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The changes of technetium-99m-labeled annexin-V in delayed anesthetic preconditioning during myocardial ischemia/reperfusion

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Abstract This study was designed to use real-time imaging to test the hypothesis that delayed cardiac protection induced by volatile anesthetics inhibits apoptosis. Rats were divided into two groups. One group was exposed to 120 min of 33 % O₂ [control group (CON group)] and the other group was exposed to 2.5 % sevoflurane in 33 % O₂ for 120 min [sevoflurane group (SEVO group)]. Both groups were allowed to return to their cages for 24 h. After 24 h recovery, all rats underwent 30 min myocardial ischemia by occluding coronary artery followed by 2 h of reperfusion. After reperfusion, technetium-99m-labeled annexin-V was administered intravenously to identify apoptosis. Left ventricular samples were obtained to measure infarct size and radionuclide imaging and caspase-3. Radionuclide imaging indicated that apoptosis was reduced in SEVO group (0.78 % ± 0.82) when compared with the CON group (1.15 % ± 0.61), and the infarct size was also decreased in the SEVO group (40 % ± 7). The transferase dUTP nick end labeling (TUNEL)-positive cardiomyocytes

in the SEVO group (16 % ± 6) were significantly decreased in the peri-infarct zone when compared with the CON group (28 % ± 4). After reperfusion, caspase-3 expression was significantly blunted in the SEVO group than in CON group (50 % ± 11 vs. 68 % ± 10, $p < 0.05$). This study used technetium-99m-labeled annexin-V of real-time imaging to detect cardiomyocyte apoptosis and the results were confirmed by the TUNEL assay and caspase-3 expression. We concluded that delayed volatile anesthetic preconditioning (APC) protects against I/R in vivo. The method of technetium-99m-labeled annexin-V of real-time imaging can be used to detect cardiomyocyte apoptosis in delayed APC during ischemia/reperfusion.

Keywords Anesthetic preconditioning · Ischemia/reperfusion · Radionuclide imaging

Introduction

Post-ischemic injury of organ such as heart, brain and kidneys represents a major problem in clinical medicine. Studies have shown the delayed preconditioning may arise 12–24 h after treatment and last up to 3 days [1–3]. It might be more useful in clinical practice for the patient who has coronary artery disease. Our previous studies have also demonstrated that the volatile anesthetic sevoflurane offers cardiac protection in both acute and delayed preconditioning against myocardial ischemia/reperfusion (I/R) injury [3–5]. Many mechanisms contribute to the protection such as the reactive oxygen species, mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels, nuclear factor-kappa B (NF-κB) and anti-apoptosis [6–10]. It has been reported that volatile anesthetic preconditioning (APC) can reduce apoptotic cells and apoptosis regulatory

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protein both in vivo and in vitro [11, 12]. Kehl et al. [13] reported that, unlike ischemic preconditioning, isoflurane does not produce a second window of protection (SWOP) 24 h after administration in dogs. However, others have reported that isoflurane has delayed cardiac protective effects using in vivo mouse myocardial I/R model [6] and delayed APC with sevoflurane has a similar effect on myocardial protection [2–4].

Myocardial I/R injury results from several factors that include the duration of ischemia, oxygen demand by cardiomyocytes at the time of ischemia, and the presence of collateral blood flow to the ischemic area. Importantly, the injury can also occur during the reperfusion. In addition to myocardial necrosis, apoptosis makes a significant contribution to cell death following acute myocardial ischemia and it is particularly enhanced during reperfusion [14, 15]. Phosphatidylserine, a phospholipid that is normally confined to the inner leaflet of cell membrane bilayer, becomes exteriorized during the process of apoptosis. Annexin-V is an endogenous protein that has a high affinity for binding to phosphatidylserine. Technetium-99m-labeled annexin-V is a promising method to assess the degrees of apoptosis [15–17]. By using 3 different technologies, real-time imaging of technetium-99m-labeled annexin-V, transferase dUTP nick end labeling (TUNEL) assay, and caspase-3, we test the hypothesis that delayed APC with sevoflurane can attenuate myocardial apoptosis during I/R.

Methods

This study was approved by the Animal Care Committee of the Medical College of Soochow University, and all experiments were conducted in accordance with the same guidelines for animal care of the National Institutes of Health (NIH publication vol. 25 no. 28, revised 1996) and policies of Soochow University.

