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Inhibitive effects of microRNA-34a on protecting against ischemia-reperfusion injury of vital organs in hemorrhagic shock pregnant mice

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Abstract. – OBJECTIVE: Hemorrhagic shock is a common vital condition in obstetrics, and major treatment consists of bleeding control and liquid resuscitation. MicroRNA (miR) has been found to regulate multiple diseases. However, its expression profile in hemorrhagic shock or effects on the ischemia-reperfusion injury in pregnant mice has not been reported yet.

PATIENTS AND METHODS: This study generated rat hemorrhagic shock pregnant model, on which real-time quantitative PCR was used to measure miR-34a expressions. MiR-34a inhibitor was applied to specifically suppress miR-34a expression. Serum malondialdehyde (MDA) and superoxide dismutase (SOD) levels were measured by using the commercial kit. Ischemia-reperfusion injury on rat kidney, lung, liver and intestine tissues was evaluated by using hematoxylin-eosin (HE) staining.

RESULTS: In a hemorrhagic shock pregnant rat model, miR-34a expression level was significantly elevated compared to the Normal group ($p < 0.05$). Application of miR-34a inhibitor effectively suppressed the miR-34a expression in rat kidney, lung, liver and intestine tissues ($p < 0.05$ compared to normal group). Model rats also had significantly elevated serum MDA and significantly lower SOD levels compared to Normal group ($p < 0.05$). miR-34a inhibitor reversed this abnorma lity to certain extents ($p < 0.05$ compared to model group). HE results showed ischemia-reperfusion damage in rat kidney, lung, liver and intestine tissues. miR-34a inhibitor improved such injury.

CONCLUSIONS: Suppression of miR-34a could alleviate multi-organ damage after re-perfusion of hemorrhagic shock pregnant rats, probably due to the suppression of oxidative stress. Suppression of miR-34a might work as the treatment target treating multi-organ damage caused by hemorrhagic shock.

Key Words:
miR-34a, Postnatal hemorrhagic shock, Ischemia-reperfusion.

Introduction

Hemorrhagic shock is the most common subtype of shock in obstetrics, and is one critical condition in clinics[1]. Due to significantly increased body blood volume and hyper-coagulated status of pregnant body, plus decreased hemorrhagic sensitivity during pregnancy due to placenta-derived hormones, clinical symptoms of hemorrhagic frequently present only until late stage. Therefore, even with timely resuscitation, a certain number of hemorrhagic shock cases cannot survive at pregnant stage[2]. As a result, postnatal hemorrhagic shock is one major challenge and critical condition for clinicians. Moreover, re-perfusion after long-term ischemia may also lead to secondary multi-organ failure, causing ischemia-reperfusion damage[2]. Therefore, the investigation of ischemia-reperfusion damage and protective mechanism, as well as possible treatment approach is necessary for clinics.

MiRNA (miR) is one non-coding RNA consisting of 19-25 nucleotides, and can bind with 3'-untranslated region of target mRNA to mediate target gene expression[3]. MiRNA has been recognized to be related to multiple pathological and physiological processes, including tissue/organ development, organogenesis, cancer, and other diseases[4]. In recent years, further investigation of miRNA revealed its role in tissue injury and protection, among which ischemia-reperfusion is one critical aspect. MiR-34a was firstly identified by scholars in Southwestern Medical Center, Texas University, and has been found to have pluripotent physiological activities[5], covering from osteoclast formation[6], non-alcoholic fatty liver[7], and ischemia-reperfusion of intestine[8]. However, its role in ischemia-reperfusion damage of vital organs after post-natal hemorrhagic shock has not been reported yet.

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This study aims to investigate the role of miR-34a in ischemia-reperfusion damage on vital organs under postnatal hemorrhagic shock. We employed a rat model to mimic human postnatal hemorrhagic shock and reperfusion, and elucidated the role of miR-34a in ischemia-reperfusion damage at animal level with possible mechanisms.

Materials and Methods

Major Materials and Reagents

Chloralose and urethane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Total protein extraction kit was purchased from Kaiji Biotech. Ltd. Co. (Jiangsu, China). Assay kits for malondialdehyde (MDA) and superoxide dismutase (SOD) were purchased from Jiancheng Biotech. Co. (Nanjing, China). Reverse transcription and real-time quantitative PCR kits were purchased from Toyobo Co., Ltd., (Osaka, Japan). Hematoxylin-eosin (HE) staining kit was purchased from Zhongshan Biotech. Co., (Beijing, China).

