

Original Article

Propofol inhibits microRNA-17 expression in vascular endothelial cells during ischemia-reperfusion

Zhijie Xu, Zhou Wang, Zhigang Wang, Wenbo Yi, Huanliang Wang, Jingui Yu

Department of Anesthesiology, Qilu Hospital of Shandong University, Jinan 250012, P. R. China

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Abstract: This study is to investigate the role of miR-17 in propofol induced protective effects in vascular endothelial cells. Hypoxia-reoxygenation (H/R) model was established in human umbilical vein endothelial cells (HUVECs) to mimic the *in vivo* situation of ischemia-reperfusion, and the cells were treated with propofol or not. Expression of miR-17 was analyzed using Real-time PCR. HUVECs were transfected with miR-17 inhibitor or scramble control and subjected to H/R. Cell viability and apoptosis was detected by CCK8 assay and flow cytometry. Western blot was performed to analyze apoptosis-related proteins. Target of miR-17 was predicted by bioinformatics analysis and verified by Dual-luciferase reporter gene assay. Expression of miR-17 was decreased after propofol treatment. Blocking endogenous miR-17 significantly increased cell viability and decreased apoptosis rate, which was consistent with the effect of propofol treatment. Blocking miR-17 expression and propofol treatment after H/R inhibited the expression of Bax but upregulated Bcl-2 expression. In addition, 3'untranslated region (3'UTR) of signal transducer and activator of transcription 3 (STAT3) has a putative binding site for miR-17. MiR-17 was able to directly bind to the STAT3 3'UTR and negatively regulate the expression of STAT3 protein. Taken together, these data show that propofol inhibits miR-17 expression and further alleviates vascular endothelial cell injury during ischemia-reperfusion by reducing apoptosis of vascular endothelial cells *in vitro*.

Keywords: Signal transducer and activator of transcription 3 (STAT3), miR-17, endothelial cells, ischemia/reperfusion (I/R) injury

Introduction

Reperfusion of ischemic tissues or cells is the most common pathologic process in operative surgery, leading to widespread microvascular dysfunction and tissue/cell injury which in turn may affect prognosis and may directly endanger the patients' life [1, 2]. Nutrients carried in the blood are released to tissues via the permeable endothelium of blood vessels, and blood vessel damage is the basic pathologic process in ischemia/reperfusion (I/R) injury [3]. Endothelial cells are important barriers between the blood vessels and tissues. Studies have shown that the vascular endothelium is a crucial site that is affected by I/R injury, and the apoptosis of endothelial cells prior to that of tissue cells [4-6]. Damage of vascular endothelial cells leads to the infiltration of intravascular inflammatory mediators and inflammatory cells directly into the tissue, and as a consequence aggravates tissue damage [7]. Therefore, it is

of great clinical significance to study the molecular mechanism of I/R injury.

Propofol is a common intravenous anesthetic, and it is widely used because of the minimal side effects, a quick onset, fast metabolic rate *in vivo* and controllable anesthetic state [8]. Evidences have shown that propofol is involved in the protection of many organs during surgery, including brain, lung, spinal cord, cardiovascular, kidney and etc [9-11]. Although many progresses have been gained in the molecular mechanisms of propofol's protective effects on tissue I/R injury, there are still many aspects to be investigated. For example, investigation of genes involved in this process is of great significance for drug development and clinical application in ischemia-reperfusion injury.

MicroRNAs (miRNAs) are an evolutionarily conserved family of short noncoding RNAs at the length of 18-22 nt, which regulate genes via

binding to the 3'-untranslated region (UTR) of their target mRNAs [12]. Studies provide an overview of the role of miRNAs in changing cell activities induced by propofol treatment. For example, propofol can effectively induce apoptosis of epithelial ovarian cancer (EOC) cells by upregulating miR-let-7i [13]. In addition, propofol may have antitumor potential in osteosarcoma, which is partly due to the downregulation of MMP-13 expression by miR-143 [14]. However, miRNAs that are associated with the protective effect of propofol on I/R injury remain largely unknown.

