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ORIGINAL RESEARCH

Isoflurane ameliorates oxygen-glucose deprivation-induced cardiomyocyte injury through SIRT6/DNMT1 pathway

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Abstract

The incidence of cardiovascular diseases is on the rise in the world, which poses a significant threat to human health. Myocardial ischemia can cause heart disease. Therefore, it is necessary to avoid myocardial hypoxia/reoxygenation (H/R) injury to attenuate the risk of heart disease. The present study focuses on the protective effect of isoflurane on H/R-induced cell injury through the Sirtuin 6 (SIRT6)/DNA (cytosine-5)-methyltransferase 1 (DNMT1) pathway. Quantitative reverse transcription PCR (RT-qPCR) and Western blot analysis were used to measure protein levels and mRNA expression in H9c2 cells. Cell Counting Kit-8 assays (CCK8 assay) was used to determine cell viability. The expression levels of pro-inflammatory molecule were assessed using commercial Enzyme-linked immunosorbent assay (ELISA) Kits. The ratio of cellular apoptosis was determined by flow cytometry. The contents of Lactate dehydrogenase (LDH), Cardiac Troponin I (cTnI), and Creatine Kinase MB (CK-MB) were detected using colorimetric assays. This study shows that Isoflurane reduces the expression of DNMT1 by activating SIRT6 in oxygen-glucose deprivation (OGD)-induced H/R injury. The damage of cardiomyocyte was decreased after Isoflurane treatment under OGD exposure condition. In addition, Isoflurane ameliorates OGD-induced inflammatory responses and cellular apoptosis in H9c2 cell *via* interaction with the SIRT6/DNMT1 pathway. Taken together, this study suggested the protective effect of Isoflurane on the process of OGD-induced damage and provided a new mechanism of action for Isoflurane in the treatment of H/R-induced cardiomyocyte injury.

Keywords

Isoflurane; OGD; SIRT6; DNMT1; Hypoxia/reoxygenation

1. Introduction

The incidence of cardiovascular diseases in the world is escalating year by year, such as coronary artery disease, arteriosclerosis and ischemic heart disease, which poses a major threat to human health and is one of the causes of death worldwide [1, 2]. Although modern medical research has made important progress in the treatment of cardiovascular diseases and improving the quality of life of patients, there is currently no effective therapy for the prevention of cardiovascular diseases. Isoflurane (ISO) is one of the frequently used anesthetics [3], which has a protective effect in some cardiovascular diseases [4]. Previous studies have indicated that isoflurane attenuated pathological damage to cardiomyocytes and protects rat hearts from hypoxia/reperfusion injury [5]. Numerous studies have suggested that both pre-conditioning and post-conditioning with isoflurane can prevent reperfusion injury after myocardial ischemia both *in vitro* and *in vivo* [5, 6]. Sirtuins are recognized in yeast as silent information regulators 2, which plays a role in histone and non-histone proteins nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylation and thereby participates in multiple pathophysiological progressions [7]. The sirtuins family comprises seven subunits (SIRT1-SIRT7) in mammals that share a conserved core catalytic domain, but their tissue distribution and cell localization are diverged [8, 9]. Among them, the nuclear-distributed SIRT6 plays a critical role in managing aging, gene stability, and stress response [10]. SIRT6 has a protective effect against cardiac injury, which could protect cardiomyocytes from ischemia/reperfusion injury. SIRT6 has the limit of expression in H/R-induced cardiomyocytes, and the expression of SIRT6 was enhanced after isoflurane treatment [11, 12]. DNA methyltransferase 1 (DNMT1) plays a role in various heart diseases, for example, knockdown of DNMT1 can protect against heart failure [13]. DNMT1 inhibitors suppress myocardial ischemia/reperfusion-related injury and ameliorate cardiac performance [14]. Previous studies have indicated that SIRT6 suppresses DNMT1 transcription through physical interactions and deacetylates DNMT1 proteins to destabilize its protein. SIRT6 inhibitor

could improve the expression level of DNMT1 [15]. The present study was to explore the protective effect of isoflurane on hypoxia-reoxygenation induced cardiomyocyte injury through the SIRT6/DNMT1 pathway.

