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Original Research Article

Effect of sevoflurane delayed-postconditioning on ischemia-reperfusion injury in isolated rat hearts

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Abstract

Purpose: *To evaluate the protective effect, effective time window, and mechanism of protection of sevoflurane post-conditioning and late sevoflurane post-conditioning on heart muscle ischemia-reperfusion (I/R) lesion in isolated rat heart.*
Methods: *Langendorff isolated perfusion model was established in Sprague-Dawley (SD) rats. After 15 min of stabilization, the rats were assigned to 10 groups, based on the treatments given: control group (TTC), I/R group, sevoflurane post-processing group (SpostC), 0.5 min-, 1 min-, 5 min-, 10 min-, 20 min-, 30 min- delayed sevoflurane post-processing group (S0.5, S1, S3, S5, S10, and S20). The levels of mitochondrial membrane permeability, area of myocardial infarction, myocardial apoptosis, and p-GSK-3 beta level were determined.*
Results: *Myocardial infarction areas in Spost, S0.5, S1, S3, S5, S10 and S20 groups were significantly than in I/R group ($p < 0.05$), while expression levels of myocardial p-Akt were significantly higher in Spost, S0.5, S1, S3, S5, S10, S20, and S30 groups than in I/R group ($p < 0.05$). GSK-3 phosphorylation level was significantly higher in Spost, S1, S10, S20, and S30 groups than in I/R group ($p < 0.05$). Expression of p-GSK-3β in S30 group decreased, relative to that in Spost group ($p < 0.05$).*
Conclusion: *Sevoflurane post-conditioning and delayed sevoflurane post-conditioning mitigates I/R lesions in isolated rat hearts. The protective effect against ischemia-reperfusion injury is related to the levels of phosphorylation of Akt and GSK-3β. Thus, this treatment approach may be useful in the management of cardiac ischemia-reperfusion injury.*

Keywords: *Sevoflurane postconditioning, Delayed sevoflurane postconditioning, Protein kinase B, Glycogen synthase kinase-3β, Mitochondrial permeability transition pore (MPTP)*

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INTRODUCTION

Delayed postconditioning is based on the concept of ischemic postconditioning (Post C) which addresses the treatment of cardiac ischemia-reperfusion injury [1]. Inhaled anesthetics which are widely clinically used as general anesthetic drugs are known to protect against myocardial I/R damage and are used in delayed post-treatment [2]. However, in clinical practice, due to inadequate lung perfusion, a time window is required for inhaled narcotic

drugs to reach their corresponding targets in order to play protective roles.

Phosphatidylinositol-3-kinase protein kinase B (PI3K-Akt)/glycogen synthase kinase 3β (GSK3β) is a signaling pathway of reperfusion injury salvage kinase (RISK). It is crucial for protection against I/R injury in heart muscle. The MPTP is important in cell apoptosis and necrosis. Some studies have shown that MPTP opening and GSK-3β are related [3,4]. It has been observed that postconditioning of inhaled anesthetic sevoflurane provided significant mitigation of I/R lesions in isolated rabbit hearts, in a previous study [5].

The present study was carried out as a further investigation of the protective window and the related mechanisms involved in treatment with delayed sevoflurane postconditioning.

EXPERIMENTAL

Animals and model drugs

Adult male SD rats (n = 100) weighing 250 - 350 g were obtained from Beijing Vito Lihua Co. Ltd. (Production license number: SCXK (Beijing) 2014-0006). The rats were maintained on standard laboratory rodent feed at controlled room temperature and humidity of 20 - 25 ^{o}C and 70 %, respectively. This research was approved by the Animal Ethical Committee of Fuwai Cardiovascular Hospital (approval no. FWCH) and was performed in accordance with the guidelines of "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [6]. Sevoflurane was purchased from Maruishi Pharmaceutical Company, Japan (production Lot 0624).

Reagents

Sodium chloride (NaCl), KCl, $MgSO_4$, $NaHCO_3$, KH_2PO_4, Glucose, HEPES, and $CaCl_2$ were purchased from Beijing Chemical Reagent Company. 2,3,5-Chlorotriphenyltetrazole (TTC) was bought from Sigma, Inc., while the BCA protein concentration assay kit was purchased from Beijing Puli Li Gene Technology Co., Ltd. Polyacrylamide (10 %), SDS-PAGE separating gel, concentrated gum, phosphorylated and total protein kinase B (Akt), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and membrane washing buffer (tris-buffered saline Tween, TBST) were purchased from Sigma Company. Antibodies for phosphorylated Akt and glycogen synthase kinase (GSK)-3 β were purchased from CST.