Surgical instrumentation

In this in vivo study, a total of 64 male Sprague–Dawley rats (270–350 g) were randomly divided into two groups by using pre-determined numbers for each protocol. One group was exposed to 120 min of 33 % O₂ [control group (CON group)] and the other group was exposed to 2.5 % sevoflurane in 33 % O₂ for 120 min [sevoflurane group (SEVO group)]. Both groups were allowed to return to their cages for 24 h (Fig. 1). After the 24 h recovery, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and received additional anesthetic (15–25 mg/kg) to ensure there were no pedal and palpebral reflexes

throughout the experiments. Two heparin-filled catheters were inserted into the right jugular vein and the right carotid artery arterial for fluid or drug administration and blood pressure monitoring, respectively. The rats were then intubated via tracheostomy and ventilated with a rodent ventilator (Shanghai Alcott Biotech Co., Shanghai, China) using 33 % oxygen. A left thoracotomy was performed via left fifth intercostal space and the heart was suspended in pericardial cradle. A 6-0 Prolene (Product manager-Ethicon, Shanghai, China) ligature was placed around the proximal left descending coronary artery (LAD). The ends of the ligature were passed through a propylene tube to form a snare. The snare was clamped to cause coronary artery occlusion. It was confirmed ischemia when the appearance of epicardial was cyanosis. At the end of ischemia, the snare was unclamped to achieve reperfusion and verified by observing the epicardial hyperemic response [4]. The hemodynamic and artery blood gas data were monitored during the whole experiment. (Tables 1, 2).

Detection of radioactivity distribution in rats

All image studies were done with MultiSPECT2 dual-head gamma cameras (Siemens, Hoffman Estate, IL, USA) with low energy, high-resolution collimators, an energy peak of 140 keV, and a window of 20 %.

After 2 h of reperfusion, seven rats from each group were intravenously injected with technetium-99m-labeled annexin-V and imaged. The regions of interest were analyzed (counts per pixel) with subsequent calculation of the percentage difference in radioactivity between the experimental and control groups. The focal uptake of radioactivity at the experimental infarct areas with a target/background (T/B) ratio was recorded at 30 min after technetium-99m-labeled annexin-V injection.

Determination of myocardial infarct size

In another 16 rats (eight from each group), the hearts were stained with Evans blue (Sinopharm Chemical Reagent Co. Ltd., Beijing, China) at the end of the 2 h reperfusion. The heart was removed rapidly. LV was cut into six 2 mm cross-sectional slices. The staining separated the non-stained at risk area from the blue-stained normal area. The LV tissues were incubated at 37 °C for 15 min in 1 % 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer adjusted to pH 7.4. All tissues were fixed in 10 % formaldehyde overnight, and the infarct tissue was carefully separated from the area at risk using a dissecting microscope and areas were calculated. Infarct size was expressed as a percentage of the LV area at risk.

Fig. 1 Schematic illustration of experimental protocols used in the experiments. *CON* control group, *SEVO* sevoflurane group, *OCC* occlusion