2. Methods

2.1 Cell culture

The cardio myoblast H9c2 cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (Sigma, USA) at 37 °C. In order to establish H/R model, H9c2 cells were maintained in non-glucose and serum-free DMEM at 37 °C for 8 hours under hypoxic conditions (1% O_2/95% N_2). After hypoxic exposure, H9c2 cells were placed to reoxygenation at 37 °C in a normal incubator (95% air/5% CO_2) and divided into five groups: Control, OGD, OGD + ISO, OGD + ISO + OSS_128167 (20 μM), OGD + ISO + DNMT1. 3 hours after OGD treatment, cells were exposed to 1.5 % of isoflurane (ISO, Baxter Healthcare Corporation, Deerfield, IL, USA) for 30 min at 2 L/min. For overexpression of DNMT1, the Lipofectamine 2000 transfection reagent (Invitrogen) was used to transfected the plasmids to the H9c2 cells.

2.2 Cell Counting Kit-8 (CCK8) assay

The viability of H9c2 cell was measured by CCK8 assay (Abcam, Cambridge, USA). H9c2 cells were cultured into 96-well plates (1500 cells/well). Cell viabilities at 0, 24, 48 and 72 hours were subsequently detected. CCK8 solution (10 μL/well) was added for 1.5 hours at 37 °C. The optical density of the viability curve was quantitated at 450 nm by a microplate reader.

2.3 Western blot

The total protein from H9c2 cells was collected by RIPA buffer and centrifuged at 12,000 g. 30 μg protein samples were separated *via* 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and the nitrocellulose membranes (Sigma-Aldrich, St. Louis, MO, USA) were used to transfer proteins. The primary antibodies against SIRT6 (1:1000, ab62739, Abcam, Cambridge, MA, USA), DNMT1 (1:2000, sc-271729, Santa Cruz Biotechnology, Santa Cruz, Ca, USA), Bax (1:5000, ab182734, Abcam), Bcl-2 (1:3000, 2876, Cell Signaling Technology, Inc., Danvers, Massachusetts, USA) and β-actin (1:10,000, sc-47778, Santa Cruz Biotechnology) overnight at 4 °C. The membrane was cultured with horseradish peroxidase-labeled secondary antibody at 37 °C for 1 hour, and the band of proteins was measured by the enhanced chemiluminescence (Sigma-Aldrich). The internal control was β-actin.

2.4 Quantitative real-time PCR

H9c2 cells were harvested and the TRIZOL reagent (Invitrogen) was used to extract the total RNA. A total RNA was reversed by using universal cDNA synthesis and SYBR Green Master Mix kits incorporation on Roche Light-Cycler480 Real Time PCR system (Roche, Penzberg, Germany). The SIRT6 and DNMT1 primers were as follows:

SIRT6:
Forward (5′-3′): GCCGTCTGGTCATTGTCA;
Reverse (5′-3′): AGCCTTGGGTGCTACTGG;
DNMT1:
Forward (5′-3′): GTTCCTCCTTCTGCCATCAAT;
Reverse (5′-3′): CGTCTCATCATCGTCCTTAGC.

2.5 Measurements of Lactate dehydrogenase (LDH), Creatine Kinase MB (CK-MB) and Cardiac Troponin I (cTnI) levels

The levels of LDH (Abcam), CK-MB (BioVision, Palo Alto, USA) and cTnI (My Biosource, CA, USA) from heart tissue, serum and culture medium were quantitative by the commercial kits according to the manufacturer's protocols.

2.6 Measurements of TNF-α, IL-1β and IL-6 levels

The levels of pro-inflammatory molecule expression were assessed from cultured H9c2 cells, using commercial Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), and IL-1β ELISA Kits (ab46087, ab178013, ab46052, Abcam, Cambridge, MA, USA) according to the manufacturer's protocols. The plates were analyzed at the absorbance of 450 nm.

