong Technology Industrial Co., Ltd., Shanghai, China). The absorbance at 450 nm was detected by a microplate reader, and all the results were standardized using a standard curve.

2.4 Cell viability evaluation (MTT assay)

Approximately 1×10^4 cells were inoculated in a 96-well plate, which was subsequently placed in a 5% CO_2 cell incubator at 37°C to culture for 24 hours. Appropriate concentrations of the tested compounds were added into the culture solution under suitable conditions and then cultured for 24 hours. Afterwards 1× MTT (50 μL) was added into each well and incubated at 37°C for 4 hours. The supernatant was removed, 150 μL of DMSO was added into each well, and the optical density at 570 nm in each well was determined by a microplate reader.

2.5 Real-time quantitative PCR (RT-qPCR)

A randomized selection of 6 rat kidney macrophages were performed the relative expression levels of miR-132, AChE, AChE-R and AChE-S mRNA were determined by RT-qPCR. TRIzol kit (Invitrogen, USA) was used to extract the total RNA of the renal macrophages. The absorbance of RNA was tested at 260 and 280 nm, and its expression level and purity were determined using an ultraviolet spectrophotometer. Reverse transcription was performed using PrimeScript RT reagent Kit (TaKaRa Biotechnology Co. Ltd., Shanghai, China), total system 10 μL per reaction. RT-qPCR was completed using SYBR Premix Ex Taq II kit (Applied Biosystems, Foster City, CA, USA), total system 20 μL per reaction. PCR amplification was completed using a one-step quantitative PCR system, and U6 snRNA and β-actin were used as standard internal references for the expression levels of miR-132, AChE, AChE-R and AChE-S mRNA. The 2–ΔΔCt method was used for graphical representation of data, and the difference in the samples was evaluated. The kits mentioned above were used in accordance with the manufacturer's instructions. The primers for miR-132, AChE, AChE-R and AChE-S U6 snRNA, and β-actin are listed in table 1. All of the experiments were repeated three times.

2.6 Western blot analysis

To test the AChE and control GAPDH protein, we extracted the total protein of renal macrophages using RIPA, and the same amount of protein sample was used for loading. The total proteins were incubated with blocking buffer for 2 hours after electrophoresis, decolorized with TBST at room temperature, and then washed twice on a shaking table for 10 minutes each time after the AChE antibody Abcam, Cambridge, MA, USA, ab188358 and GAPDH antibody (*Santa* Cruz, USA, sc-365062) primary antibody was diluted with TBST to 1:500 and 1:1000 prior to incubation at room temperature for 1–2 hours. The total protein was washed again with TBS for 10 minutes, incubated with secondary antibody dilution buffer (1:800) labeled with HRP at room temperature for 1 hour, decolorized with TBST at room temperature, washed three times on a shaking table for 10 minutes each time, and finally tested with ECL luminescence kit. The results were quantitatively analyzed using Gel pro4.0 gel optical density analysis software. In addition, the integrated optical density was determined.

2.7 Statistical analysis

All analyses were performed using SPSS13.0 software. The results are presented as mean ± SD. Comparison between two groups was performed using one-way ANOVA. P values of <0.05 were regarded as statistically significant.

3 Results

3.1 Effect of ACh and Gal on OGD model of renal macrophages

The OGD model of renal macrophages was constructed to test the effect of miRNA-132 in renal macrophages