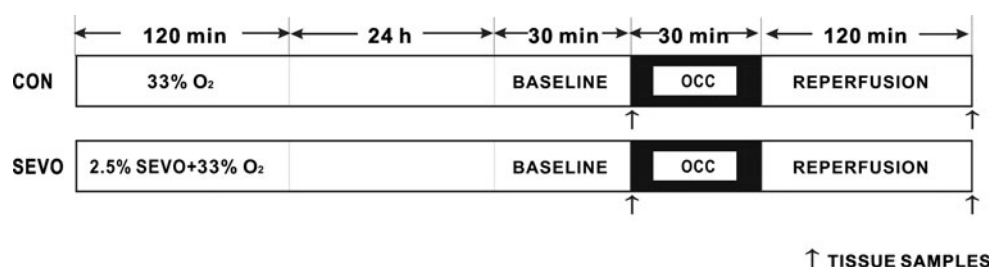


Table 1 Hemodynamic data

	Baseline	Pre-occlusion	End of CAO 30 min	End of reperfusion	
				1 h	2 h
HR (beats/minute)					
CON	380 ± 45	372 ± 38	367 ± 38	348 ± 65	334 ± 43
SEVO	367 ± 52	359 ± 41	378 ± 46	361 ± 53	348 ± 36
MAP (mm Hg)					
CON	112 ± 15	107 ± 21	98 ± 21	75 ± 14*	72 ± 13*
SEVO	108 ± 17	120 ± 13	105 ± 11	82 ± 15*	77 ± 18*
RPP (min ^{−1} * mm Hg × 10 ^{−3})					
CON	50.2 ± 10.5	48.4 ± 9.5	45.1 ± 10.3	35.4 ± 12.6*	30.8 ± 13.1*
SEVO	49.3 ± 12.7	51.7 ± 12.4	55.4 ± 8.7	36.1 ± 11.4*	34.3 ± 7.9*

Data presented as mean ± SD

CAO coronary artery occlusion, HR heart rate, RPP rate pressure product

* Compare to baseline, $p < 0.05$ is considered as statistically significant

Table 2 Artery blood gas data

	Baseline	Pre-occlusion	End of CAO 30 min	End of reperfusion	
				1 h	2 h
pH					
CON	7.32 ± 0.14	7.35 ± 0.10	7.40 ± 0.05	7.42 ± 0.06	7.37 ± 0.12
SEVO	7.41 ± 0.21	7.39 ± 0.08	7.38 ± 0.07	7.40 ± 0.11	7.41 ± 0.06
PaO ₂ (mm Hg)					
CON	92.4 ± 2.85	93.5 ± 1.26	91.8 ± 3.79	90.4 ± 3.09	91.6 ± 1.36
SEVO	90.1 ± 3.44	92.0 ± 2.06	92.8 ± 1.38	92.0 ± 2.45	93.5 ± 1.59
PaCO ₂ (mm Hg)					
CON	37.4 ± 3.42	36.5 ± 3.87	38.3 ± 1.14	36.2 ± 2.36	36.8 ± 4.02
SEVO	38.2 ± 4.10	37.2 ± 2.56	36.4 ± 3.68	35.9 ± 3.49	37.1 ± 2.08
SO ₂ (%)					
CON	95.2 ± 3.78	93.4 ± 4.56	95.4 ± 3.16	92.1 ± 4.96	92.8 ± 1.57
SEVO	93.5 ± 4.26	95.1 ± 5.02	93.8 ± 2.48	95.0 ± 5.23	93.4 ± 3.04
TCO ₂ (mmol * L-1)					
CON	21.9 ± 2.85	20.6 ± 4.21	21.5 ± 1.34	22.4 ± 1.04	23.7 ± 1.39
SEVO	22.1 ± 1.46	23.4 ± 2.47	23.8 ± 1.20	21.6 ± 2.47	22.6 ± 3.02

Data are presented as mean ± SD

CAO coronary artery occlusion

TUNEL assay

Another 16 rats were used for this assay. Heart samples were also collected after reperfusion for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay ($n = 8$ in each group). To determine myocardial apoptosis in a quantitative manner, the apoptosis cell death was evaluated by TUNEL method using an apoptosis detection kit, ZK-8005, (Zhongshan Goldenbridge Biotechnology, Beijing, China), as previously described [4]. Left ventricle tissues were removed after reperfusion rapidly. It was washed in ice-cold phosphate-buffered saline solution, and then incubated with 10 % paraformaldehyde overnight. After fixed and embedded in paraffin, the sections were cut to 4- μ m thicknesses and mounted on glass slides. After hematoxylin and eosin staining, the necrotic areas were simply confirmed from the morphologic appearance of the tissue. According to the manufacturer's instructions, immunohistochemical procedures were performed. For each slide, 10 fields were randomly selected. Nuclei with brown staining indicated TUNEL positive cells. Cell apoptosis index was expressed as a percentage of total cells counted.