2.7 Flow cytometry

H9c2 cells were detached with trypsin and harvested with a binding buffer. Then, H9c2 cells were cultured with Annexin V/FITC and propidium iodide from Dead Cell Apoptosis Kit (Thermo Fisher Scientific, San Jose, CA, USA) following the manufacturer's protocols. The fluorescence signal was analyzed by a flow cytometer (BD Biosciences, San Jose, CA, USA). Each experimental group's portion of apoptotic cells was calculated as the sum of early and late apoptosis.

2.8 Statistical analysis

All data were analyzed using Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) software package. Data were expressed as mean $\pm$ standard error of the mean (SEM). The differences between groups were performed with one-way Analysis of variance (ANOVA) followed by Tukey's post hoc test. p value < 0.05 was defined as statistically significant.

3. Results

3.1 Isoflurane down-regulates the expression of DNMT1 by activating SIRT6 in H9c2 cells with H/R injury

To investigate the effect of Isoflurane on oxygen-glucose deprivation (OGD) treated H9c2 cells, the level of SIRT6 and DNMT1 were analyzed by western blot and qPCR, respectively. To induce OGD, H9c2 cells were exposed to H/R conditions. SIRT6 expression was significantly suppressed, while

DNMT1 was enhanced in the OGD group compared with that of the control group (Fig. 1A,B). Conversely, compared with the OGD group, the Isoflurane treatment groups remarkably induced SIRT6 expression and attenuated DNMT1 expression. Furthermore, the effect of Isoflurane in OGD-treated H9c2 cells was reversed by SIRT6 inhibitor OSS_128167. The results indicate that Isoflurane inhibits DNMT1 expression by SIRT6 activation.

3.2 Isoflurane ameliorates OGD-induced H9c2 cell injury through SIRT6/DNMT1 pathway

To determine whether Isoflurane contributes to the myocardial protective effect against H/R-induced cellular damage, the level of cardio injury-related proteins LDH, CK-MB, cTnl, and cell viability were assessed. CCK8 assay was used to examine cell viability. CCK8 assay and western blot analysis revealed that OGD remarkably suppressed cell viability and enhanced the levels of cardio-injury-related proteins in H9c2 cells (Fig. 2A,B). Conversely, cotreatment with Isoflurane significantly enhanced cell viability and down-regulated cardio-injury-related proteins level compared with those in the OGD group. Additionally, these above changes were reversed after cotreatment with SIRT6 inhibitors or overexpression of DNMT1 (Fig. 2A,B). These results suggest that Isoflurane ameliorates OGD-induced cellular damage through SIRT6/DNMT1 pathway.

3.3 Isoflurane improves OGD-induced inflammatory damage in H9c2 cell through SIRT6/DNMT1 pathway

To explore the effect of Isoflurane on OGD-induced inflammatory damage, the levels of cytokines of inflammatory response were detected by commercial ELISA kits. Compared with the OGD group, the OGD + ISO group showed lower levels of TNF-α, IL-1β, and IL-6. Additionally, these above changes were reversed after cotreatment with SIRT6 inhibitors or overexpression of DNMT1 (Fig. 3). These results indicated that Isoflurane ameliorates OGD-induced inflammatory damage through SIRT6/DNMT1 pathway.

3.4 Isoflurane ameliorates OGD-induced cellular apoptosis in H9c2 cell through SIRT6/DNMT1 pathway

To clarify the potential mechanism underlying the effects of Isoflurane on inhibiting OGD-induced apoptosis, cell apoptotic rate was measured by cell apoptosis kit with Annexin V-FITC and western blot analysis. The results from flow cytometry assay determined that the apoptotic rate of the OGD group was obviously higher than that of the OGD + ISO group, while the apoptotic rate was significantly increased after cotreatment with SIRT6 inhibitors or overexpression of DNMT1 (Fig. 4A). Western blot analysis showed consistent results, showing that the expression level of Bax in OGD + ISO group was decreased while the expression level of Bcl-2 was suppressed, compared with that in the OGD group. Cotreatment with SIRT6 inhibitors or overexpression of DNMT1 group exhibited significantly higher expression level of Bax and a lower level of Bcl-2 expression (Fig. 4B). These results strongly indicate that Isoflurane improves OGD-induced cellular apoptosis in H9c2 cell through SIRT6/DNMT1 pathway.