Major Equipment

PCLAB-UE biomedical signal processing system was purchased from Weixin Sida Tech (Beijing, China). Gel imaging system UVP Multispectral Imaging System (UVP, Sacramento, CA, USA). PS-9 semi-dry transfer electrophoresis apparatus was purchased from Jingmai Int. (Jiangsu, China). CO_2 incubator and thermos-354 microplate reader was purchased from Thermo Fisher Scientific (Waltham, MA, USA). HE staining and slices were prepared by Pathology Department of our hospital.

Experimental Animal

Male and female Sprague-Dawley (SD) rats (2 months old, n=30) were provided by Laboratory Animal Center of Fourth Military Medical University. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Fengxiang County Hospital.

Experimental Group and Model Preparation

Normal female SD rats (n=30) were mated with normal SD male rats at 1:1 ratio. On the next morning, virginal smear was prepared by saline for microscopic observation, and those found sperms were designated as day 0. On pregnant day 14, females were randomly assigned into three groups: normal pregnant group (A), hemorrhagic shock model group (B) and miR-34a inhibitor treatment group (C). Rats were anesthetized with chloralose and urethane, and a longitudinal incision was made in the middle of lower abdomen after stable blood pressure and heat beat. The uterus was dissected to remove the fetus, and was sutured for closing the abdominal cavity after controlling hemorrhage. The pregnant model of hemorrhagic shock was prepared as previously described. Cannulation was performed on bilateral femoral arteries. One cannula was connected to barometer for monitoring average artery pressure. Blood was collected from the other cannula to replicate hemorrhagic shock model. Blood drainage was performed until reaching 40-45 mmHg for maintenance of 30 min. Blood was heparinized for back-perfusion. 30 min after hemorrhage, heparinized blood was mixed with 2× volume of Krebs solution for back-transfusion via external jugular vein until the blood pressure reached above 80 mmHg. Group C rats received miR-34a inhibitor 2 h before surgery, and were processed as identical in group B.

Real-time Quantitative PCR

Rat kidney, lung, liver, and intestine tissues (100 mg each) were collected in 1 ml TRIzol for 5 min iced incubation, followed by tissue homogenization. The supernatant was collected after centrifugation. Supernatant from each sample was transferred into 1.5 ml Eppendorf (EP) tubes, which were added with 200 μl chloroform for 15 s vigorous mixture. After 3 min room temperature and 12000 ×g centrifugation for 15 min at 4°C, the upper aqueous phase was saved and mixed with 500 μl isopropanol for 10 min room temperature. After 12000 × g centrifugation for 15 min at 4°C, the supernatant was discarded, and the pellet was rinsed for three times in 1 ml ethanol. The supernatant was carefully removed, and mRNA was dissolved in 20 μl DEPC treated water.

MiR-34a primer was designed and synthesized by Sigma-Aldrich (St. Louis, MO, USA), and U6 RNA was used as the internal reference. Primer sequences were shown in Table I. PCR was performed in a 50 μl system following the manual instruction of test kit. Reaction parameters were: 50°C for 30 min and 95°C for 5 min, followed by 40 cycles each consisting of 95°C for 30 s, 55°C for 30 s and 72°C for 50 s, ended with 72°C elongation for 5 min. Amplification and dissolving

Table I. Primer sequence.

Name	Forward primer	Reverse primer
MiR-34a	5'-CGT CAC CTC TTA GGC TTG GA-3'	5'-CAT TGG TGT CGT TGT GCT CT-3'
U6	5'-CTC GCT TCG GCA GCA CAT ATA CT-3'	5'-ACG CTT CAC GAA TTT GCG TGT TC-3'

curves were confirmed, and relative expression level was calculated based on Ct values of target gene and housekeeping gene. Gene expression was quantified by $2^{-\Delta\Delta Ct}$ approach.

Assays for MDA and SOD Levels

The MAD level was quantified by thiobarbituric acid method[9], while SOD was measured by xanthinoxidase method[10] following manual instruction of test kit. A microplate reader was used to determine the absorbance value. Serum MDA and SOD levels were measured from rat serum in triplicates, and results were presented as mean ± standard deviation (SD).

Plotting of Survival Curves

24 h survival time of rats was recorder after ischemia-reperfusion treatment for plotting survival curves.