As a member of the miR-17/92 cluster, miR-17 was initially found abnormally amplified in diffuse cell lymphomas. Subsequent studies reveal that miR-17 acts as an oncogene which is related to the development of many tumors [15, 16]. On the other hand, miR-17 is highly expressed in embryonic cells and has an important role in the development of many tissues including heart, lung and brain [17, 18]. In this study we intend to investigate the molecular mechanism of miR-17 in the protective effect of propofol on vascular endothelial cell I/R injury and to provide experimental evidence for clinical application of propofol as a protect drug for suppressing I/R injury.

Materials and methods

Construction of H/R model in HUVEC

For hypoxia, the culture media was replaced by synthetic ischaemia solution as described before [19] and the HUVECs were then placed in hypoxic conditions with 5% CO₂ and 95% N₂ at 37°C for 24 h. After hypoxia, the medium was washed off, and the HUVECs were returned to reperfusion solution as described before [19] and cultured with 5% CO₂ and 95% O₂ for 6 h. At the same time, prepared propofol was added to the medium at the concentration of 150 µM.

RNA extraction and real-time PCR assay

The total RNA from each treatment was extracted using TRIzol® isolation reagent (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacture's construction. Following gel electrophoresis verification of RNA integrity and quantification using UV spectrophotometer, 0.5 µg of total RNA was reverse transcribed

into cDNA using a miRNA cDNA Kit (TAKARA, Dalian, China) with specific primers. The expression of small nuclear U6 was used as internal control. Quantitative Real-time PCR was performed using KAPA SYBR FAST qPCR Kits (Kapa biosystems, Boston, Massachusetts, USA). Forward primer used for miR-17 amplification was 5'-CAAAGTGCTTACAGTGCA-3', and the reverse primer is provided by kit. Primers used for amplification of U6 are as follows, forward: 5'-CTCGCTTCGGCAGCAC-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'. The relative expression levels were evaluated using the 2^{-ΔΔCt} method.

MiRNA transfection of HUVEC

Human HUVECs were maintained in DMEM (Thermo Fisher Scientific, Vilnius, Lithuania) supplemented with 10% FBS. Cells were seeded at 24-well plate and incubated to a contingency of 70%-90% prior to transfection with 31.25 µM of miR-17 mimics or miR-17 inhibitor using Lipofectamine 2000 (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer's instructions. Negative control (NC) without transfection was set up. Cells were collected at 48 h post-transfection for further experiments or subjected to H/R treatment.

CKK-8 assay

After hypoxia culture, HUVECs were returned to reperfusion solution and cultured with 5% CO₂ and 95% O₂ for 6 h. After washing twice with PBS, cells were suspended in fresh DMEM containing 10% of Cell Counting Kit-8 (Biyuntian, Beijing, China) and incubated for 1 h at 37°C. The absorbance values at 450 nm in each well were measured with ELx800 (BioTek, Winooski, Vermont, USA).

Annexin V-FITC staining and flow cytometry analysis

Cells were harvested after trypsin digestion, washed with PBS twice and stained by Annexin V-FITC Apoptosis Detection Kit I (BD bioscience, San Jose, CA, USA) according to the manufacturer's instruction. Cells were immediately analyzed by flow cytometry. Cells undergoing apoptosis were Annexin V positive and PI negative, and cells that were necrosis were Annexin V negative and PI positive. Cells observed to be Annexin V and PI positive were in end stage apoptosis or already dead.

Propofol suppresses miR-17 to ameliorates endothelial cell injury

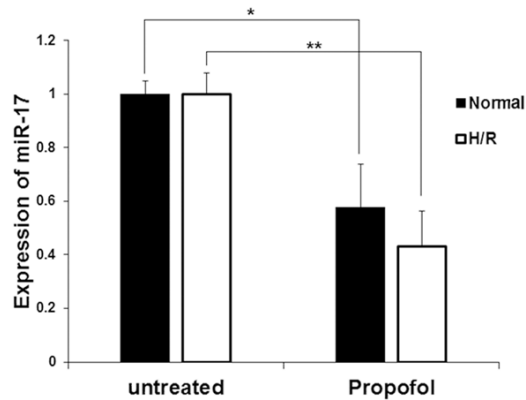


Figure 1. Expression level of miR-17 in HUVECs after propofol treatment. Normal HUVECs or HUVECs administrated with H/R were treated with propofol or left untreated, and miR-17 expression was analyzed by Real time PCR. * $P < 0.05$, ** $P < 0.01$, t test, compared with untreated cells.