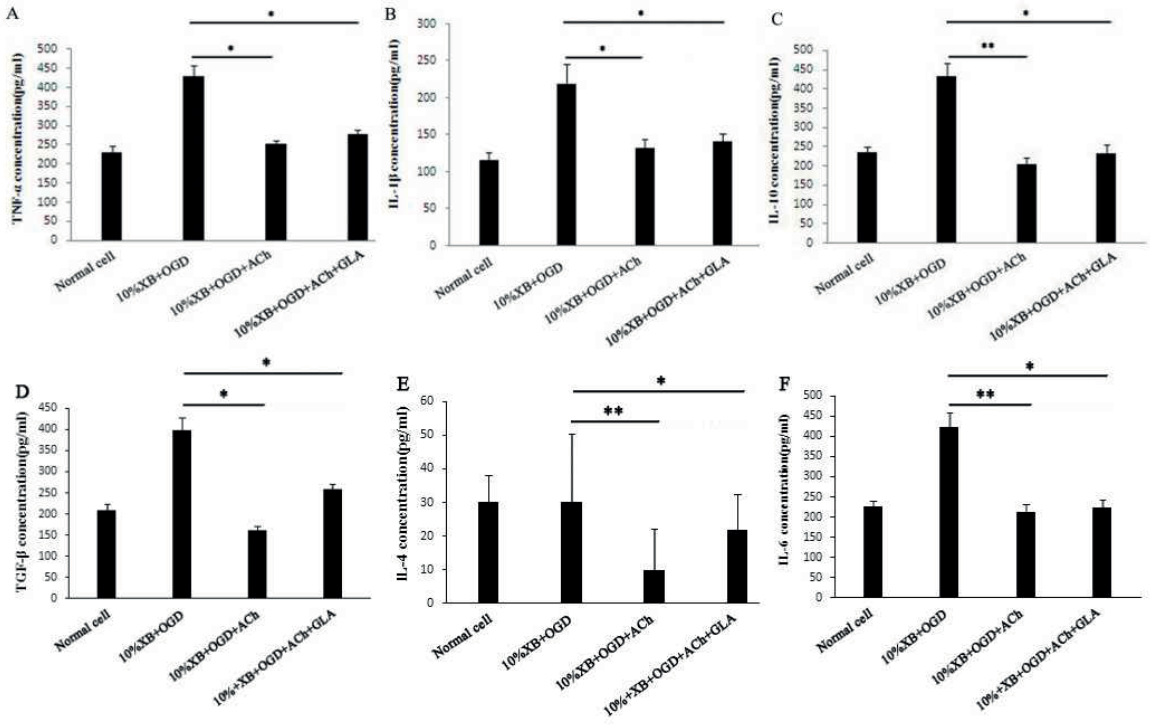

Figure1: Expression of inflammatory factors in renal macrophages of rats after being processed using different methods: (A) TNF-α, (B) IL-1β, (C) IL-10, (D) TGF-β, (E) IL-4 and (F) IL-6. The results are presented as mean ± SD, * P < 0.05, ** P < 0.01.

Table 1: Sequences of primers for reverse transcription and real-time quantitative. PCR

Gene	Primer sequences
miR-132	RT primer: 5'-GTC GTA TCC AGT GCA GGGTCC GAG GTA TTC GCA CTG GAT ACGACC GAC CA-3' PCR primer F: 5'-GCC GCT AAC AGT CTA CAGCCA T-3' PCR primer R: 5'-GTG CAG GGT CCG AGG T-3'
U6	RT primer: 5'-AAC GCT TCA CGA ATT TGCGT-3' PCR primer F: 5'-CTC GCT TCG GCA GCACA-3' PCR primer R: 5'-AAC GCT TCA CGA ATT TGCGT-3
AChE	PCR primer F: 5'-AAA CAT GCA GAA GAT GAGGAT-3' PCR primer R: 5'-GAC CAC TAT AGC AAG CAGGAA C-3'
AChE-R	PCR primer F: 5'- CCCCTGGACCCCTCTCGAAAC-3' PCR primer R: 5'- ACCTGGCGGGCTCCCACTC-3
AChE-S	PCR primer F: 5'- CGGGTCTACGCCTACGTCTTTGAACACCGTG-CTTC-3' PCR primer R: 5'- CACAGGTCTGAGCAGCGATCCTGCTTGCTG-3'
β-actin	PCR primer F: 5'-TAC TGC CCT GGC TCC TAGCA-3' PCR primer R: 5'-TGG ACA GTG AGG CCA GGATAG-3'

under AKI caused by hemorrhagic shock. Macrophages subjected under hypoglycemia and hypoxia were processed for 24 hours with ACh and Gal as antagonists, and the expression levels of TNF-α, IL-1β, IL-10, TGF-β, IL-4 and IL-6 were tested in cell culture suspension. The results demonstrated that the expression levels of TNF-α, IL-1β, IL-10, TGF-β, IL-4 and IL-6 after treatment with ACh and Gal were all significantly lower than those in the group of macrophages subjected under hypoglycemia and hypoxia (P < 0.05 or P < 0.01) (Figures 1A–F).