Western blot

Sixteen rats were used for this analysis. Western blot analyses were used to detect the caspase-3 protein expression before ischemia and after reperfusion. LV tissue samples were obtained before ischemia and 2 h after reperfusion. Tissues were homogenized in ice-cold cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing a complete protease inhibitor, phenylmethyl sulfonylfluoride (Beyotime Institute of Biotechnology, Haimen, China) to isolate total protein. Protein concentrations were measured using the measurement kit (Beyotime Institute of Biotechnology, Haimen, China). Equivalent amounts of protein were mixed with loading buffer and heated at 100 °C for 5 min, separated by 10–12 % SDS-PAGE, and then transferred onto nitrocellulose membrane (Pall Corporation, East Hills, USA) which was stained with 0.1 % Ponceau S (Beyotime Institute of Biotechnology, Haimen, China) in 5 % acetic acid for 5 min to verify equal loading of lanes using the method previously described [4]. Nonspecific reactivity was blocked in 5 % non-fat dry milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 % Tween-20) for 60 min at room temperature. The membrane was then incubated overnight at 4 °C with 5 % milk and 1:500 dilution of goat polyclonal antibody (caspase-3, Cell Signaling, Danvers, MA, USA), followed by reaction with secondary antibody (Cell Signaling, Danvers, MA, USA).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most common housekeeping genes used in

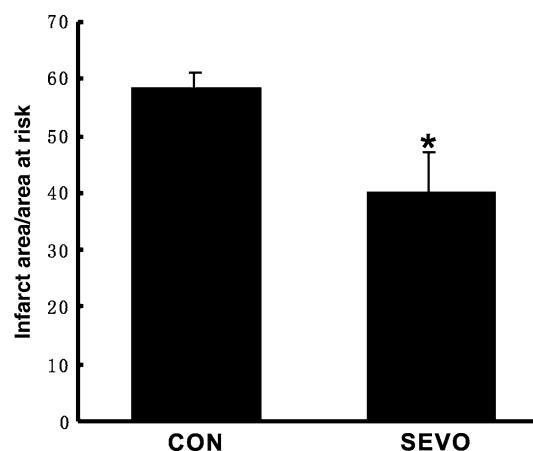


Fig. 2 The illustration of Infarct size. *CON* control group, *SEVO* sevoflurane group. *Significantly ($p < 0.05$) different from *CON* group, $n = 8$ per group

comparisons of gene expression data. GAPDH was used as a reference for the quantitative analysis. Reactive protein was detected by using ECL Chemiluminescence System (Merck, Darmstadt, Germany). Quantitative analysis of the band densities was performed using Sigma pro5.0 (Sigma-Aldrich, Inc, Saint Louis, MO, USA).

Statistical analysis

SAS 8.1 (SAS Institute Inc, Cary, NC, USA) software was used for statistical analysis. All values are expressed as mean $\pm$ SD. Data between groups were tested using paired *T* test and a p value < 0.05 was considered significant.

Results

All 62 rats were instrumented to obtain successful experiments protocol with the exception of two due to intractable arrhythmia that occurred during coronary artery occlusion. The analysis of endpoints was blinded.

The technetium-99m-labeled annexin-V revealed a real-time imaging of cardiac apoptosis in the rats, indicating that this is a useful tool to study apoptosis in animal models. Its biodistribution in rats showed rapid blood and renal clearance with gradual excretion in other organs or tissues including heart, liver, lungs, and muscle. Low uptake by the brain was also observed throughout the course of the study. The results show that the T/B ratio of radioactivity at the infarct areas in the SEVO group ($0.78 \% \pm 0.82$) was significantly lower than in the CON group ($1.15 \% \pm 0.61$, $p < 0.05$).

Figure 2 demonstrated that Infarct size was significantly reduced in the SEVO group ($40 \% \pm 7$) when compared

Fig. 3 Representative photomicrographs of TUNEL assay (40 × objective). The red arrow indicates the brown nucleus for apoptotic cells and black arrow shows the normal myocardial nucleus. Cell apoptosis index values are mean ± SD. CON control group, SEVO sevoflurane group. *Significantly ($p < 0.05$) different from CON group, $n = 8$ per group. (Color figure online)