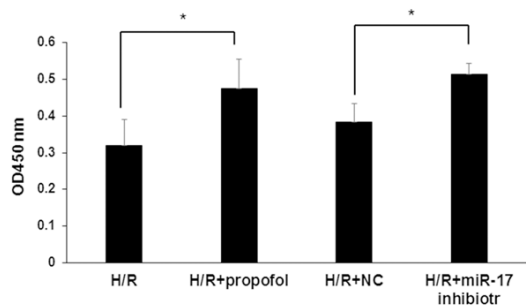


Figure 2. Suppression of miR-17 increases HUVEC cell viability. HUVECs administrated with H/R were treated with propofol (H/R+propofol) or left untreated (H/R). In the latter two groups, HUVECs administrated with H/R were transfected with miR-17 inhibitor (H/R+miR-17 inhibitor) or scramble (H/R+NC). Cell viability was analyzed by CCK8 assay. * $P < 0.05$, t test.

Western blot analysis

Cells after propofol treatment or transfection were collected and re-suspended in RIPA lysis buffer with 1% PMSF to extract the total protein. Each sample was centrifuged at $12,000 \times g$ for 10 min at 4°C for collecting the supernatant. Protein assay kit (Biyuntian, Beijing, China) using bovine serum albumin (BSA) as the standard was used to measure the total protein. Protein abundance of Bcl-2, Bax, Stat3 and GAPDH were measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting.

Dual-luciferase reporter gene assay

Luciferase reporter plasmids were generated by insertion of wildtype or mutant 3'UTR sequences of STAT3 into the multiple cloning site (Spe-1 and HindIII) downstream of the luciferase reporter gene in the pMIR-REPORT™ Luciferase (Thermo Fisher Scientific, Vilnius, Lithuania). The miR-17 seed-site in the 3'UTR of STAT3 was mutated to remove miR-17 binding site. HUVECs were seeded at a concentration of 5×10^4 cells per well in 24-well plates and transfected with 1 μg constructed luciferase reporters and 10 ng pMIR-REPORT™ β -gal Control Plasmid as an internal control for transfection efficiency. And 100 nmol miRNA mimics or negative control RNA were also transfected. Luminescence was measured at 24 h after transfection using the Dual-Luciferase® Reporter Assay System (Sigma Chemical Co., St. Louis, MO, USA) according to the manufacturer's instructions. Measurements of luminescence were performed on the luminometer (Gloma $\times 20/20$, Promega, Madison, WI, USA).

Statistical analysis

All data were presented as mean $\pm$ S.D. of three experiments. Statistical comparisons between groups were performed using the Student's t-test. A p -value of less than 0.05 was considered statistically significant.

Results

MiR-17 expression after propofol treatment

We first evaluated the expression of miR-17 upon propofol treatment. Using Real-time PCR analysis, we found that miR-17 expression was not obviously changed in normal HUVECs and HUVECs subjected to H/R. However, its expression was significantly reduced after propofol treatment in both normal cells and cells stimulated with H/R (**Figure 1**) ($P < 0.05$). These results indicated that miR-17 may play a role in the protection of invascular endothelial cell injury induced by H/R.

Mir-17 increased the viability of HUVECs

CCK-8 assay showed that after exposure to H/R, propofol treatment significantly increased cell viability ($P < 0.05$). Transfection with miR-17 inhibitor resulted in upregulation of cell viability

