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Original Article

Effect of combination therapy of propofol and sevoflurane on MAP2K3 level and myocardial apoptosis induced by ischemia-reperfusion in rats

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Abstract: To investigate the mechanism of combination therapy of propofol and sevoflurane on MAP2K3 level and myocardial apoptosis induced by ischemia-reperfusion (IR) in rat. A total of 30 SD rats were randomly separated into 3 groups: normal, IR (ligation of left coronary artery), and IR+ propofol and sevoflurane (IR+P+S). Different methods were used to detect the serum index associated IR injury. TUNEL assay was used to analyze the apoptotic cells of rat heart tissues. qRT-PCR was used to analyze the mRNA levels of cell apoptosis related proteins such as Bcl-2, Bax, and MAP2K3. Western blotting was used to detect the expression of Bcl-2, Bax, MAP2K3, and Caspase-3 of heart tissues. Compared with normal group, serum LDH, cTnI, and CK-MB levels in IR group were significantly increased with time increasing ($P<0.05$), while that in IR+P+S group were significantly decreased compared with that in IR group ($P<0.05$). The percentage of apoptotic cells of heart tissue in IR+P+S group was larger than that in IR group ($P<0.05$). Compared with IR group, mRNA expression of MAP2K3 and Bax were significantly decreased with Bcl-2 was significantly increased in IR+P+S group ($P<0.05$). Also, expression of MAP2K3, Caspase-3, and Bcl-2 in IR+P+S group were statistically lower while Bax was statistically higher than that in IR group ($P<0.05$). Our study suggested that combination therapy of propofol and sevoflurane may protect myocardial cells from damage during IR through decreasing MAP2K3 level and reducing cell apoptosis via Bcl-2/Bax pathway.

Keywords: Ischemia-reperfusion, MAP2K3, cell apoptosis, propofol and sevoflurane

Introduction

Ischemia-reperfusion is an injury that caused by the blood resupply when tissue cells with perfusion, and is usually occurred in some ischemia animal bodies such as cardiac surgery, coronary artery bypass, viscera recanalization after blood supply infarction, and organ transplantation [1]. Cardiac injuries that caused by ischemia-reperfusion are permanent to tissues and are inevitable during surgeries and have brought huge damage to patients even deaths [2]. Recently, lots of researches have referred the injury molecular mechanisms of ischemia-reperfusion [3, 4].

Numerous studies have demonstrated that anesthetics such as the widely used inhaled sevoflurane and intravenous anesthetic injected propofol played crucial protective roles in heart injuries that caused by ischemia-reperfusion during some cardiac surgeries. For instance, Chen and his colleagues proved that efficacy of sevoflurane post-conditioning gradually weakened with increasing ischemia duration in rabbits *in vivo* [5], and Li *et al.* experimented that sevoflurane could pre-condition ameliorates neuronal deficits through suppressing MMP-9 expression after ischemia-reperfusion in rats [6]. Besides, recent study refers that propofol could attenuate small interstinal ischemia-reperfusion injury via inhibiting NADPH oxidase mediated mast cell activation [7]. Tao *et al.* reported that anesthetic propofol protected against ischemia-reperfusion injury by reducing oxidative stress induced by reactive oxygen species in rats [8].

Meanwhile, increasing evidences have demonstrated that cell apoptosis functions as an

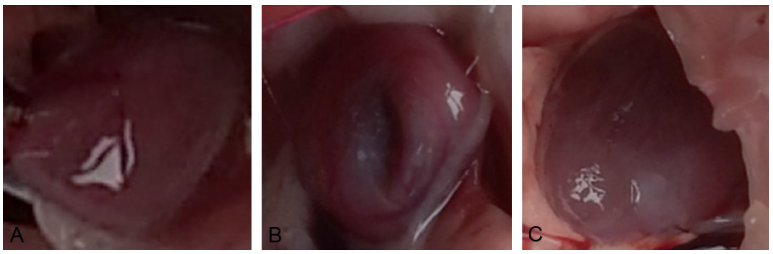

Figure 1. Model of myocardial ischemia-reperfusion in rat. A: Normal heart of rat (Normal); B: Heart with ischemia-reperfusion of rat (IR); C: Heart with IR given with the propofol and sevoflurane (IR+P+S).