4. Discussion

The ischemia-reperfusion injury of heart is one of the ischemic cerebrovascular diseases, which can seriously damage heart tissue and cause significant loss of cardiomyocyte biological functions. The pathological process of ischemia-reperfusion injury is complex, including ischemia and hypoxia of heart tissue. Reperfusion-induced cardiac injury also induces damage to reactive oxygen species, white blood cells accumulation, and inflammatory cytokines overexpression [16]. As cardiomyocyte damage progressively aggravated, cTnl, LDH, and CK-MB released by cardiomyocytes escalate [17, 18]. These factors interact to one another and eventually lead to crucial cardiomyocyte injury, apoptosis, and inflammatory response. This study focused on the role of Isoflurane in OGD-induced cardiomyocyte injury. Isoflurane was found to suppress DNMT1 expression by stimulating SIRT6. Significantly, Isoflurane ameliorated OGD-induced cell damage, inflammatory response, and cellular apoptosis in H9c2 cells. Therefore, this study proposes that Isoflurane improves OGD-induced cardiomyocyte injury through SIRT6/DNMT1 pathway.

Oxygen-glucose deprivation (OGD) could induce ischemic disease and usually occurs when blood supply to some tissues is abruptly blocked, which is also a commonly used model to mimic ischemic cardiovascular disease *in vitro* [19]. The pathogenesis of ischemic cardiovascular disease is related to oxidative stress, myocardial remodeling, and inflammatory response, and inflammation thought to be a primary contributor [20, 21]. Previous research have reported that the degree of inflammation had been shown to be correlated positively with the severity of myocardial injury [22, 23]. Numerous studies have indicated that Isoflurane is a regulator of inflammation in cardiovascular diseases [24, 25]. Moreover, Isoflurane also suppresses injury-induced inflammatory factors secretion, such as IL-6 and TNF-α [26]. Inflammatory cytokines specifically mediate the inflammatory responses to heart tissue injury during ischemic cardiovascular disease. The present study has demonstrated that treatment of isoflurane prior to OGD-induced H/R injury could inhibit TNF-α, IL-1β, and IL-6 expressions, and have a myocardial protective effect on H9c2 cells.

Moreover, cell apoptosis, cell damage, and heart tissue necrosis are fundamental reasons for heart failure and ventricular dysfunction with myocardial H/R injury. Prevention of cell apoptosis is essential for the therapy and prevention of myocardial ischemic injury. Isoflurane is associated with anti-apoptosis during OGD-induced H/R injury and promotes the translation of Bcl-2 expression [27, 28]. Both Bcl-2 and Bax are members of the Bcl-2 family and prevent cell apoptosis by increasing Bcl-2 expression and attenuating Bax expression. This study reports that Isoflurane attenuated OGD-induced apoptosis by suppressing Bax and enhancing Bcl-2. The results of this study support these previous findings and illustrate the protective effects of isoflurane against cell apoptosis by OGD-