Animal grouping and treatments

Unified disposal method

All experimental animals were assigned to the control group (TC), I/R group, sevoflurane post-processing group (SpostC), 0.5 min-delayed sevoflurane postprocessing group (S0.5), 1 min-delayed postprocessing group (S1), 3 min-delayed postprocessing group (S3), 5 min-delayed postprocessing group (S5), 10 min-delayed postprocessing group (S10), 20 min-delayed postprocessing group (S20), and 30 min-delayed postprocessing group (S30), with 10 rats per group.

Establishment of Langendorff ex vivo perfusion model

SD rats were intraperitoneally injected with heparin at a dose of 1000 IU/kg. Ten (10) min later, the rats were anesthetized with chloral hydrate drug. The effect of anesthesia was determined *via* caudal reflex. The hearts of the anesthetized SD rats were excised and immersed in a pre-cooled solution at 4 ^{o}C without calcium phosphate buffer and suspended in the Langendorf perfusion device within 30 s.

Criteria for the successful establishment of in vitro model

The rat hearts were suspended in a Langendorf perfusion device for < 30 sec and allowed to stabilize for 15 min. The normal heartbeat rhythm was > 250 times/min and left ventricular systolic blood pressure was > 75 mmHg. The model was considered a failure if any of the following events occurred: (a) a decrease in heart rate > 20 % or frequent ventricular premature beats, ventricular tachycardia, or ventricular fibrillation; (b) balloon rupture or manometric failure; (c) appearance of red spots or large pale areas on the surface of the heart after perfusion, and (d) leakage at the junction between the aortic root and perfusion needle.

Treatment of groups

Following effective induction of the cardiac model of perfusion, the following measures were taken in each group:

TC group: Continuous constant perfusion current was applied for 120 min after stabilization of pre-oxygen KH liquid.

I/R group: After the stabilization of constant cardiac perfusion for 30 min, the constant

perfusion current flow perfusion was restored to full in preoxygenation KH solution for 120 min.

SpostC group: After the onset of reperfusion, the KH solution was fully saturated with 3 % sevoflurane. Then, the rats were continuously perfused for 15 min, followed by perfusion with KH solution at a constant flow for 105 min.

S0.5 group: After 0.5 min of reperfusion, the KH solution was fully saturated with 3 % sevoflurane, after which the rats were continuously perfused for 15 min, followed by perfusion with KH solution at constant flow for 105 min.

S1 group: After 1 min of reperfusion, the KH solution was fully saturated with 3 % sevoflurane. Then, the rats were continuously perfused for 15min, followed by perfusion with KH solution at a constant flow for 104.5 min.

S3 group: Following 3 min of reperfusion, the KH solution was fully saturated with 3 % sevoflurane. Then, the rats were continuously perfused for 15 min, followed by perfusion with KH solution at a constant flow for 102 min.

S5 group: After 5 min of reperfusion, the KH solution was fully saturated with 3 % sevoflurane. Thereafter, the rats were continuously perfused for 15 min and then perfused with KH solution at a constant flow for 100 min.

S10 group: After reperfusion for 10 min, the KH solution was fully saturated with 3 % sevoflurane, and the rats were continuously perfused for 15 min, followed by perfusion with KH solution at a constant flow for 95 min.

S20 group: After 20 min of reperfusion, the KH solution was fully saturated with 3% sevoflurane. Then, the rats were continuously perfused for 15 min, followed by perfusion with KH solution at a constant flow for 85 min.

S30 group: Following 30 min of reperfusion, the KH solution was fully saturated with 3 % sevoflurane, and then the rats were continuously perfused for 15 min, followed by perfusion with KH solution at a constant flow for 75 min.