HE Staining

Harvested tissues were fixed in 4% paraformaldehyde for 24 h, and were dehydrated in gradient ethanol (100%, 95%, 85%, and 75%). Tissues were permeabilized in turpentine, and were embedded in paraffin for embedding. Tissue blocks were sectioned (4 μm thickness). After dewax in xylene and re-hydration by gradient ethanol, tissues were rinsed twice in distilled water, and were stained wiyh hematoxylin. Tissues were rinsed under tap water, and were differentiated in 1% HCl-ethanol, followed by eosin staining, and tap water rinsing. Gradient ethanol was used for dehydration and permeabilization in turpentine. After mounting the cover-slips, tissues were observed under a microscope.

Statistical Analysis

All test data came from at least three independent experiments. Data were presented as mean ± standard deviation. The Student *t*-test was employed for comparison between two groups. The multi-group comparison was performed using one-way analysis of variance. The paired comparison was performed by SNK approach. A statistical significance was defined when $p < 0.05$.

Results

Rat Pregnancy, Experimental Group and General Conditions

A total of 30 pregnant rats were randomly assigned into 3 groups (n=10 each), including normal pregnant group (A), hemorrhagic shock model group (B) and miR-34a inhibitor pre-treatment group (C). Before model preparation, all pregnant rats presented good living status without mortality.

Elevated microRNA-34a Expression in Hemorrhagic Shock Pregnant Rats

Real-time quantitative PCR was employed to test miR-34a expression in kidney, lung, liver and intestine in all hemorrhagic shock model pregnant rats. As shown in Figure 1, tissue expression of miR-34a was expressed to certain extents in group B, with statistical significance compared to those in group A.

Specific Inhibition of microRNA-34a in Hemorrhagic Shock Model and microRNA-34a Expression Level

To demonstrate successful inhibition of miR-34a in hemorrhagic shock model, we measured miR-34a expression level in kidney, lung, liver,

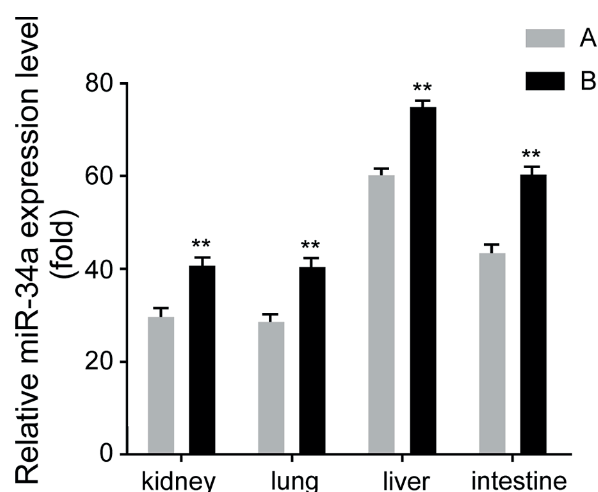

Figure 1. MiRNA-34a expressional profile in hemorrhagic shock pregnant rat model. Normal pregnant group *(A)*. Hemorrhagic shock model group *(B)*. **, $p < 0.05$ compared to group A.

and intestine tissues. As shown in Figure 2, compared to control group, miR-34a specific inhibitor application suppressed miR-34a expression level to different extents.

Serum MDA and SOD Level After miR-34a Suppression

As shown in Figure 3, compared to normal control group, ischemia-reperfusion pregnant rats had significantly elevated serum MDA level but lower SOD levels, all with significant difference against normal control group. After applying miR-34a inhibitor, serum MDA and SOD levels were significantly elevated and suppressed, respectively, compared to hemorrhagic shock plus reperfusion pregnant rats. These results showed that suppression of miR-34a can potentiate anti-oxidation potency of cells and tissues to certain extents.

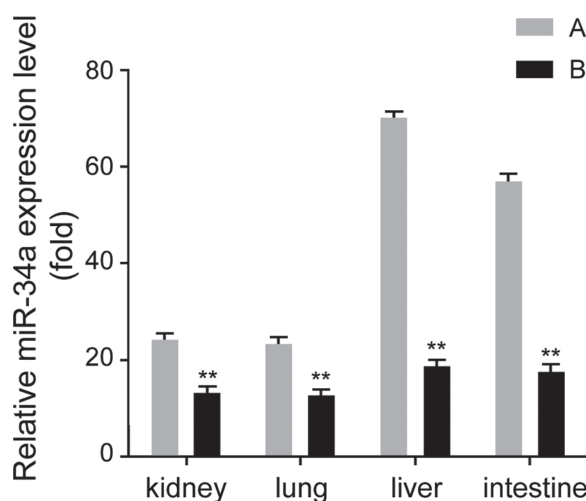

Figure 2. MiR-34a expression after specific inhibitor application. Normal pregnant group *(A)*. Hemorrhagic shock model group *(B)*. **, $p < 0.05$ compared to group A.