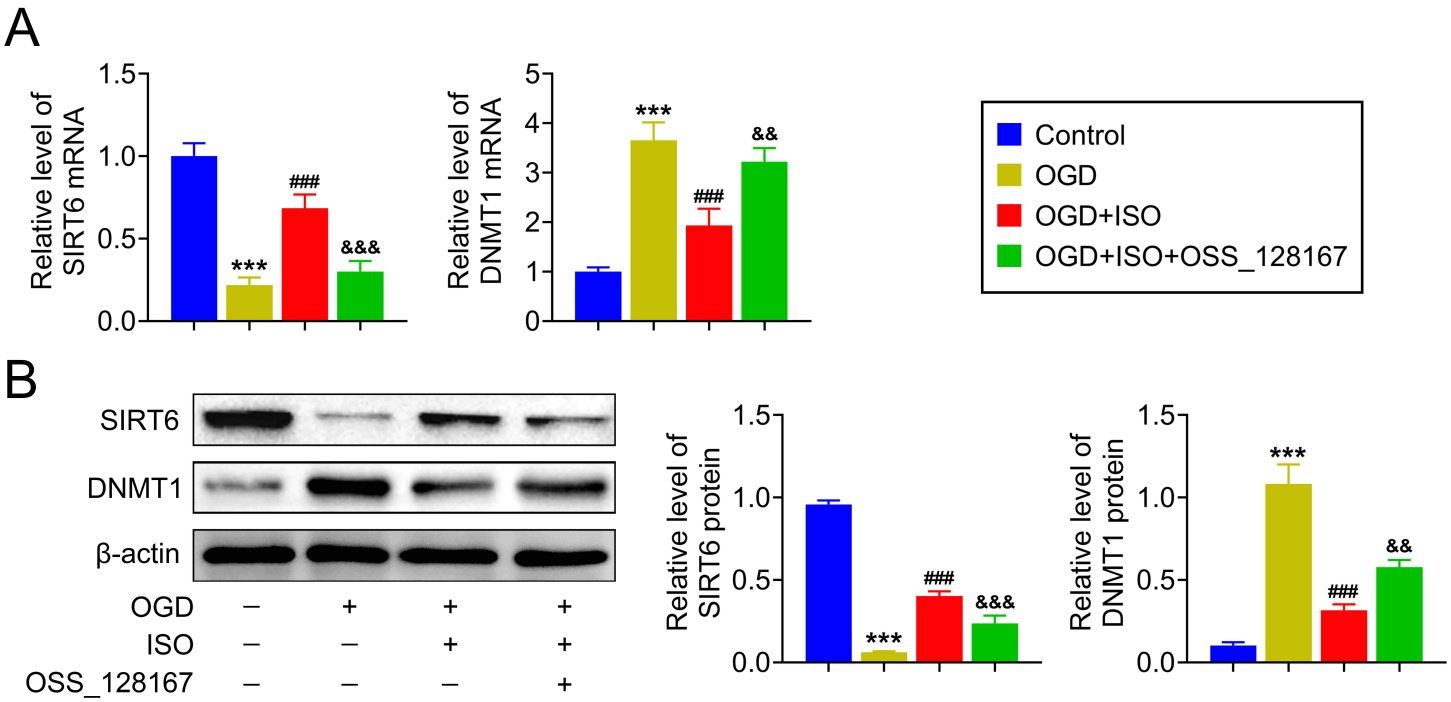

FIGURE 1. Effects of isoflurane on SIRT6/DNMT1 expression after OGD exposure in H9c2 cells. (A) The mRNA expressions of SIRT6 and DNMT1 were analyzed by qRT-PCR. (B) The protein expressions of SIRT6 and DNMT1 were evaluated by western blot analysis. *** $p < 0.005$ *vs.* Control. ### $p < 0.005$ *vs.* OGD. && $p < 0.001$ *vs.* OGD + ISO. &&& $p < 0.005$ *vs.* OGD + ISO. Data are expressed as mean ± SEM.