3.2 Expression of miRNA-132 in renal epithelial cells

To further verify the effect of miRNA-132, we constructed its expression plasmid and then transfected in renal epithelial cells. We found that 3 days after its transfection, miRNA-132 was stably expressed and the fluorescence intensity was higher than that in the control group (Plasmids were not inserted into the miRNA-132 group) (Figures 2A, B)

3.3 Analysis of cell viability

Analysis of cell viability of each experimental group, using the MTT method showed that the cell viability of renal macrophages was significantly reduced in the 10%XB+OGD+miRNA-132 group compared to the 10%XB+OGD group (P < 0.01). Moreover, the cell viability

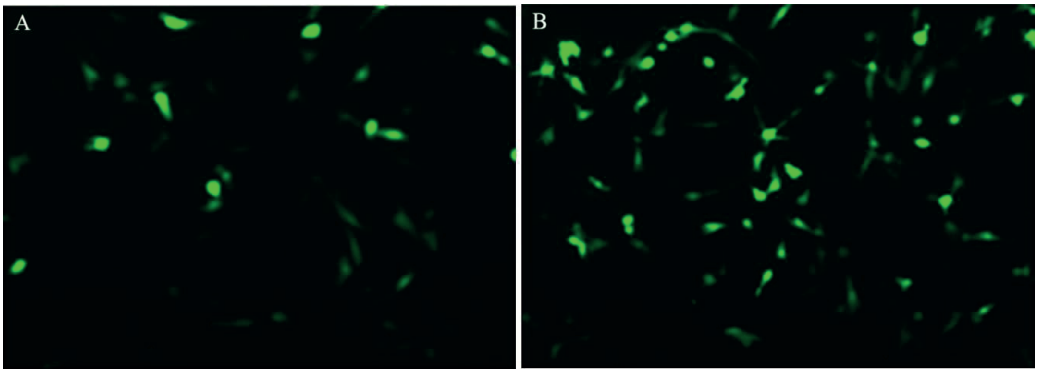

Figure 2: MiRNA-132 expression after transfection of renal epithelial cells in rats through miRNA-132 plasmids; (A) miRNA-132 nc and (B) miRNA-132 . Scale bar = 50 μm.

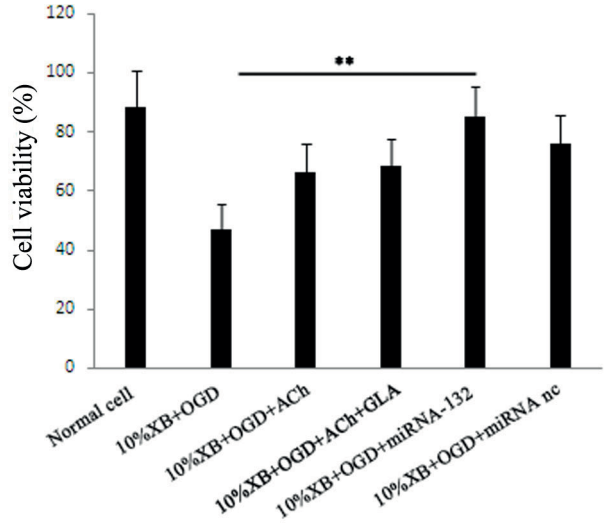

Figure 3: Analysis of cell viability of renal macrophages in rats using the MTT method after processing through different methods. The results are presented as mean ± SD, ** P < 0.01.

of macrophages in double cell culture system with expression of miRNA-132 was similar to that of normal cells (Figure 3).

3.4 Effect of miRNA-132 on expression of TNF-α, IL-1β, IL-10, TGF-β, IL-4 and IL-6

The effect of miRNA-132 was further verified in the double cell culture system of renal macrophage–renal epithelial cell, and the expression levels of TNF-α, IL-1β, IL-10, TGF-β, IL-4 and IL-6 were analyzed in the double cell culture system, in which miRNA-132 was transfected and stably expressed. Analysis showed that the expression levels of TNF-α, IL-1β, IL-10, TGF-β, IL-4 and IL-6 in the cell suspension with transfected and stably expressed miRNA-132 were significantly lower than that in the macrophages of the hypoglycemia and hypoxia group ($P < 0.05$ or $P < 0.01$), demonstrating that miRNA-132 inhibits inflammation (Figures 4A–F).