Evaluation of parameters/indices

Hemodynamic parameters

The rat heart in each group was fully fixed in the perfusion device, and a small incision was made on the left atrial appendage of the heart. A latex balloon containing a pressure sensor, and filled with KH buffer was inserted into the left ventricle. The left ventricular end-diastolic pressure (LVEDP) was set to 0 - 10 mmHg by adjusting the balloon volume, and the volume of the balloon remained stable. A computer was connected to the pressure sensor at the end of the balloon. Then, the Powerlab system was used for recording indices such as LVDP, heart rate (HR), LVDP maximum rate of rise (+dp/dt), and LVDP maximum descent rate (-dp/dt) in each group.

Cardiac troponin level in coronary outflow

At the heart stabilization stage and after 120 min of reperfusion, the KH fluid (1 mL) was taken from each of the 10 groups of rats. Cardiac troponin (cTnI) levels were measured using ACS:180 automated chemiluminescence immunoassay system (US, BECKMAN) and Roche kit A.

Myocardial infarct size

The hearts of each group of SD rats were numbered, and 5 samples were taken from each group using the random number method. The magnitude of the infarct was determined with TTC staining. Following TTC treatment, the heart samples were fixed overnight in 4 % formalin and photographed after 24 h. Images were analyzed with Image J 1.37 software. The infarct size of the heart was calculated in terms of the percentage of the total necrotic area of the left ventricular section.

Phosphorylation levels of Akt and GSK-3β

After measuring the myocardial infarct sizes in the 10 groups of SD rats, 30 mg of myocardial tissue taken from each of the remaining 5 heart samples was subjected to Western blot assay for protein expression levels of p-Akt in TC, I/R, SpostC, S0.5, S1, S3, S5, S10, S20 and S30 groups, as well as protein levels of p-GSK-3β in TC, I/R, SpostC, S1, S10, S20, and S30 groups.

Myocardial cell apoptosis

After I/R for 120 min, complete single apoptotic body and apoptotic nuclei in left ventricular myocardial tissues in isolated heart risk areas of TC, I/R, SpostC, S1, S10, S20 and S30 groups (n=5) were stained *in situ* using transferase-mediated TUNEL assay. The numbers of normal cells and apoptotic cells in each high-power field (x400) were calculated. Generally, 10 high-power fields were examined per heart sample. TUNEL-positive cells appeared brownish yellow, indicating apoptosis of cardiomyocytes, while TUNEL-negative cells (normal and healthy

cardiomyocytes) were colored blue. The apoptotic index (AI) was calculated as a percentage of the number of apoptotic cells in the total number of cells.

Degree of opening of MPTP

After 30 min of reperfusion, 30-mg myocardial tissues were obtained from the left ventricle areas of 5 rats in each group (TC, I/R, SpostC, S1, S10, S20, and S30 groups). Absorbance at 340 nm was read using fluorescence measurement. The oxidant NAD^+ exhibits transference in cells. When MPTP is open, mitochondrial function is lost, resulting in the efflux of high levels of NAD^+ from mitochondria into the blood flow to the peripheral circulation during reperfusion. Therefore, when the ischemic tissue NAD^+ content is decreased, it is an indication that the MPTP is open.

Statistics

All results are presented as mean ± SD. The SPSS version 13.0 software was used for statistical analysis. Two-group comparisons were done for non-normal distribution data using one-way ANOVA and LSD methods. Values of $p < 0.05$ indicated that differences were statistically significant.

RESULTS

Effects of delayed sevoflurane postconditioning

As shown in Table 1, there were no significant differences in baseline values amongst the groups. However, compared with the baseline values, there were decreases in HR, LVDP, dp/dt, and -dp/dt at 30, 60, and 120 min after reperfusion in the other 9 groups except for TC ($p < 0.05$). Levels of HR, LVDP, dp/dt, and -dp/dt at 30, 60, and 120 min after reperfusion were decreased in the other 9 groups, relative to the TC group ($p < 0.05$). There were significant increases in HR, LVDP, dp/dt, and -dp/dt in the Spost, S0.5, S1, S3, S5, and S10 groups at 30, 60, and 120 min of reperfusion, relative to values in I/R. However, values of HR, LVDP, dp/dt, and -dp/dt in S30 and I/R groups at the same time points were comparable. Moreover, there were no statistically significant variations in these parameters between the Spost group and the S0.5, S1, S3, S5, S10, and S20 groups at 30, 60, and 120 min after reperfusion, while their levels were significantly reduced in the S30 group, relative to Spost group ($p < 0.05$).