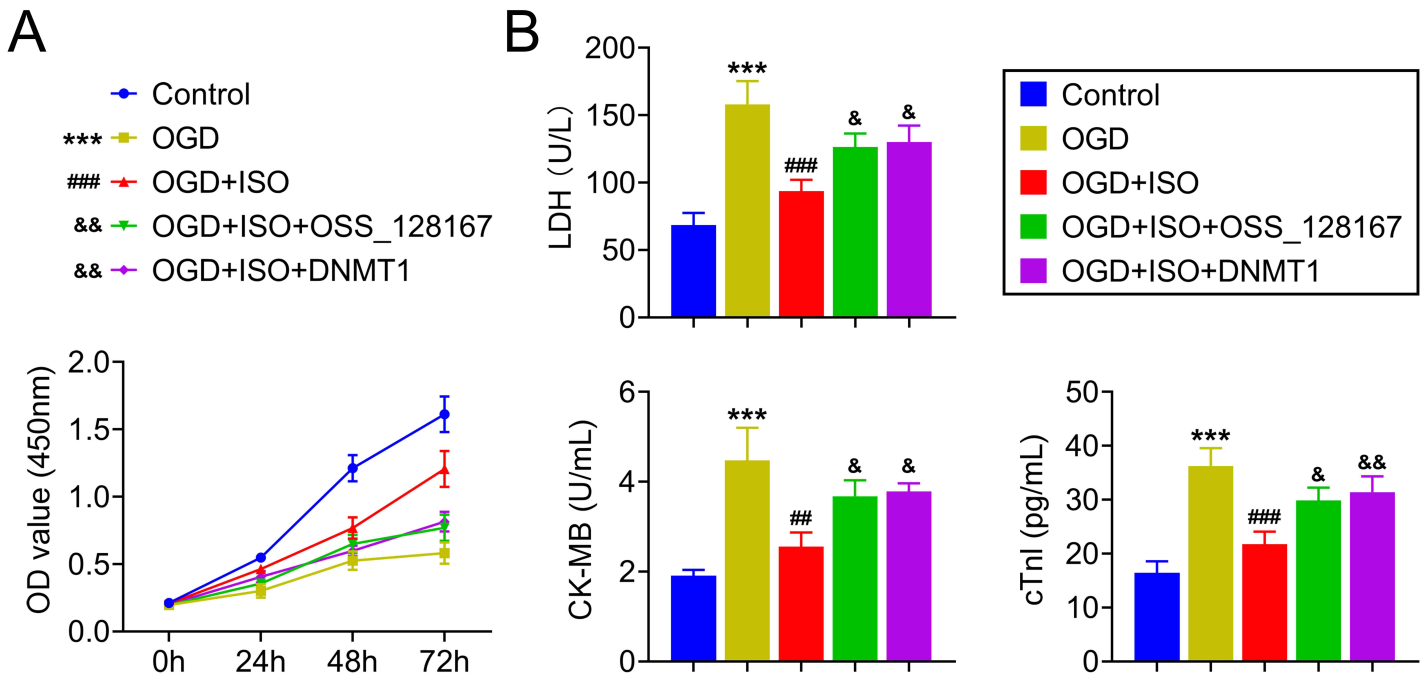

FIGURE 2. Effects of isoflurane on OGD-induced H9c2 cell injury through SIRT6/DNMT1 pathway. (A) After OGD exposure, cell viability in different groups was determined by CCK8 assay. (B) The protein expressions of LDH, CK-MB and cTnI were assessed by using commercial kits. *** $p < 0.005$ *vs.* Control. ### $p < 0.005$ *vs.* OGD. & $p < 0.05$ *vs.* OGD + ISO. && $p < 0.001$ *vs.* OGD + ISO. Data are expressed as mean ± SEM.