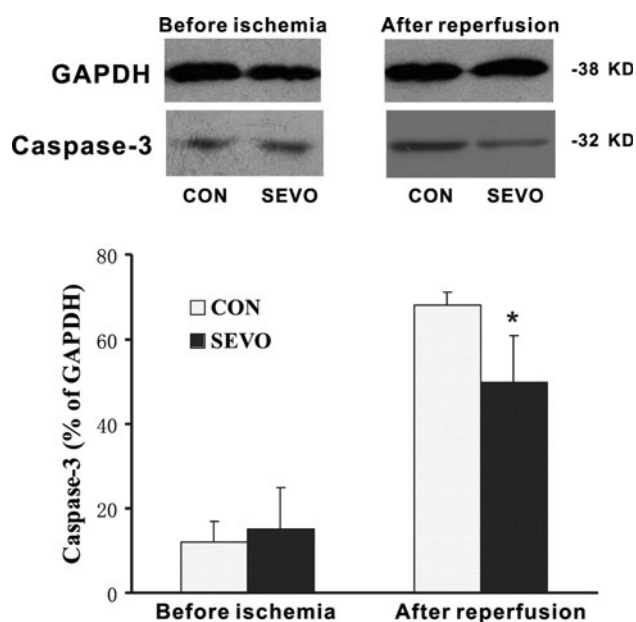
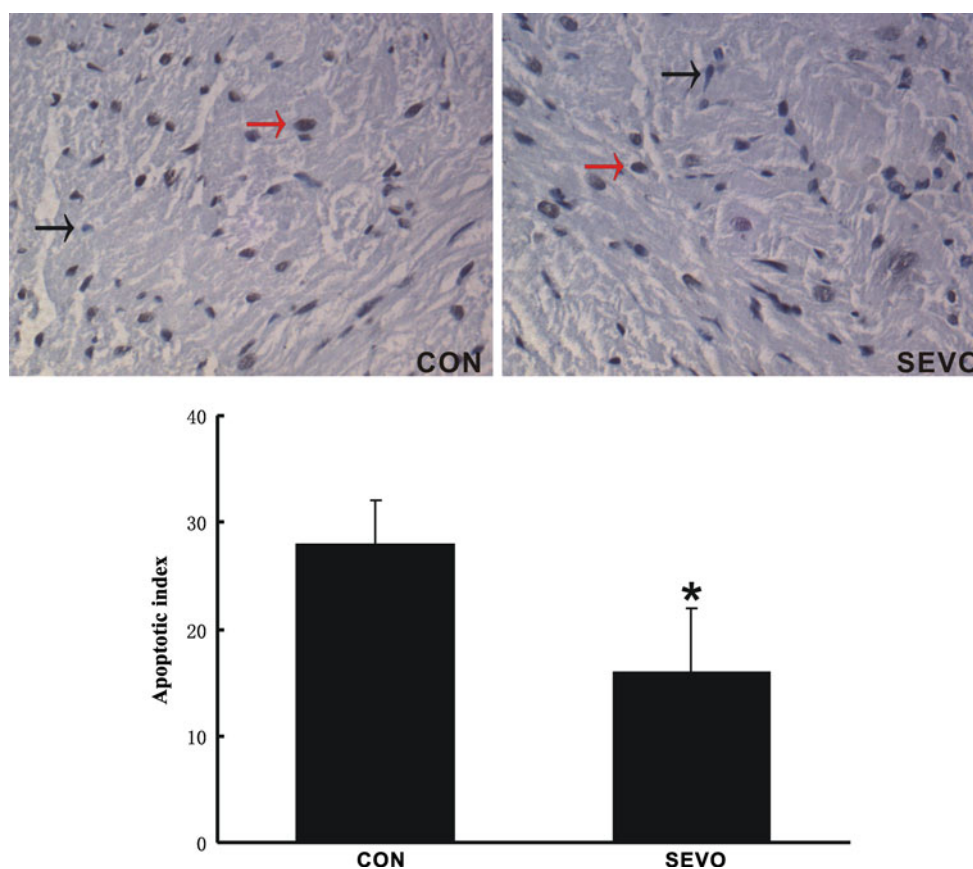


Fig. 4 Representative immunoblots (top panel) of caspase-3 in rats before and after reperfusion in each group. Histograms illustrating caspase-3 corresponded to the pictures. All data are mean ± SD. CON control group, SEVO sevoflurane group. *Significantly ($p < 0.05$) different from CON group after reperfusion, $n = 4$ per group

with the CON group ($58 \% \pm 3$, $p < 0.05$) and the TUNEL assay (Fig. 3) demonstrated that it also reduced the apoptotic index from ($28 \% \pm 4$) in CON group to ($16 \% \pm 6$) in SEVO group ($p < 0.05$).