Effect of delayed sevoflurane postconditioning on the level of cTnI

There were no significant differences in the level of cTnI in the coronary effluent amongst the 10 groups ($p > 0.05$). However, the level of cTnI in coronary effluent was significantly increased in each of the other 9 groups, relative to the TC group ($p < 0.05$). In contrast, the levels of cTnI were significantly decreased in Spost, S0.5, S1, S3, S5, S10, and S20 groups at 120 min after reperfusion, relative to I/R model, but cTnI levels in coronary effluent amongst the S0.5, S1, S3, and S10 groups were comparable to those in Spost group. However, there were increased levels of cTnI in coronary effluent in the S5, S20, and S30 groups, when compared with the Spost group ($p < 0.05$). These results are shown in Figure 1.

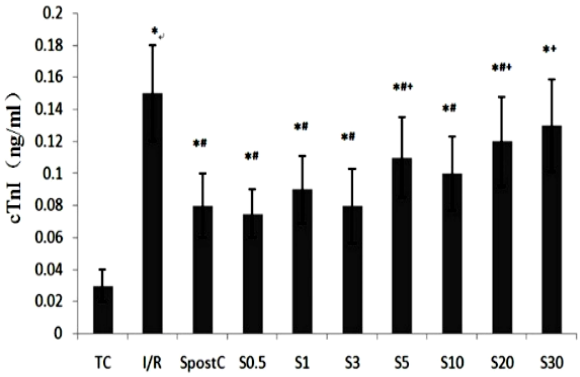

Figure 1: Cardiac troponin levels in coronary effluents of the 10 groups. $^{*,\#,+}P < 0.05$: *vs TC; $^{\#}$vs I/R; $^{+}$vs SpostC

Effect of delayed sevoflurane postconditioning on isolated cardiac infarct size

In the TC group, cardiac infarct size was not very obvious. Thus, statistics was not done on infarct size of this group. In contrast, pale areas were visible in the other groups. The degrees of infarction in I/R, Spost, S0.5, S1, S3, S5, S10, S20 and S30 groups were 44 ± 5.8 %, 20 ± 2.8 %, 23 ± 5.7 %, 22 ± 4.7 %, 25 ± 3.9 %, 32 ± 5.8 %, 30 ± 5.8 %, 34 ± 4.2 % and 39 ± 4.9 %, respectively. Relative to I/R group, infarct size in Spost, S0.5, S1, S3, S5, S10 and S20 was significantly decreased ($p < 0.05$). However, although there were no significant differences in infarct size amongst S30 group and the other groups, there were significant differences amongst S5, S10, S20, S30 and SpostC groups ($p < 0.05$; Figure 2).