important factor in IR injuries [9, 10]. MAPK family proteins are associated with triggering cell apoptosis through activating the MAPK cascades and some signaling pathways in many biological processes [11]. Recent paper shows that sevoflurane induces the delayed neuroprotection by enhancing p38MAPK phosphorylation during ischemia-reperfusion [12] and p38MAPK phosphorylation was increased by sevoflurane during ischemia [13]. Also, propofol protects cells against ischemia-reperfusion injury via activating MAPK related pathway [14]. Yang *et al.* predicted the effect of combination therapy of propofol and sevoflurane on cardiac tissues using the DNA microarrays [15]. Although many researches have devoted to the protective mechanisms exploration of anesthetics on heart injuries resulted from ischemia-reperfusion, the protective mechanism of combine therapy of propofol and sevoflurane in protecting heart from damaged by ischemia-reperfusion has not been fully described.

In this study, we constructed a rat model of ischemia-reperfusion and treated the MI rat with propofol and sevoflurane. Comprehensive biological experimental methods were used to detect the serum level of cTnI, LDH, and mRNA and protein levels of MAP2K3 and MAPK in MI and MI+ propofol and sevoflurane models compared with the normal groups. Further experiments were conducted to detect the cell apoptosis protein levels of Bax, and Bcl-1. This study aimed to explore the potential protective effect of combination therapy of propofol and sevoflurane on ischemia-reperfusion. Our study may provide basis for the future research of combination therapy propofol and sevoflurane on protecting heart injuries that resulted from ischemia-reperfusion during some cardiac surgeries.

Materials and methods

Rat and modeling

All experimental procedures in this study were approved by the Institute of Health Services Research at Hebei Medical University on the protection of animal used for scientific purpose. The manuscript was prepared according to the guidelines of the declaration of Helsinki for biomedical research. The male SD (Sprague-Dawley) rats (Medical experimental center of Hebei University, Laboratory animal center of Hebei University) weighting 220-300 g were individually housed in a rodents feeding room with the adjustable temperature, humidity, light, and pressure. The environment was maintained at a 12/12 h light/dark cycle (lights at 150-200 LX), with the relative humidity at 55%-75%, temperature set at 23 ± 1°C, and noise <50 dB. The total 30 male SD mice were randomly separated into 3 groups (each group with 5 repeats): (1) normal, (2) ischemia-reperfusion (IR), (3) ischemia-reperfusion + sevoflurane and propofol (IR+S+P).

Before modeling, anesthesia was conducted using the anesthesia apparatus and maintained with 25 $\mu mol \cdot L^{-1}$ of propofol or 2% of sevoflurane. Endotracheal intubation was completed via oral, and then rats were connected with HSE-HA MiniVent (USA). Chest at the fourth rib was open to exposure the heart, the left coronary artery was ligated (with the 8.0 nylon suture) about 1 mm from their origin. The immediate color change of the heart surface (pale appearance about 1 min) and the slower movement of ventricular wall were the signs of successful coronary ligation (**Figure 1**). The diaphragm was sutured with an absorbable 5.0 suture starting at the muscular segment towards its tendinous part, and final suture was tightened after the air was expelled from thoracic cavity. After modeling, rats in each group were maintained for 2 weeks.

Cell apoptosis analysis

After being treated for 6 h, heart tissues in the three experimental groups of normal, IR, and

Table 1. Primers used for the targets amplification in this study

Target	Primer	Sequence (5'-3')
Bcl-2	sense	5'-TTGTGGCCTTCTTTGAGTTCGGTG-3'
	anti-sense	5'-GGTGCCGGTTCAGGTACTCAGTCA-3'
Bax	sense	5'-ATGGACGGGTCCGGGGAG-3'
	anti-sense	5'-TCAGAAAACATGTCAGCTGCC-3'
MAP2K3	sense	5'-GACCTTCATCACCATTGGAG-3'
	anti-sense	5'-CGTAGAAGGTGACAGTGTAG-3'
GAPGH	sense	5'-GGTGGAGGTCGGGAGTCAACGGA-3'
	anti-sense	5'-GAGGGATCTCGCTCCTGGAGGA-3'