3.5 Effect of miRNA-132 on expression levels of AChE mRNA and protein in renal macrophages

The effect of transfected miRNA-132 was investigated, and the results demonstrated that miRNA-132 expression in rat renal macrophages of double cell culture system with transfected expression plasmids was significantly higher than that in other experimental groups (Figure 5A). Moreover, the effects of transfection and expression of miRNA-132 on AChE mRNA and protein and their activities were investigated in the double cell culture system. The results demonstrated that expression of AChE mRNA was significantly inhibited by overexpression of miRNA-132 in renal macrophages (Figure 5B, ** $P < 0.01$). The results also showed that the expression of AChE protein in the cells was significantly inhibited (Figure 5C). Expression of AChE protein was then quantitatively analyzed (Figure 5D). The effects of transfection and expression of miRNA-132 on AChE-R mRNA in the double cell culture system. We found the expression of AChE-R mRNA was significantly inhibited by overexpression of miRNA-132 (Figure 5F, ** $P < 0.01$), and there was no obvious change in the expression of AChE-S. The results show that miRNA-132 regulates inflammation by inhibiting the expression of AChE-R protein in renal macrophages. This has some protective effect on renal injury (Figure 6).

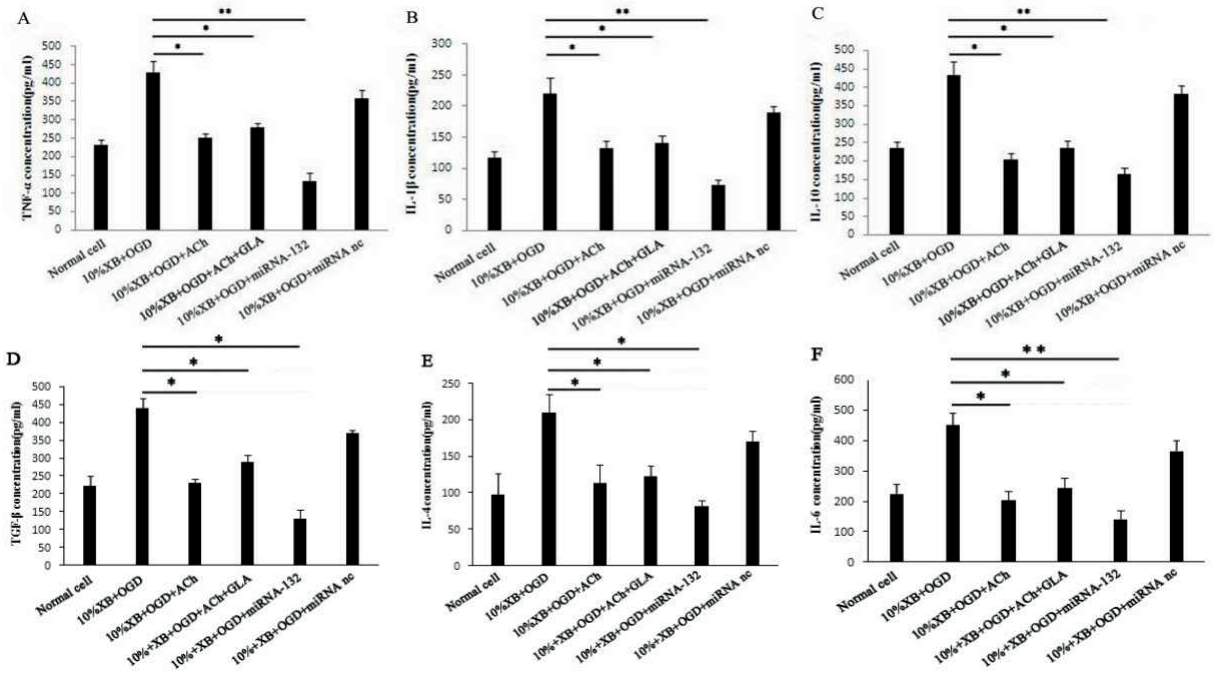

Figure 4: Testing of inflammatory factors in the double cell culture system 24 h after miRNA-132 was transfected and stably expressed: (A) TNF-α, (B) IL-1β, (C) IL-10, (D) TGF-β, (E) IL-4, and (F) IL-6. The results are presented as mean ± SD, * P < 0.05, ** P < 0.01.