Table 1: Levels of hemodynamic parameters of the experimental groups

Group	HR (beat/min)	LVDP (mmHg)	+dP/dt$_{max}$ (mmHg/s)	-dP/dt$_{max}$ (mmHg/s)
Baseline				
TC	290.2±14.2	110.4±6.0	3280.5±213.3	2382.2±216.3
I/R	285.4±20.1	96.5±13.3	3172.2±312.4	2326.7±298.0
SpostC	295.6±25.3	102.2±18.1	3189.3±303.2	2249.5±209.4
S0.5	293.5±18.6	106.3±14.0	3233.4±229.1	2128.5±211.6
S1	282.3±21.0	112.2±10.1	3082.7±368.5	2253.4±268.4
S3	287.4±16.2	111.5±9.0	3312.1±289.0	2264.2±278.3
S5	294.0±8.4	110.3±12.3	3308.2±292.1	2196.3±232.2
S10	286.4±12.3	113.2±11.0	3079.4±321.3	2182.0±241.1
S20	290.1±6.0	107.4±13.2	2936.3±227.2	2267.6±281.5
S30	289.3±13.2	115.1±6.0	2859.6±232.5	2329.1±200.1
Reperfusion for 30 min				
TC	289.3±19.3	76.3±17.9	2565.4±200.2	1654.3±152.4
I/R	250.3±12.0*	25.4±6.2*	1526.3±202.5*	603.6±119.9*
SpostC	280.5±21.2#	52.1±8.0*#	2103.2±102.1#	1152.3±100.2#
S0.5	279.4±19.5#	58.3±7.5#	2219.3±134.0#	963.4±84.5*
S1	275.4±18.1#	49.1±10.0*#	2103.5±86.3*#	924.3±80.3*#
S3	270.5±20.3*#	45.4±10.9*#	2064.1±79.0*#	899.1±69.2*
S5	265.1±10.5*	38.3±7.0*#	2111.3±93.2#	1024.2±92.1*#
S10	254.3±13.2*+	35.1±6.9*+	2010.1±93.0#	938.0±75.1*#
S20	261.2±12.1*	30.4±10.2*+	1964.3±85.2*#	826.6±73.5*+
S30	259.3±14.2*	28.1±9.0* +	1863.5±80.4*#+	796.1±68.1*+
Reperfusion for 60 min				
TC	285.2±18.2	70.0±15.6	2254.2±259.0	1506.2±148.3
I/R	246.4±10.1*	28.3±6.1*	1550.2±214.4*	698.4±98.7*
SpostC	279.3±20.0#	58.2±9.1*#	2210.0±213.9#	1179.2±113.1#
S0.5	275.2±18.3#	55.0±8.1*#	2354.4±221.1#	1054.3±90.4*#
S1	260.3±17.0*#	46.7±10.6*#	1998.3±150.1*	931.2±79.2*#
S3	249.4±16.2*	47.3±9.8*#	1952.2±103.1*#	902.0±74.1*#
S5	261.0±13.4*	40.2±6.9*#	2092.1±102.0#	996.2±90.1*#
S10	252.2±11.0*	37.1±8.0*+	2024.3±95.2#	950.3±82.4*#
S20	253.3±12.1*	38.4±9.2*+	2000.1±92.0*#	859.4±80.3*#+
S30	250.1±15.0*	30.1±8.9*+	1953.3±100.2*#	861.0±60.0*+
Reperfusion for 120 min				
TC	280.2±15.1	65.3±12.0	2060.3±172.1	1494.1±125.2
I/R	241.3±12.0	26.3±5.4*	1450.1±220.3*	592.2±83.5*
SpostC	275.2±20.9#	55.7±8.3#	2009.2±201.1#	927.3±100.2#
S0.5	269.2±15.3#	51.6±8.0#	2296.3±198.0#	1000.2±85.3*#
S1	263.2±14.0#	43.2±9.3*#	1869.2±138.1*#	926.2±80.0*#
S3	258.3±16.1*#	42.1±9.1*#	1858.2±94.1*#	864.0±72.1*#
S5	248.1±13.5*	36.0±6.5*#	1993.3±100.2#	957.2±89.1*#
S10	250.2±10.0*	35.1±7.9*	1986.1±91.0#	931.3±80.4*#
S20	246.3±14.1*	31.3±8.9*	1908.0±89.9*#	843.4±76.3*#
S30	247.1±12.0	28.1±8.3*+	1843.1±92.0*#+	714.1±72.2*+

*,#,+P < 0.05, *vs TC; #vs I/R; +vs SpostC

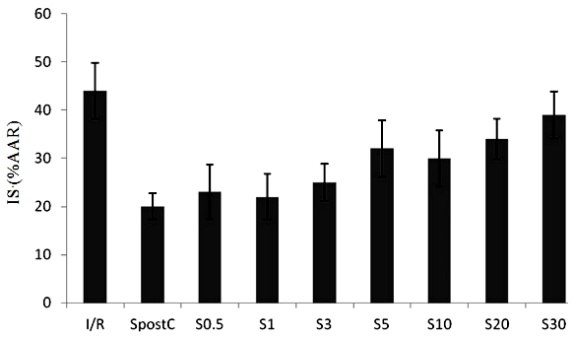

Figure 2: Myocardial infarct size in each group. #,+,P < 0.05: #vs I/R; +vs SpostC (IS: cardiac death area)

Effect of delayed sevoflurane postconditioning on p-Akt and p-GSK-3β levels

The p-Akt expression level in the TC group was 0.26 ± 0.03 units, while the expression level of p-Akt in the I/R group was 0.42 units. Compared with the TC group, the relative concentrations of p-Akt in the other 9 groups were significantly increased (p < 0.05). Moreover, p-Akt expression was significantly up-regulated in the Spost group, S0.5, S1, S3, S5, S10, S20, and S30 groups, when compared to the I/R group (p < 0.05). The expression level of p-Akt in the Spost group was

comparable with the corresponding levels in S0.5, S1, S3, S5, S10, S20, and S30 groups ($p > 0.05$; Figure 3). The expression levels of p-GSK-3β in I/R, Spost, S1, S10, S20, and S30 groups were significantly increased, relative to the TC group ($p < 0.05$). Compared with the I/R group, there were significantly increased expressions of p-GSK-3β in the Spost, S1, S10, S20, and S30 groups ($p < 0.05$; Figure 3 and Figure 4). However, the expression of p-GSK-3β was comparable in the Spost, S1, S10, and S20 groups.