IR+P+S groups were obtained, and apoptosis of cardiomyocytes was detected using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay. Briefly, apoptosis cells from heart tissues were identified using an *in situ* cell death detection kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Nuclei with brown staining indicated TUNEL positive cells. For each group, 20 randomly selected fields (5 hearts per group, 4 fields per heart) were observed. Also, the apoptotic index (AI), namely the percentage of apoptotic nuclei (TUNEL-positive) vs. total number of nuclei was measured.

Measurement of serum LDH, CPK-MB, and cTnI

Before anesthesia and modeling, blood was collected from each rat in different groups by artery intubation at different time point and stored in liquid nitrogen at -80°C. The extracted blood was centrifuged at 3000 r/min for 15 min to obtain the supernatant. Then the total myocardial enzymes of LDH, cTnI, and CK-MB were detected using the automatic biochemical analyzer (Hitachi 7600, Japan).

Quantitative RT-PCR

The fresh heart tissues of rats collected at 6 h were grinded in the liquid nitrogen and then washed with the PBS buffer (PH 7.4). Total RNA was extracted from the collected heart tissues using Trizol reagent as described previously [16]. The extracted RNA was treated with RNse-free Dnase I (Promega Biotech), and the concentration and purity of extracted RNA were tested using SMA4000 UV-VIS spectrophotometer (Merinton, Shanghai, China). The purified RNA that adjusted to 0.5 μg/μL with nuclease-free ddH_2O was used for cDNA synthesis using the cDNA Synthesis Kit (Takara, China). Primers for the targets amplification were shown in **Table 1**. Then RT-PCR was carried out in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). The reaction system of 20 μL volume containing 1 μL cDNA from the above PCR, 10 μL SYBR Premix EX Taq, 1 μL each of the primers (10 μM), and 7 μL ddH_2O. The PCR program was as follows: denaturation at 95°C for 2 min; followed by 45 cycles of 95°C for 10 s, 59°C for 20 s, and 72°C for 30 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. The primers for target genes such as Bax, Bcl-2, MAP2K3, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were shown in **Table 1**. GAPDH was the internal control that used for data normalization to determine the relative mRNA expression of target genes. The $2^{-\Delta\Delta Ct}$ method was used to stand for the gene expression value.

Western blotting analysis

Heart tissues from rats that harvested at 2 days post-ischemia-reperfusion were lapped in RIPA (radioimmunoprecipitation assay, Sangon Biotech) lysate containing PMSF (phenylmethanesulfonyl fluoride) and then were centrifuged at 12,000 rpm/min for 10 min at 4°C. Supernatant was collected to measure the concentration of proteins by BCA (bicinchoninic acid) protein assay kit (Sigma, USA). Briefly, a total of 20 μg protein per cell lysates was subjected to a 10% SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) and then transferred onto a Polyvinylidence fluoride (PVDF) membrane (Millipore). The membrane was blocked in TBST (Tris Buffered Saline Tween) with 5% non-fat milk for 1 h, and subsequently incubated with rabbit anti-human MAP-2K3, Bcl-2, Bax, Casepase-3, and Casepase-8 monoclonal antibodies (1:100 dilution) overnight at 4°C, followed by incubation with horse-