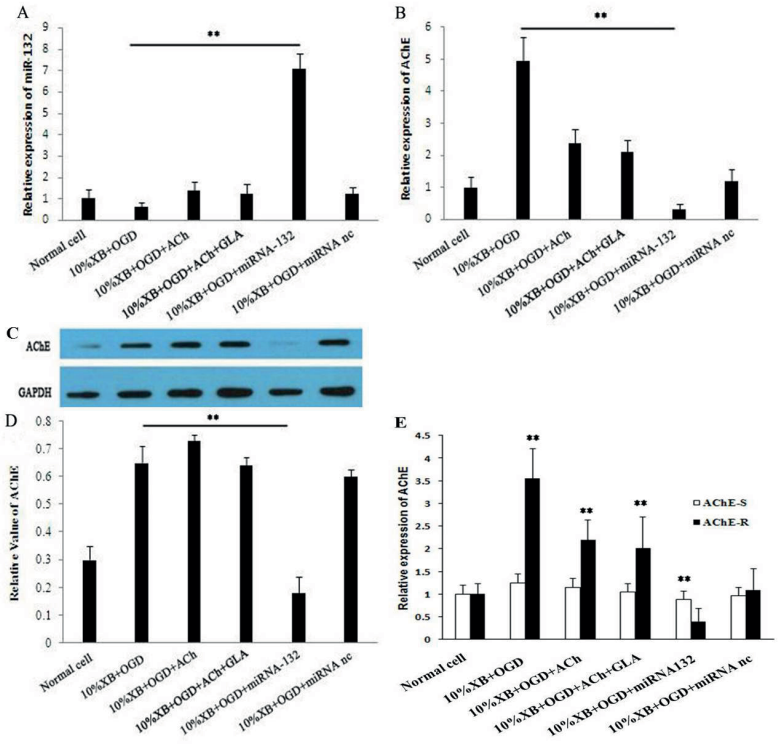

Figure 5: Investigation on the regulatory mechanism of miRNA-132 on the inflammatory pathway in rat renal macrophages of the hypoglycemia and hypoxia groups by testing (A) the relative expression of miRNA-132 in macrophages by using quantitative PCR, (B) the relative expression of AChE mRNA in renal macrophages, (C) the relative expression of AChE protein in renal macrophages, (D) The expression level of AChE protein was quantitatively analyzed, and the results are presented as mean ± SD, * P < 0.05, ** P < 0.01.

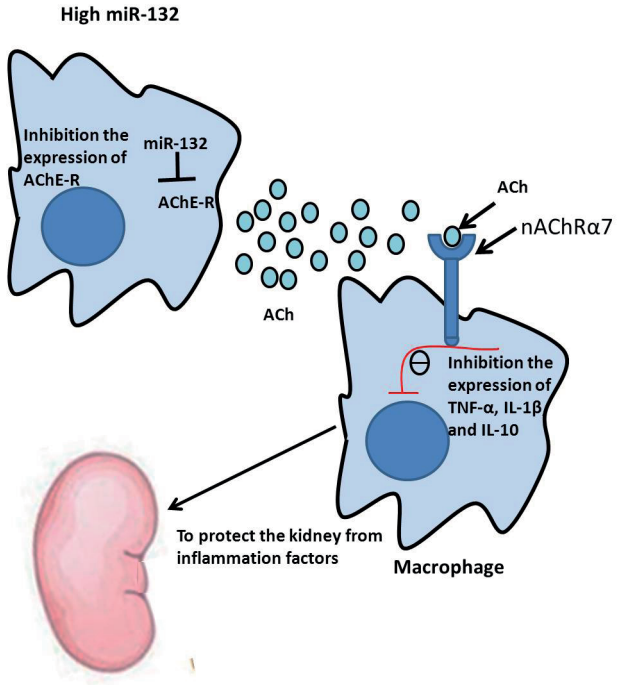

Figure 6: A schematic of the cholinergic anti-inflammatory impact. ACh-mediated anti- inflammatory response was increased by miRNA-132 in the renal macrophage. MiRNA-132 can target the 3'-UTR of AChE mRNA to inhibit AChE expression and decrease the hydrolysis of ACh. nAChRα7 in monocyte– macrophages is activated by ACh to subsequently inhibit the activation of monocyte– macrophage and reduce the release of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-10. MiRNA-132 can inhibit macrophage activation through the role of the above mentioned and protect the kidney.