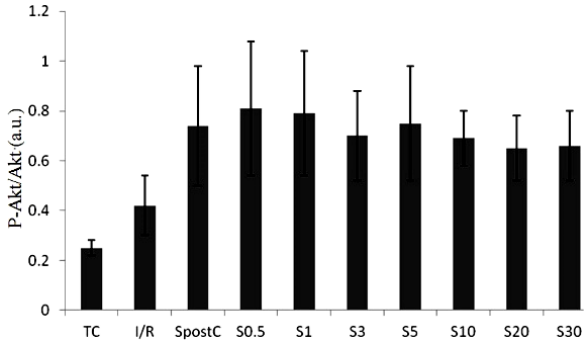

Figure 3: Expression levels of phosphorylated protein kinase B. $^{*,\#}P < 0.05$: *vs TC; #vs I/R. (p-Akt: phosphorylated protein kinase β; Akt: total protein kinase β)

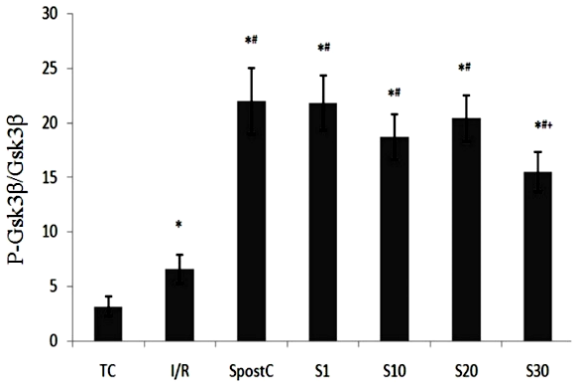

Figure 4: Expression levels of phosphorylated glycogen synthase kinase 3β. $^{*,\#,+}P < 0.05$: *vs TC; #vs I/R; +vs SpostC

Effect of delayed sevoflurane postconditioning on myocardial apoptosis index

The apoptosis index of heart muscle cells in the I/R group was 14.3 ± 1.9 %, while the apoptotic index values of myocardial cells in Spost, S1, S10, S20, and S30 groups were 8.6 ± 1.2, 7.5 ± 1.4, 9.2 ± 1.5, 14 ± 2.1, and 12.7 ± 1.9 %, respectively. Myocardial apoptosis index in Spost, S1 and S10 groups were significantly lower than that in the I/R group ($p < 0.05$). However, the myocardial apoptosis index was comparable in I/R, S20, and S30 groups (Figure 5).

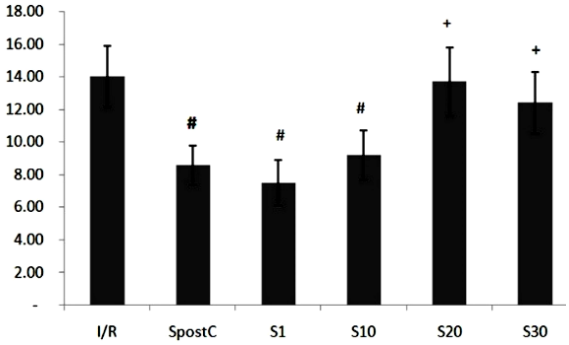

Figure 5: Myocardial cell apoptosis index values. $^{\#,+}P < 0.05$: #vs I/R; +vs SpostC

Effect of late sevoflurane post-priming on NAD+ content

The content of NAD+ in the left ventricular risk area was 162.39 ± 12.56 nmol/g. The contents of NAD+ in the myocardium of I/R, Spost, S1, S10, S20, and S30 groups were significantly lower than the corresponding level in the TC group. Significant reductions were evident in the content of NAD+ in myocardial tissues of the left ventricle. However, NAD+ levels in myocardial tissue of the left ventricular myocardium in the Spost, S1, S10, and S20 groups were significantly increased, when compared to the I/R group ($p < 0.05$). Relative to the Spost group, significant reductions were seen in NAD+ levels in the myocardium of the left ventricular risk area in the S10, S20, and S30 groups ($p < 0.05$, Figure 6).