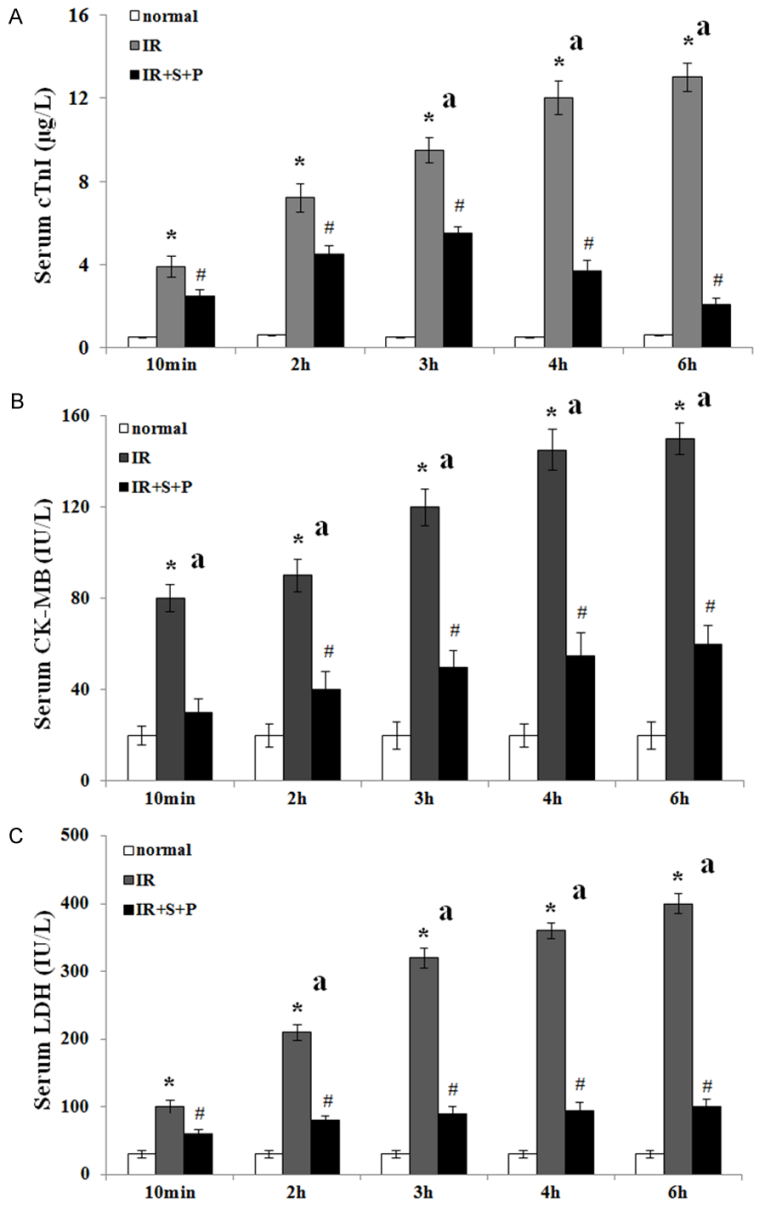

Figure 2. Serum levels of LDH, cTnI, and CK-MB in different groups. A: Measurement of serum cTnI in three groups; B: Measurement of serum LDH in three groups; C: Measurement of serum CK-MB in three groups. *stands for the significant difference between normal group and IR group (P<0.05); #stands for significant difference between normal group and IR+P+S group (P<0.05); a stands for the significant difference between IR group and IR+P+S group (P<0.05).

radish peroxidase labeled goat anti-rat secondary antibody (1:100 dilution) at room temperature for 1 h. The PVDF was washed using the 1×TBST buffer for 10 min with 3 times. Detection was performed using the development of X-ray after chromogenic substrate with an enhanced CEL (chemiluminescence) method. In addition, ß-actin (Sigma, USA) served as the internal control.

Statistical analysis

The data was calculated as mean ± standard error of mean (SEM). The differences among groups (normal vs. IR, IR vs. IR+P+S) were calculated using the independent sample t-test method with Prism 5.0 software (GraphPad Prism, San Diego, CA). The P<0.05 was considered as statistically significant.