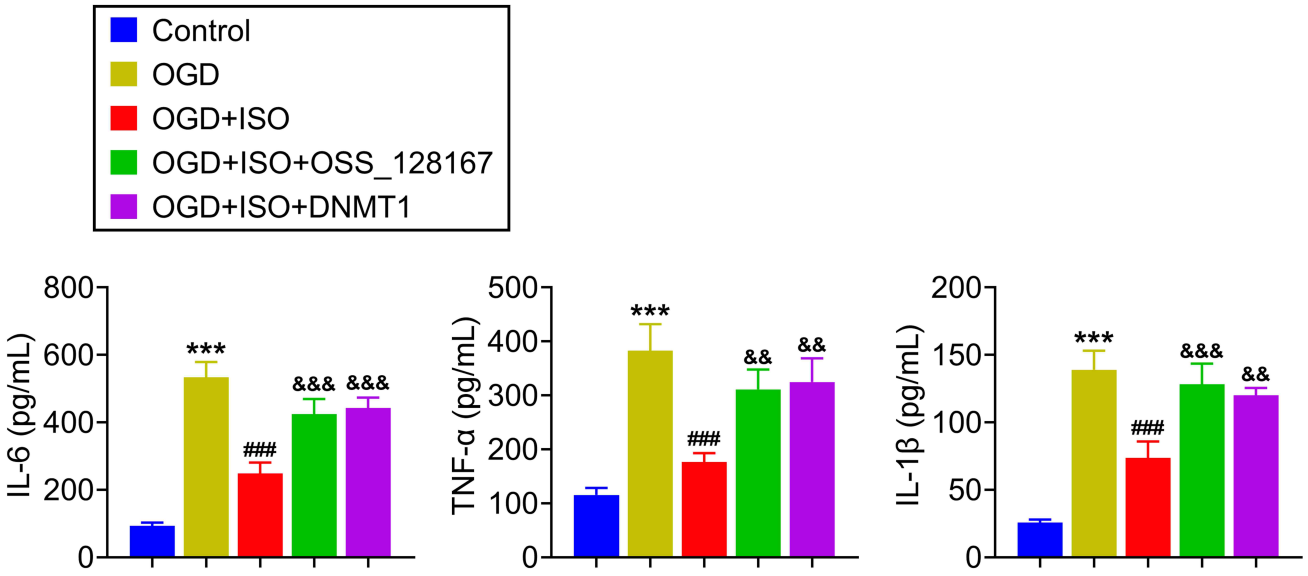

FIGURE 3. Effects of isoflurane on OGD-induced inflammatory damage in H9c2 cell by SIRT6/DNMT1 pathway. After OGD exposure, the TNF-α, IL-6 and IL-1β levels were assessed by using ELISA assay. *** $p < 0.005$ *vs.* Control. ### $p < 0.005$ *vs.* OGD. && $p < 0.001$ *vs.* OGD+ ISO. &&& $p < 0.005$ *vs.* OGD + ISO. Data are expressed as mean ± SEM.