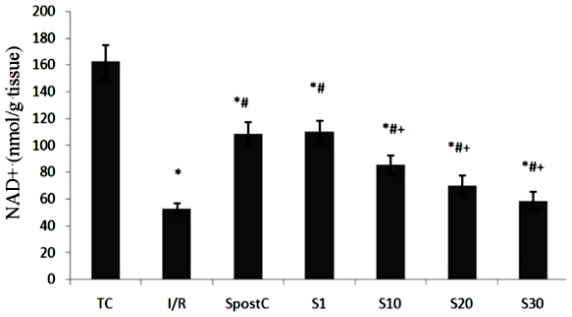

Figure 6: NAD+ contents in myocardial tissues. $^{*,\#,+}P < 0.05$: *vs TC; #vs I/R; +vs SpostC

DISCUSSION

Studies have confirmed that the pretreatment and postconditioning of a volatile anesthetic such as sevoflurane produce cardioprotective effects [7]. Previous studies focused on the protective effects of sevoflurane pretreatment and postconditioning on the myocardium, and the mechanisms involved. The traditional view

suggests that the implementation of postconditioning is associated with a narrow effective time window. Moreover, the implementation of postconditioning within the effective time window plays a key role in myocardial protection. Delayed postconditioning of sevoflurane and other volatile anesthetics results in a protective effect that has not received adequate research attention [8]. Therefore, this research was done to study the protective influence and mechanism of delayed sevoflurane postconditioning so as to further enrich knowledge in the area of myocardial protection by volatile anesthetics.

The results of this study showed that HR, LVDP, dp/dt, and -dp/dt in perfused hearts from Spost, S0.5, S1, S3, S5, and S10 groups at 30, 60, and 120-min groups were significantly improved, when compared with I/R group, suggesting enhanced ventricular, systolic and diastolic functions. Although sevoflurane has a direct inhibitory effect on the human heart, this study was aimed at ischemia and reperfusion of the rat heart which has a very different normal heart physiology in that the effect of sevoflurane on the myocardium L-type calcium channel may be directly inhibited, thereby maintaining myocardial calcium homeostasis. Sevoflurane reduces the accumulation of ROS during reperfusion, and it protects myocardial function during reperfusion. However, it should be noted that postconditioning of sevoflurane has a time limit for the improvement of hemodynamics in the heart of rats with ischemia-reperfusion. A 10-min delay in postconditioning improved systolic and diastolic function in isolated hearts, but when sevoflurane postconditioning was delayed by 20 or 30 min, its effect on cardiac hemodynamics was not significant. Moreover, it had no significant effect on cardiac systolic and diastolic function. Therefore, to protect myocardial function during reperfusion, sevoflurane postconditioning must be implemented within 10 min after reperfusion.

Under normal physiological conditions, due to the integrity of the cell membrane, cardiac troponin I (cTnI) only exists in normal cardiomyocytes. However, when the myocardium is damaged, myocardial cell membrane integrity is destroyed, resulting in cTnI release into the blood. This causes an increase in peripheral tissue cTnI levels. Skeletal muscle troponin I (sTnI) and cTnI have different amino acid sequences. Therefore, cardiac troponin I is a specific marker of heart muscle lesions when the concentration of cTnI is determined in the absence of cross-reactions which affect the test results [9]. Cardiac infarct size was determined using TTC staining in which normal myocardial tissue gives a brick red color due to the reduction of TTC by succinate dehydrogenase reaction. In contrast, in myocardial infarction areas, there is a reduced level of ischemic myocardial dehydrogenase activity, resulting in the pale color of the myocardium. Therefore, the area of pale color of the heart is used to determine the specific size of the infarction [10].

Ischemic postconditioning improves cardiac function during reperfusion, reduces the incidence of reperfusion-induced arrhythmias, and reduces the area of cardiac infarction [11]. This study showed that this protective effect on the heart was time-limited: the time taken to increase its protective effect was affected by postconditioning delays. The results of this study showed that the levels of cTnI in the coronary effluent were significantly lower in the Spost, S0.5, S1, S3, S5, S10, and S20 groups after 120 min of reperfusion, indicating that postconditioning and delayed postconditioning of sevoflurane produced remedial effects.