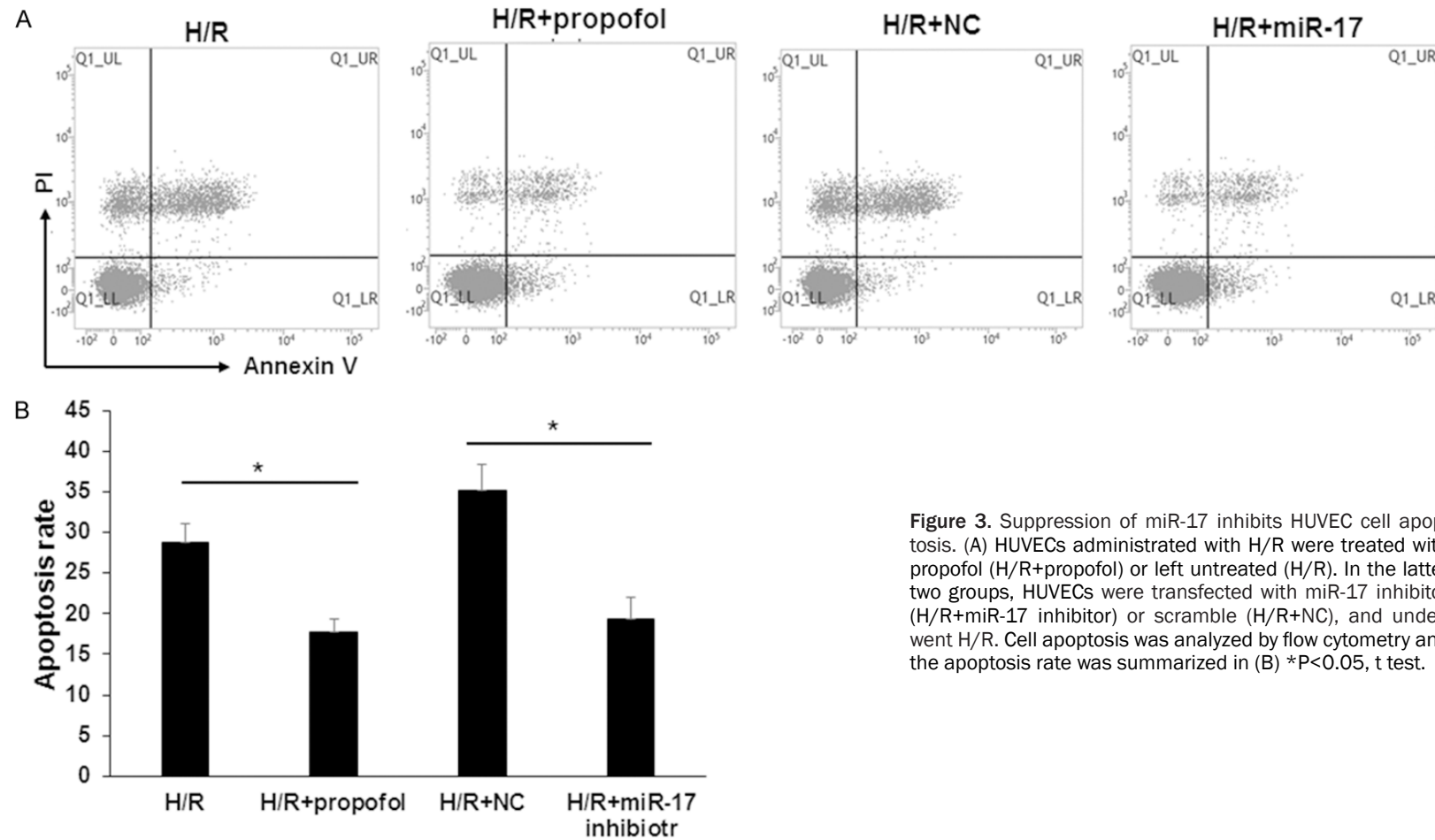


Figure 3. Suppression of miR-17 inhibits HUVEC cell apoptosis. (A) HUVECs administrated with H/R were treated with propofol (H/R+propofol) or left untreated (H/R). In the latter two groups, HUVECs were transfected with miR-17 inhibitor (H/R+miR-17 inhibitor) or scramble (H/R+NC), and underwent H/R. Cell apoptosis was analyzed by flow cytometry and the apoptosis rate was summarized in (B) *P<0.05, t test.