Results

Measurement of serum cTnI, LDH, and CK-MB

Our data showed that the ischemia-reperfusion model of rat was successfully constructed (**Figure 1**). Different methods were used to analyze the significant serum index of ischemia-reperfusion of rat (**Figure 2**). The results showed that cTnI level was significantly increased in IR group compared to that of rats in normal groups with time increasing (P<0.05), otherwise, cTnI level was significantly decreased in IR+P+S group compared with that in IR group, indicating that combination therapy of propofol and sevoflurane can improve the cardiac cells from damaging by IR (**Figure 2A**). With the same tendency of serum cTnI, serum level of CK-MB in IR group was significantly increased compared to the normal group with time increasing (P<0.05), while this phenomenon was opposite in IR+P+S group (P<0.05) (**Figure 2B**). Additionally, serum level of LDH was also statistically increased in IR group compared with normal group with time increasing (P<0.05), and LDH level was significantly decreased in IR+P+S group compared with that in IR group (P<0.05) (**Figure 2C**). Besides, the results displayed that serum levels of the three index in IR+P+S group were higher than that in normal group till 6 h (P<0.05), suggesting that

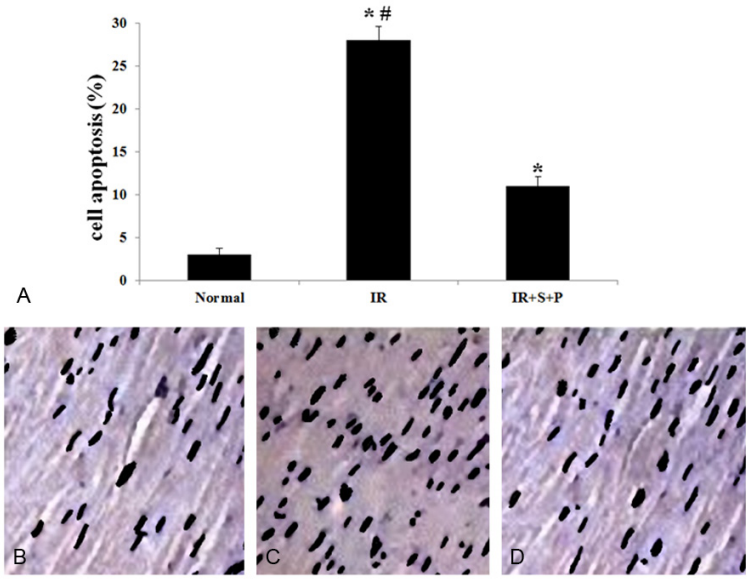

Figure 3. TUNEL analysis of cell apoptosis in different groups. A: The percentage of apoptotic cells in three groups, *stands for the significant difference between IR or IR+P+S groups and the normal group (P<0.05); #stands for the significant difference between IR+P+S group and IR group (P<0.05). B: Apoptotic cells of heart tissues in normal group; C: Apoptotic cells of heart tissues in IR group; D: Apoptotic cells of heart tissues in IR+P+S group.

the combination therapy of propofol and sevoflurane could certainly suppress the IR injury to cardiac cells, but not absolutely as the normal tissues.

Cell apoptosis analysis

TUNEL method was used to determine the influence of combination therapy of propofol and sevoflurane on cardiac cell apoptosis during IR injury (**Figure 3**). Compared with the normal groups, our data showed that cell apoptosis of cardiac tissues was apparently observed in IR group, while the apoptotic cells of cardiac tissues in IR+P+S groups were less than that in IR group (**Figure 3A**), suggesting that cell apoptosis of cardiac tissues may be suppressed by the combination therapy of propofol and sevoflurane during IR injury (**Figures 3B-D**).

mRNA levels of MAP2K3 and cell apoptosis related proteins

We used qRT-PCR to analyze the mRNA levels of MAP2K3 and cell apoptosis related proteins such as Bcl-2 and Bax in three groups (**Figure 4**). Our data showed that the mRNA level of Bcl-2 in IR group was significantly decreased (**Figure 4A**) while MAP2K3 and Bax mRNA level were significantly increased (P<0.05) (**Figure 4B** and **4C**). Otherwise, mRNA expression of Bcl-2 in IR+P+S group was significanly increased while the MAP2K3 and Bax mRNA level were significantly decreased compared with the IR group (P<0.05).