Survival Curve After miR-34a Inhibition

After generating hemorrhagic shock and reperfusion model, we recorded rat survival conditions and plotted survival curve as shown in Figure 4. Normal pregnant rats had good survival conditions without any mortality. In both hemorrhagic shock model and miR-34a inhibitor pre-treatment group, mortality was found. Nevertheless, miR-34a inhibitor significantly elevated rat survival rate.

Tissue Morphology Change of Vital Organs in Hemorrhagic Shock Pregnant Rats After miR-34a Inhibition

We employed HE staining approach to observe morphological change of kidney, lung, liver, and intestine tissues. Tissue sections of kidney were shown in Figure 5A. In brief, group A showed normal gross morphology, whilst group B showed glomerulus deformation, edema, and vacuoles, accompanied with inflammatory cell infiltration, mesenchymal widening; group C showed significant improvement compared to group B.

Lung tissues sections were shown in Figure 5B. Group A had normal solid and mesenchymal tissue structures of lung, with complete vacuolar cavity and no change of intra-alveolar mesenchyme. However, group B showed partial destruction of alveolar structure, with edema and thickening of intra-alveolar mesenchyme, plus prominent in-

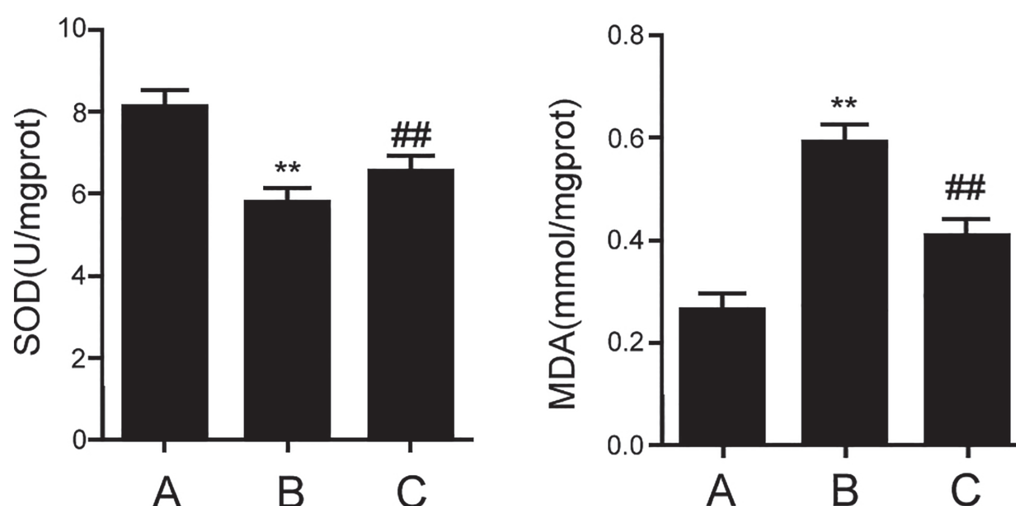

Figure 3. Serum MDA and SOD levels after miR-34a inhibition. Normal pregnant group *(A)*. Hemorrhagic shock model group *(B)*. miR-34a inhibitor pre-treatment group *(C)*. **, $p < 0.05$ compared to group A. $^{##}$, $p < 0.05$ compared to group B.

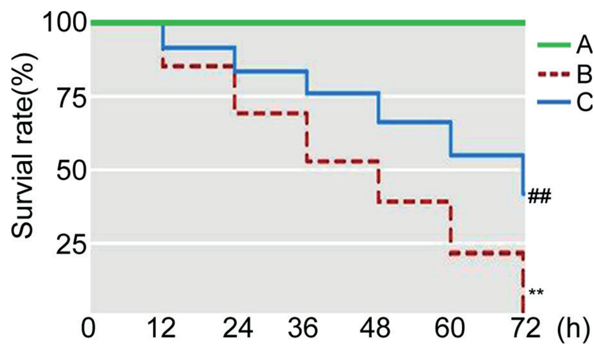

Figure 4. Rat survival curves after miR-34a inhibition. Normal pregnant group *(A)*. Hemorrhagic shock model group *(B)*. miR-34a inhibitor pre-treatment group *(C)*. **, $p < 0.05$ compared to group A. ##, $p < 0.05$ compared to group B.

filtration of neutrophils. Inside alveolar cavity, few erythrocyte and tissue exudate fluid can be observed. Compared to group B, group C rats showed weakening of alveolar mesenchymal edema, tissue thickening, neutrophil infiltration, and alveolar cavity bleeding or exudate, as well as improved condition of alveolar destruction.