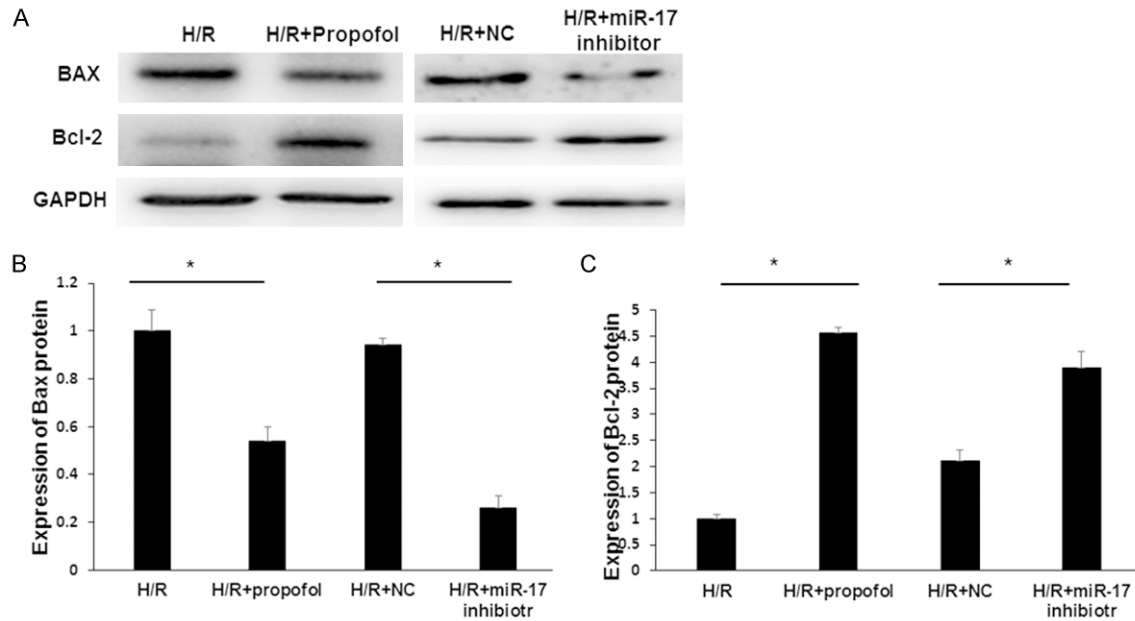


Figure 4. Western blot analysis of apoptosis-related proteins. A. HUVECs administrated with H/R were treated with propofol (H/R+propofol) or left untreated (H/R). HUVECs were transfected with miR-17 inhibitor (H/R+miR-17 inhibitor) or scramble (H/R+NC), and underwent H/R. Cell lysates were prepared and analyzed by Western blot for Bax1, Bcl-2 and GAPDH. B. Relative Bax1 protein expression. C. Relative Bcl-2 protein expression. * $P<0.05$, t test.

after exposure to H/R (Figure 2). These results suggested that administration of propofol could protect HUVECs against H/R, and this protection may dependent on the regulation miR-17 expression.

Effect of miR-17 on apoptosis

To confirm the inhibitory effect of miR-17 inhibitor on cell apoptosis, HUVECs were transfected with miR-17 inhibitor or scramble, stained with Annexin V-FITC and propidium iodide, and then analyzed by flow cytometry after exposure to H/R. There was an obvious reduction in the number of apoptotic cells when propofol was administrated compared with the untreated H/R group ($P<0.05$) (Figure 3A and 3B). A similar reduction in the number of apoptotic cells was observed when cells were transfected with miR-17 inhibitor compared with that transfected with scramble (Figure 3A and 3B). We then examined the apoptosis-related proteins by Western blot. After H/R exposure, apoptotic protein Bax was significantly downregulated while anti-apoptotic protein Bcl-2 was upregulated upon propofol treatment (Figure 4A-C). In addition, the expression of these proteins was in the same tendency upon miR-17 inhibitor transfection after hypoxia treatment (Figure

4A-C). Overall, inhibition of miR-17 expression induced apoptosis of HUVECs, which may contribute to the protection effect of propofol.

STAT3 is a target of miR-17

Predicted by computational screening (Target-Scan, Whitehead institute for Biomedical Research, www.targetscan.org), we found that the 3'UTR of STAT3 contains multiple potential binding sites for miR-17. It is reported that STAT3 activation results in dysregulation of cell cycle control and abnormal expression of apoptosis genes, leading to cell apoptosis. Therefore, miR-17 may inhibit endothelial cell apoptosis through targeting STAT3. To determine whether miR-17 could directly target STAT3 in HUVECs, we performed Western blot and found that expression of STAT3 protein is significantly downregulated upon miR-17 mimic transfection (Figure 5, $P<0.05$).