Studies have demonstrated the importance of the RISK pathway in ischemic preconditioning and postconditioning, with respect to myocardial protection. At the same time, the protective effects of narcotic drug pretreatment and postconditioning on the myocardium are also closely related to the RISK pathway [12]. In a previous study, sevoflurane postconditioning was found to be mediated by activation of the RISK pathway in a rat model of ischemic care [13]. In the present study, it was observed that in the *in vitro* perfusion rat model, sevoflurane postconditioning and delayed postconditioning also significantly protected the heart from ischemia-reperfusion-mediated damage, and also significantly increased the expression of p-Akt in cardiomyocytes. These data indicate that the cardioprotective influence of sevoflurane postconditioning and delayed postconditioning were generated through the activation of the RISK signaling pathway. In this study, the expression level of p-Akt in the S30 group was significantly higher than that in the I/R group, but the infarct size of the heart was not decreased, indicating that the RISK path was activated at this time. However, the delayed postconditioning interval was too long, and it exceeded the time window required for protection by late sevoflurane post-priming. In order to achieve this postconditioning-associated protection, there is a need to implement the sevoflurane postconditioning within 20 min of reperfusion.

The GSK-3 is present in various cellular components such as cytoplasm, mitochondria, and nuclei. In addition to glycogen synthase,

GSK-3 plays an important role in various responses within the cell, especially in protection against myocardial ischemia-reperfusion injury. Although MPTP decreases the target site of ischemia-reperfusion, its opening has a direct relationship with GSK-3β phosphorylation [14,15]. In a previous study, it was found that sevoflurane postconditioning increased the phosphorylation of GSK-3β and reduced the opacity of mPTP [16]. In this study, sevoflurane postconditioning was also found to increase GSK-3β phosphorylation levels and delayed sevoflurane postconditioning groups showed significant increases in GSK-3β phosphorylation levels. However, compared with the sevoflurane postconditioning group, the GSK-3β phosphorylation level in the S30 group was significantly reduced. We hypothesize that this may be related to the phosphorylation of GSK-3β with time.

Mitochondrial non-specific MPTP is the central hub of mitochondrial information exchange. When large numbers of mitochondrial MPTPs are open, the metabolic environment is destroyed. A study has demonstrated that irreversible changes resulted in cells in which MPTP continued to remain open, leading to cell necrosis [17]. When a short-term ischemic heart does not cause MPTP to open, its opening occurs during the reperfusion period, resulting in increased damage in the ischemic myocardium [18,19]. In this study, we chose to indirectly reflect the openness of MPTP by measuring the levels of NAD^+ in the mitochondria of cardiomyocytes in line with extant literature [20]. The levels of NAD^+ in the myocardium of the left ventricles in Spost, S1, S10, and S20 groups were significantly higher, relative to the I/R group, indicating that postconditioning and sevoflurane late postconditioning inhibited the opening of MPTP. The MPTP is a key feature in myocardial ischemia/reperfusion injury. Inhibition of MPTP opening reduces myocardial damage due to ischemia-reperfusion. However, with an extension of the duration of delayed sevoflurane postconditioning, the number of open MPTPs gradually increased, resulting in irreversible damage and loss of the protective effect of sevoflurane postconditioning.

CONCLUSION

Sevoflurane postconditioning and delayed postconditioning produces protective effects from I/R lesion *ex vivo* in rat heart. This results in an improvement in the hemodynamics of the heart after reperfusion, reduces the release of cTnI from the myocardium, decreases infarct size, and reduces the degree of cardiomyocyte apoptosis. At the same time, the protective effect has a time window of < 30 mins. The protection of isolated rat hearts due to delayed post-priming with sevoflurane may be related to the phosphorylation levels of Akt and GSK-3β and inhibition of cardiomyocyte MPTP opening. Thus, this treatment approach may be useful in the management of cardiac ischemia-reperfusion injury.

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Ethical approval

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Li-huan Li designed the study, supervised the data collection, and analyzed the data. Gang Cheng interpreted the data and prepared the manuscript for publication. Gang Cheng supervised the data collection, analyzed the data, and reviewed the draft of the manuscript.

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