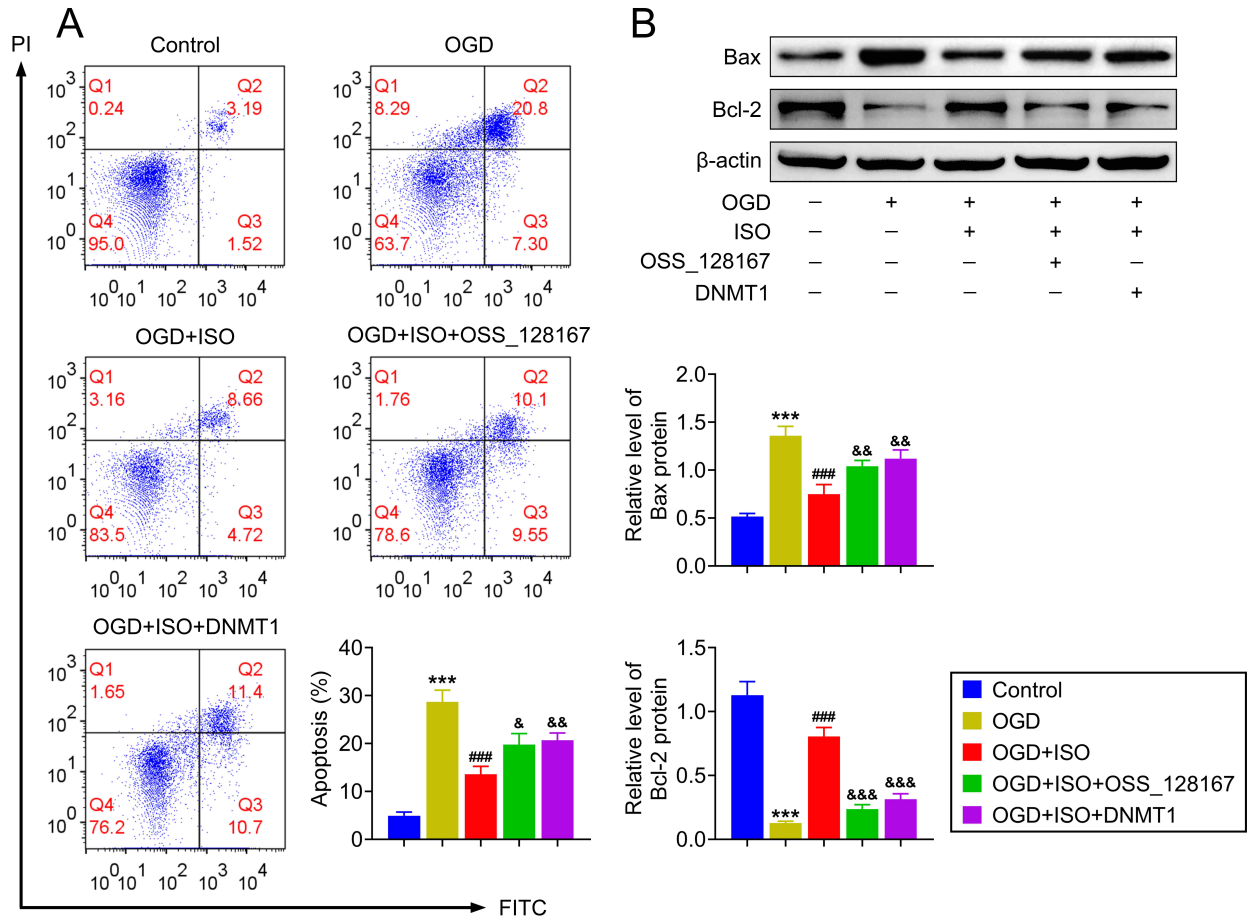

FIGURE 4. Effects of isoflurane on OGD-induced H9c2 cell apoptosis by SIRT6/DNMT1 pathway. (A) Flow cytometry assay was used to measure the apoptotic rate of H9c2 cell after isoflurane treatment. (B) The apoptotic related protein (Bax and Bcl-2) expressions were evaluated by using western blot analysis. *** $p < 0.005$ *vs.* Control. ### $p < 0.005$ *vs.* OGD. && $p < 0.001$ *vs.* OGD+ ISO. &&& $p < 0.005$ *vs.* OGD + ISO. Data are expressed as mean ± SEM.

induced H/R injury and attenuation of Bax protein expression on apoptosis.

Previous studies have indicated that SIRT6 promotes resistance to DNA damage and oxidative stress and maintains cardiovascular homeostasis [11, 29]. In addition, SIRT6 has been reported to block the progression of cardiac hypertrophy and play a crucial role in inhibiting reactive oxygen species production and against myocardial injury in response to cardiac H/R. However, the role of SIRT6 in isoflurane-treated cardiomyocytes during H/R remains unclear. The present study revealed that isoflurane could reduce OGD-induced H9c2 cell injury and inhibit cell viability through SIRT6/DNMT1 pathway.

5. Conclusions

In conclusion, the data from this study indicated that Isoflurane down-regulates the expression of DNMT1 by activating SIRT6 in OGD-induced H/R injury. In addition, Isoflurane ameliorates OGD-induced cardiomyocyte injury, inflammatory response and cellular apoptosis *via* interaction with the SIRT6/DNMT1 pathway. This study showed the novel finding of isoflurane in the pathogenesis of OGD-induced damage and provided a new mechanism of action for Isoflurane in the treatment of H/R-induced cardiomyocyte injury.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

QW and BSZ—designed the research study. QW and BSZ—performed the research. QW and BSZ—analyzed the data. QW and BSZ—wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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