To determine whether the observed reduction of STAT3 expression is directly driven by miR-17, reporter gene assays were performed. The 3'UTR of STAT3 was cloned into the pMIR-REPORTER Luciferase control vector. In addition, we generated a mutated reporter construct in which the miR-17 seed match sequence in

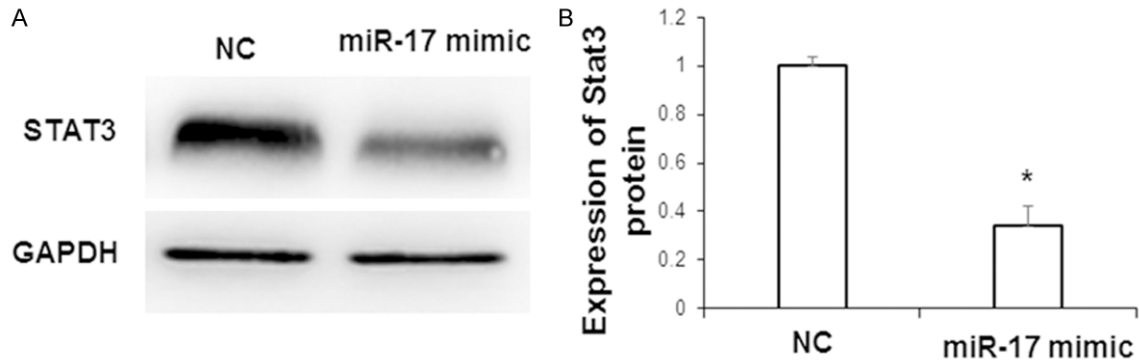


Figure 5. Western blot verification of miR-17 target. (A) HUVECs were transfected with miR-17 mimic or scramble (NC). Cell lysates were prepared and analyzed by Western blot for STAT3 and GAPDH. Relative STAT3 protein expression normalized to GAPDH expression was shown in (B). Representative Western blot results were shown. * $P < 0.05$, t test.

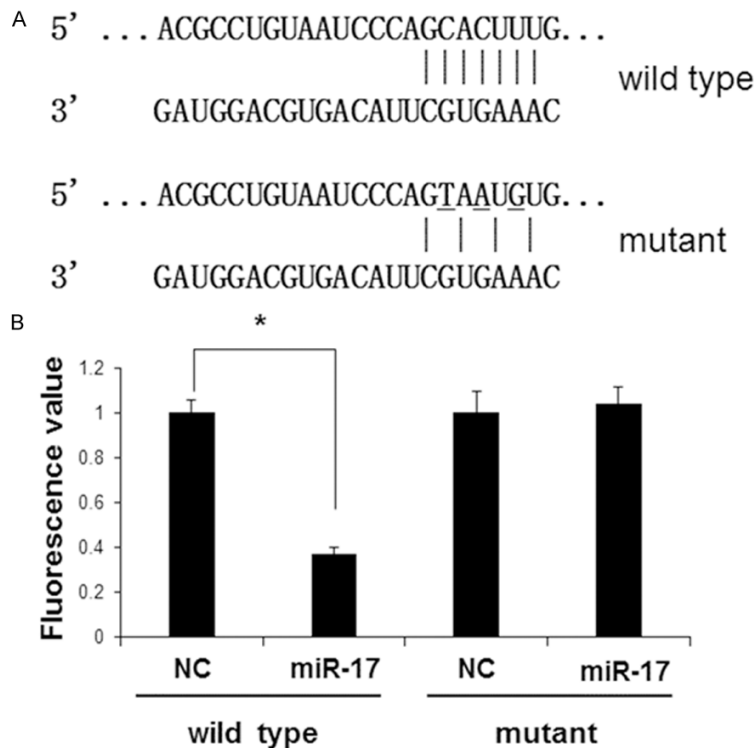


Figure 6. Luciferase reporter gene assay of miR-17 target gene. A. miR-17 binding site in the 3' UTR region of STAT3 mRNA and the mutated sequence in the luciferase reporter construct was shown, and the mutated nucleotide is shown underlined. B. The STAT3 3'UTR luciferase reporter was co-transfected with miR-17 mimic or scramble, and luciferase activities were assayed 24 h post transfection. The mutated STAT3 3'UTR luciferase reporter was co-transfected with miR-17 mimic or scramble, and luciferase activities were assayed 24 h post transfection. * $P < 0.05$ compared with NC, t test.