Western blotting analysis

To identify the association of combination therapy of propofol and sevoflurane on MAP2K3, Bcl-2, and Bax in heart tissue during IR injury, western blotting analysis was used to further detect their protein expressions (**Figure 4**). The relative expression levels of MAP2K3, Caspase-3, and Bax in IR group was higher while protein level of Bcl-2 in IR group was lower than that in normal group (**Figure 4D**). However, protein levels of MAP2K3 and Bax in IR+P+S group was lower while Bcl-2 expression was higher than that in IR group, suggesting that combination application of propofol and sevoflurane could suppress the MAP2K3 and Bax expression but enhance the Bcl-2 expression during IR injury (**Figure 4E**).

Discussion

Ischemia-reperfusion is an injury that caused by the blood resupply when tissue cells with perfusion, and is usually occurred in some ischemia animal bodies during surgeries and have brought huge damage to patients even deaths [1, 2]. Many studies have demonstrated that anesthetics such as the widely used inhaled sevoflurane or intravenous anesthetic injected propofol played crucial protective roles in heart injuries that caused by IR during some cardiac surgeries [17, 18]. However, few study has described the role of combination therapy of propofol and sevoflurane on myocardial cells during IR injury. In this study, we constructed an IR injury model of rat to analyze the effect of combination therapy of propofol and sevoflurane on MAP2K3 level and myocardial cell apoptosis induced by IR in rat.

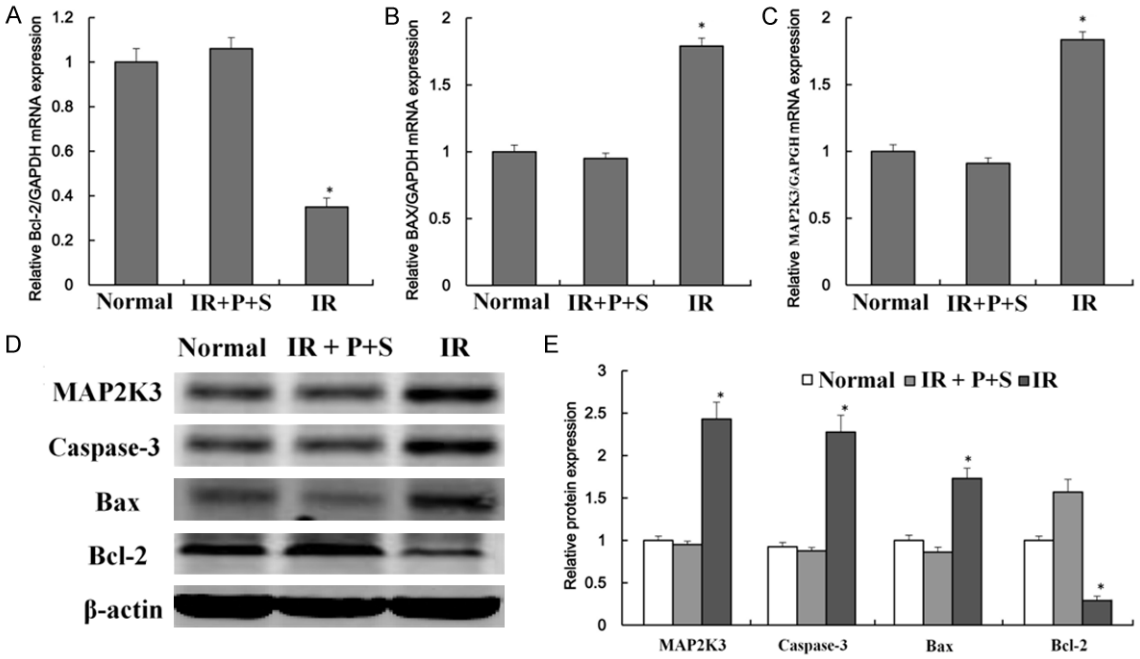

Figure 4. Expression levels of proteins in rat heart tissues in different groups. A: qRT-PCR analysis of mRNA expression of Bcl-2 in three groups; B: qRT-PCR analysis of mRNA expression of Bax in three groups; C: qRT-PCR analysis of mRNA expression of MAP2K3 in three groups; D: Western blotting analysis of protein levels of MAP2K3, Caspase-3, Bax, and Bcl-2; E: Densitometic analysis of protein levels of MAP2K3, Caspase-3, Bax, and Bcl-2 in three groups. *stands for the significant difference of mRNA expression between IR group and IR+P+S group ($P<0.05$).