Liver tissue sections were shown in Figure 5C. Group A showed regular arrangement of hepatocytes without significant edema. Group B showed prominent aqueous denature of hepatocytes with little basophilic denature. Compared to group B, group C liver tissues showed significant improvement of aqueous denaturation on hepatocytes.

Figure 5. Ischemia-reperfusion damage of rat kidney, lung, liver, and intestine after miR-34a inhibition. Normal pregnant group *(A)*. Hemorrhagic shock model group *(B)*. miR-34a inhibitor pre-treatment group *(C)*. **, $p < 0.05$ compared to group A. ##, $p < 0.05$ compared to group B. (×200).

Intestine tissue sections from all groups were shown in Figure 5D. Group A showed prominent intestine cilia development with regular arrangement. No hyperemia or inflammatory cell infiltration was observed. In group B, submucosal layer showed prominent cilia breakage or shrinkage, plus significant infiltration of inflammatory cells, with thinning of muscular layer and focal bleeding. Compared to group B, group C showed significantly alleviated ischemia-reperfusion damage.

Discussion

Hemorrhagic shock is the most common subtype of shock in obstetrics and is one major severe symptom. Reperfusion injury due to blood transfusion of postnatal hemorrhagic shock is also a major concern for obstetrics. With recent research of non-coding RNA, especially miRNA, we speculated possible involvement of miRNA in reperfusion injury after postnatal hemorrhagic shock. MiR-34a is one miRNA molecule with relatively fruitful research. It was initially identified by the Southwest Medical Center, Texas University (Dallas, TX, USA). Recent studies showed its participation in malignant tumors including breast cancer[11], oral cavity cancer[12] and acute myeloid leukemia[13], and its involvement in multiple ischemia-reperfusion injuries[14,15]. Reperfusion injury caused by postnatal hemorrhagic shock frequently manifested severer than normal reperfusion damage, as it leads to injury on more than single organ. Due to huge bleeding volume, the recovery of blood supply may cause secondary multi-organ failure. Whether the suppression of miR-34a played a protective role against such multi-organ damage thus attracted our interests. Therefore, this study utilized pregnant rats, which were dissected and bleeding until shock to establish hemorrhagic shock, followed by reperfusion, in order to mimic human postnatal major bleeding shock and consequent reperfusion injury. Real-time quantitative PCR was used to measure miR-34a expression in major tissues, and found significant elevation of miR-34a across those tissues. We thus speculated that miR-34a up-regulation might be one of the mechanisms underlying causing multi-organ damage after ischemia-reperfusion. So, we introduced miR-34a inhibitor via intravenous injection to specifically suppress miR-34a expression in following mechanistic study. Real-time quantitative PCR was employed to confirm ischemia-reperfusion damage of major tissues after miR-34a suppression. Our results showed that the inhibition of miR-34a expression significantly decreased tissue ischemia-reperfusion damage of rat kidney, lung, liver, and intestine tissues. We proposed that inhibition of miR-34a had protective roles on ischemia-reperfusion damage on vital organs of hemorrhagic shock pregnant rats.

Previous studies showed that miR-34a could sensitize wild-type p53 cancer cells for oxidative stress or treatment agent via down-regulating Sirt1/PGC-1α/Nrf2 pathway[16]. Some scholars[17-19] proposed that miR-34a expression level was probably positively correlated with oxidative stress level. Similarity, some scholars[20,21] claimed that postnatal hemorrhagic shock and consequent reperfusion produced abundant free oxygen radicals from vascular endothelial cells, and such free oxygen radicals could protect ischemia-reperfusion damage. Therefore, in this study, we measured lipid peroxidation product MDA reflecting free oxygen radicals production[22,23], and SOD level mirroring tissue clearance potency for free oxygen radicals[17,24]. MDA down-regulation and SOD potentiation all reflected body oxidation stress level and systemic injury condition of ischemia-reperfusion. Test results showed that inhibition of miR-34a could suppress oxidation stress, thus alleviating tissue injury. These results were in line with previous studies, although how miR-34a participated in the regulation of oxidative stress still requires further elucidation.

Conclusions

Suppression of miR-34a can alleviate multi-organ damage caused by reperfusion on pregnant rats with hemorrhagic shock, probably via inhibiting oxidative stress. The inhibition of miR-34a thus may work as one treatment target for multi-organ injury caused by hemorrhagic shock.

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Conflict of Interest
The Authors declare that they have no conflict of interests.

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