4 Discussion

It has become evident that microRNAs (miRNAs) are pivotal regulators of many biological processes, including inflammation [12]. Our study has proven that the ACh-mediated anti-inflammatory response was increased by miRNA-132 in renal macrophage–renal epithelial cell system of AKI caused by hemorrhagic shock in rats. Alleviation of inflammatory response, neovascularization, and tissue repair, were promoted by macrophages in the specific microenvironment [6]. The microenvironment of renal macrophages was altered resulting from the release of large number of inflammatory mediators when the kidney is chemically injured. A large nuber of inflammatory mediators, particularly some pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-10, TGF-β, IL-4 and IL-6) were all activated and released, resulting in self-destructive systemic inflammation that eventually developed into MODS [13,14]. Our study found that the expression level of inflammatory factors, such as TNF-α, IL-1β, IL-10, TGF-β, IL-4 and IL-6 was significantly higher in transfected cells than those in normal cells and cells treated with antagonists, such as ACh and Gal.

The cholinergic anti-inflammatory pathway is a neural regulatory pathway, whereas synthesis and release of inflammatory factors is effectively reduced, resulting in significant inhibition of systemic and local inflammatory responses when this pathway is activated [15,16]. nAChRα7 in monocyte–macrophages is activated to subsequently inhibit the activation of monocyte–macrophage and reduce the release of pro-inflammatory cytokines, such as TNF-α. Acetylcholinesterase is not only present in neuronal cells but also in immunocytes [17]; the highest expression was observed in macrophages [18]. Whenever an inflammatory response occurs, ACh is rapidly hydrolyzed by AChE secreted from macrophages to prevent the inhibitory effect of ACh on macrophage activation. MiRNA-132 expression is upregulated in the human monocyte–macrophage cell line THP-1 processed by LPS, whereas miRNA-132 acts as the ligand of immune response involved in regulation of TLR signal molecules and activation of NF-KB pathway, demonstrating the close relationship between miRNA-132 and inflammatory response [19]. MiRNA-132 can target the 3'-UTR of AChE mRNA to inhibit AChE expression and regulate cholinergic anti-inflammatory pathway accordingly [20].

The hydrolytic activity of AChE is presumed to be reduced by miRNA-132 via the inhibition of AChE expression in renal macrophages, improving the microenvironment in the kidney, inducing the conversion of renal macrophages into type M2, promoting repair of renal tubule and renal interstitium and improving renal function [20,21]. Our investigation found that after the renal epithelial cells with transfection and stable expression of miRNA-132 are placed over the macrophages of a kidney injured by hypoglycemia and hypoxia, the expression levels of TNF-α, IL-1β, IL-10, TGF-β, IL-4 and IL-6 in cell culture supernatant are significantly inhibited. Moreover, the expression level of miRNA-132 in the double cell culture system is significantly higher than that in other experimental groups. The cholinergic anti-inflammatory effect of miRNA-132 results from targeted inhibition of the hydrolysis of AChE on ACh, whereas our results demonstrated that the expression level of AChE mRNA and protein in renal macrophages that stably expresses miRNA-132 is significantly reduced. Several splice variants of AChE are known (e.g., AChE-S, -R), which result in forms that differ in solubility and subcellular localization. AChE-R furthermore exhibits a unique C-terminal sequence, which is responsible for nonenzymatic actions of this variant [22]. It is involved in proliferation, apoptosis and development of various

cell types in different organs, for example brain and hematopoietic cells [23]. In our study, we found increases in miRNA-132 can suppress AChE-R, whereas AChE-R suppression would elevate synaptic ACh, increasing the cholinergic anti-inflammatory effect.

Our study has proven that potential regulation of the inflammatory response by miRNA-132 in renal macrophages possibly plays a preventive, therapeutic, and protective role toward kidney injury caused by hemorrhagic shock.

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