STAT3 3'UTR was mutated (Figure 6A). Co-transfection of the STAT3 3'UTR construct and miR-17 yielded a significantly reduced rela-

tive luciferase activity (Figure 6B, $P < 0.05$). In contrast, the mutated STAT3 3'UTR construct was not affected by overexpression of miR-17 compared with NC. These data demonstrated a direct interaction between STAT3 mRNA and miR-17.

Discussion

Ischemia-reperfusion contributes to the pathophysiology of many conditions which in turn may cause widespread microvascular dysfunction and tissue/cell injury [20]. Propofol is a widely used anesthetic agent with the notable advantages of minimal side effects and controllable anesthetic state [21]. It is reported that the molecular structure of propofol is similar to endogenous antioxidants, which could bind to free radicals and prevent oxidative damage [22]. In addition, propofol acts as an L-type calcium channel antagonist to decrease peripheral resistance and initiate hypotension [23].

Nowadays it is acknowledged that the mechanism underlying the tissue protection of propofol is far more complicated [24]. Guo et al.

reported that propofol may protect the brain cells from injury through regulating the expression of chemokine (C-C motif) ligand 2 (CCL2) and C-C chemokine receptor type 2 (CCR2) in cultured hippocampal neurons [25]. Experiments by Ma demonstrated that administration of a high dose of propofol attenuated the biochemical markers of brain damage by decreasing S-100 β protein and neuron-specific enolase (NSE) levels [26]. Propofol was also reported to ameliorate acute lung injury during transplantation via activating Nrf2 pathway [27]. Recently, miRNAs were reported to play important roles in the tissue protective effect of propofol [28].

In the present study, organ protection against ischemia-reperfusion injury by the administration of propofol and the role of miR-17 during this process was discussed. Damage of vascular endothelium is the hallmark of blood vessel injury, thus hypoxia-reoxygenation (H/R) model with HUVECs was used to mimic the *in vivo* situation of ischemia-reperfusion. We found that expression of miR-17 was obviously reduced after propofol treatment in both normal cells and cells stimulated with H/R, indicating that administration of propofol could downregulate miR-17 expression. To investigate whether the decreased expression of miR-17 is related to propofol induced cell protection, HUVECs were transfected with miR-17 inhibitor to suppress endogenous miR-17 level and followed by H/R treatment. Cell viability and apoptosis analysis showed that both propofol administration and miR-17 suppression significantly increased cell viability while inhibited apoptosis. Western blot also demonstrated that apoptotic protein Bax was significantly downregulated while anti-apoptotic protein Bcl-2 was upregulated after propofol treatment or miR-17 inhibition, indicating that downregulation of miR-17 may be required for the protection effect of propofol. To further study the mechanism involved in the anti-apoptosis regulation of miR-17, the targets of miR-17 were searched and STAT3 was predicted to be a target of miR-17 by luciferase reporter assays and Western blot. STAT3 signaling pathway is related to cell apoptosis, and inhibition the activity of STAT3 induces apoptosis [29]. Therefore, in the H/R model of HUVECs, propofol treatment downregulated the expression of miR-17, which may increase STAT3 expression, upregulate the activity of STAT3 pathway, and finally suppress cell apoptosis.

In summary, propofol ameliorates invascular endothelium damage after ischemia-reperfusion, and one possible mechanism is the activation of STAT3 signal pathway mediated by downregulation of miR-17. Further studies are still needed to reveal the specific downstream molecular mechanisms.

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Disclosure of conflict of interest

None.

Address correspondence to: Jingui Yu, Department of Anesthesiology, Qilu Hospital of Shandong University, 107 Wenhua Xi Road, Jinan 250012, P. R. China. Tel: 86-531-82166460; Fax: 86-531-82166460; E-mail: Yujingui333@163.com

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