After reperfusion, caspase-3 expressions were significantly increased in both groups when compared with before ischemia, but it was significantly lower in the SEVO group than in CON group ($50 \% \pm 11$ vs. $68 \% \pm 10$, $p < 0.05$) (Fig. 4).

Discussion

This is the first study demonstrating that APC with sevoflurane produced second window of myocardial protection against I/R in vivo using technetium-99m-labeled annexin-V image, a novel approach to identify the apoptosis. Delayed APC reduced myocardial I/R injury by decreasing the infarct size and reducing apoptosis.

Studies have shown that APC produces protection against myocardial I/R injury in both the acute and delayed periods, both in vivo and in vitro. However, there is also controversy about its protective effect. In a dog model, Kehl et al. [13] found that isoflurane does not produce a second window of myocardial protection. Many

mechanisms have been extensively studied such as K_{ATP} channel, NF- κ B, 12-lipoxygenase and so on [5–7], but very few studies have focused on the role of apoptosis in delayed APC. Studies demonstrated that APC with sevoflurane protects myocardium from I/R not only protects myocardial by reduced infarct size, but also improves cardiac functional recovery, decreases post-ischemic myocardial stunning and attenuates myocardial apoptosis [3, 8, 18, 19]. In this study, we focused on the apoptosis induced by myocardial I/R. Our results are consistent with most published results that SWOP with sevoflurane blunts the I/R induced myocardial injury by decreasing infarct size and myocardial apoptosis. It is also consistent with results from acute preconditioning in myocardial protection.

Apoptosis is a process of programmed cell death. In addition to the morphological changes of apoptosis the cell membrane will also change in the process. During the process of apoptosis, phosphatidylserine groups migrate to the outer layer of the cell membrane lipid bilayer from the inner layer. This migration is the initial event of the apoptosis cascade. Annexin-V is an endogenous protein that has a high affinity for binding to phosphatidylserine. It is related to early apoptosis. Under the radioactive tracer technique the annexin-V can be radiolabeled. When it is bound to the phosphatidylserine it can be used to detect the apoptosis in vivo. Technetium-99m-labeled annexin-V is a promising method for assessing the degree of apoptosis [15–17]. In this study we used this technology to measure myocardial apoptosis with great success. Its biodistribution in rats demonstrated a rapid blood and renal clearance with gradual excretion in other organs or tissues including heart, liver, lungs, and muscle. Low uptake by the brain was also observed throughout the course of the study. The results show that the T/B ratio of radioactivity at the infarct areas 30 min after technetium-99m-labeled annexin-V injection in SEVO group were lower than in the CON group which means the degree of apoptosis is less in SEVO group. The subsequent determination by TUNEL assay supported the result that delayed sevoflurane preconditioning reduced myocardial I/R induced apoptosis and the myocardial infarct size was also significantly reduced. Caspase-3 is the most important member of the caspase family. Most of the factors that trigger apoptosis and eventually leads to apoptosis through caspase-3 mediated signal transduction pathway. In the Western blot assay, caspase-3 was reduced after reperfusion in the SEVO group when compared to the control group. This study was conducted at multi-levels (organ, cellular and protein) and the results confirmed that apoptosis was involved in the cell death and SWOP with sevoflurane inhibited apoptosis and contributed significantly to the myocardial protection against I/R injury.

Limitations

This study was conducted at multi-levels (organ, cellular and protein) to confirm that anti-apoptosis participated in the mechanisms of delayed APC with sevoflurane. There are other mechanisms involved the myocardium protection against I/R injury such as reactive oxygen and nitrogen, and K_{ATP} channels [20–22]. However, in this study we mainly focused on testing the effect of anti-apoptosis of SWOP of sevoflurane using technetium-99m-labeled annexin-V image method.

Conclusion

Sevoflurane preconditioning produced SWOP against I/R injury in vivo. The new method of real-time imaging using technetium-99m-labeled annexin-V successfully detected cardiomyocyte apoptosis and the result was confirmed by results from TUNEL assay and caspase-3 using Western blot. We conclude that anti-apoptosis plays a significant role in delayed sevoflurane preconditioning against myocardial I/R injury and thus may provide new strategy to treat myocardium post-ischemic injury.

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