In this study, serum levels of cTnI, LDH, and CK-MB in IR group were statistically higher than that in normal group, indicating our IR model was successfully constructed. CK (creatine phosphate kinase)-MB is a subtype of CK family protein and has been considered as the detective index of myocardial infarction in clinical [19], while cTnI is a myocardial-high-specificity protein with small molecular [20]. Previous studies have demonstrated that CK-MB, LDH, and cTnI would permeate into serum when the cell membrane permeability was increased during IR injury [21], and cTnI could easily permeate into serum in early stage during cardiac injury [22]. Huang *et al.* proved that cTnI and CK-MB levels in cardiac cells were low during IR injury patients who were treated with propofol [23]. Also, Law-Koune *et al.* proved that serum levels of LDH, CK-MB, and cTnI in patients treated with sevoflurane before IR injury were lower than that not treated with sevoflurane [24]. Our data showed that the serum LDH, CK-MB, and cTnI levels in IR+P+S group were statistically lower than that in IR group ($P<0.05$), indicating that combination therapy of propofol and sevoflurane can improve the myocardial cells from damage during IR.

Our results displayed that mRNA level and expression of MAP2K3 in heart tissues from IR+P+S group was lower compared with that in IR group ($P<0.05$). MAP2K3 is a dual specificity protein kinase that belongs to the MAP kinase kinase family, and phosphorylated MAP2K3 can activate p38MAPK [25]. Lots of papers have demonstrated that the phosphorylated p38MAPK resulted in cell apoptosis through activating some other signals [26, 27]. Liang *et al.* proved that sevoflurane functioned as a tumor suppressor of lung cancer through inactivating the p38MAPK signaling pathway [28], and Wu *et al.* referred that propofol was involved with the cell invasion and migration inhibition via p38MAPK [29]. In this study, our data showed that apoptotic cells in IR group were more than that in IR+P+S group ($P<0.05$), we speculated that the cell apoptosis may be induced by the activation of MAP2K3 during IR injury, and combination therapy of propofol and sevoflurane on apoptosis through MAP2K3.

Meanwhile, previous studies have demonstrated that the protein pair of Bcl-2/Bax is an important cell apoptosis related balanced factor that is related to mitochondria membrane

dynamics [30]. Tao *et al.* proved that Bcl-2 expression was low while Bax expression was high in heart tissues during IR injury [31]. The activated p38MAPK would increase the expression of Bax and decrease the expression of Bcl-2 to induce cell apoptosis [32]. Besides, Caspase-3 is a member of Caspase family that play certain roles in execution-phase of cell apoptosis [33]. Caspase-3 can be activated finally after other apoptosis enzymes activation during the cell apoptosis program and then induce cell apoptosis [34]. Contereas *et al.* said that Caspase-3 decreased IR injury of liver in mice [35]. Also, propofol could inhibit Caspase-3 expression in astroglial cells [36], and propofol decreased Caspase-3 expression during forebrain IR in rats [37]. Inamura *et al.* proved that sevoflurane could prevents Caspase-3 activation during myocardial IR [38]. Our data showed that Caspase-3 and Bax , in IR+P+S group were significantly decreased while Bcl-2 level was increased compared with that in IR group, suggesting that combination therapy of propofol and sevoflurane may reduce myocardial cell apoptosis by suppressing Caspase-3 and Bax and increasing Bcl-2.

In conclusion, this study attempts to investigate the potential role of combination therapy of propofol and sevoflurane on MAP2K3 level and myocardial apoptosis during IR injury. Combination therapy of propofol and sevoflurane may play protective roles on myocardial cells during IR through decreasing MAP2K3 level and suppressing the cell apoptosis via Bcl-2/Bax pathway. Our study may provide experimental basis for future research on the clinical use of propofol and sevoflurane during IR injury. However, further experimental studies are still needed to explore the comprehensive protective mechanism of combination therapy of propofol and sevoflurane on myocardial cells during IR.

Disclosure of conflict of interest

None.

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