Ischemic Postconditioning Reduces Ischemic Reperfusion Injury of Non–Heart-Beating Donor Grafts in a Rat Lung Transplant

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Abstract

Objectives: **This study was designed to see if ischemic postconditioning could attenuate ischemic reperfusion injury of transplanted lungs recovered from non–heart-beating donors.**

Materials and Methods: **Forty Sprague-Dawley rats were randomized into 2 groups: the control group and the ischemic postconditioning group, with 10 donor rats paired with 10 recipient rats in each group. Twenty rats underwent a left lung transplant from non–heart-beating donors with a warm ischemia time of 36.7 ± 5.62 minutes. In the ischemic postconditioning group, 5 cycles of 1-minute reperfusion and 1-minute reocclusion at the onset of reperfusion were applied as postconditioning. Arterial blood gas, wet-to-dry lung weight ratio, activities of malondialdehyde and superoxide dismutase, and expressions of apoptosis and ICAM-1 mRNA were compared.**

Results: **When compared with the control group 4 hours after reperfusion, PaO_2 was higher, and wet-to-dry lung weight ratio was lower, in the ischemic postconditioning group, and expression of apoptosis and ICAM-1 mRNA as well as activity of malondialdehyde were lower, while superoxide dismutase activity was higher in the ischemic postconditioning group.**

Conclusions: **Ischemic postconditioning can reduce ischemic reperfusion injury of lungs recovered from non–heart-beating donors and preserve lung function by reducing reactive oxygen species and inhibiting apoptosis and inflammation.**

Key words: *Ischemic postconditioning, Lung transplant, Non–heart-beating donors, Ischemic reperfusion injury, Lung protection*

Introduction

The increasing gap between the number of individuals awaiting organ replacement surgery and the supply of organs available for transplant underpins attempts to increase the number of available organs. Recently, lung transplants from non–heart-beating donors have been seen as a promising alternative to increase the donor pool.[1-3] However, ischemic reperfusion injury, as a continuing problem leading to early graft dysfunction in lung transplant[4] deserves our concern.

Ischemic postconditioning (IPo), originally introduced by Zhao and coworkers,[5] defined as *brief cycles of reperfusion alternating with reocclusion applied immediately at the onset of reperfusion*, can reduce irreversible postischemic injury. Recent studies[6-9] have demonstrated that IPo can attenuate ischemic reperfusion injury of many organs (eg, heart, brain, and lung). Because the onset of reperfusion is predictable and under a clinician's control, postconditioning is more clinically applicable than is preconditioning in unexpected acute ischemia.

This study, using a rat model of lung donation after cardiac death, was designed to investigate whether IPo can reduce ischemic reperfusion injury of lung grafts recovered from non–heart-beating donors, and how it works.

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Materials and Methods

Animal model

The study was approved by the Animal Care Committee of Central-South University, China, and was performed in accordance with National Institutes of Health guidelines for the use of experimental animals (NIH publication No. 85-23, revised 1996).

Forty Sprague-Dawley male rats weighing between 205 and 255 grams (provided by the Department of Laboratory Animals of Hunan Agricultural University) were randomized into 2 groups, with 10 donor rats paired with 10 recipient rats in each group. Donor rats were anesthetized with an intraperitoneal injection of 0.7 to 0.8 mL chloral hydrate and mechanically ventilated, with a tidal volume of 7 to 10 mL at a rate of 70 to 80 breaths/minute. Then, the abdominal aorta was cut open to lose blood. No donor rats were heparinized. Thirty minutes after cardiac arrest, the anterior wall was excised, and the trachea was clamped with the lungs fully inflated. An 18-gauge intravenous cannula was inserted into the main pulmonary artery and tightened, through which donor lungs were flushed with 4°C low-potassium dextran at a perfusion pressure of 20 cm H_2O, and the left atrial auricle was cut open at the same time for drainage of the low-potassium dextran solution. After flush, the vascular and bronchial stumps of the donor lungs were prepared by the "two-cuff-one-stent technique," first introduced by Zhang and coworkers,[10] with which we anastomosed the pulmonary arteries and veins in a traditional cuff technique and anastomosed the bronchi in a self-made stent technique. Then, the donor lungs were removed and stored in 4°C low-potassium dextran for 1 hour. These maneuvers and the subsequent operation were performed with an operating microscope at ×10 magnification.

Recipient rats were anesthetized and ventilated as described above, and anesthesia was maintained with 0.2% halothane, adjusted depending on heart rate and blood pressure. Through a neck incision, the left carotid artery was cannulated with a P60 catheter to measure arterial blood gas and monitor blood pressure via transducers. A baseline arterial blood gas level was measured from a 0.3-mL blood sample to assess the adequacy of mechanical ventilation. Through a left thoracotomy in the fifth intercostal space, the left lungs were removed, and the donor lungs transplanted by the technique above. Then, the transplanted lung was inflated to eliminate atelectasis. The success of transplant was determined by 3 items: no leakage of air and blood, full filling of pulmonary arteries and veins, and the transplanted lungs turned pink and flexible.

Experimental protocol

After transplant, in the control group, the pulmonary vessels were unclamped, and reperfusion was restored, ending ischemic time. While in the IPo group, the left pulmonary artery was given 5 cycles of 1 minute's reperfusion and 1 minute's reocclusion at the onset of reperfusion as postconditioning. Then, the recipients were ventilated and observed for 4 hours.

Preparation of specimens

Arterial blood gas was obtained after snaring the right hilum at 10 minutes, 1 hour, 2 hours, and 4 hours after the onset of reperfusion. The animals were killed with intravenous thiopental sodium, and portions of donor lungs were immediately frozen in liquid nitrogen and stored at -70°C. A piece from the midportion of the donor graft was excised and weighed. It was then dried in a 60°C oven for 48 hours and reweighed. The ratio of wet/dry weight was calculated.

Measurement of malondialdehyde and superoxide dismutase activity

Lung tissues were homogenized on ice with normal saline. The homogenates were centrifuged at 4000*g* $minute^{-1}$ at 4°C for 10 minutes. The malondialdehyde level in the supernatants was determined by measuring thiobarbituric acid-reactive substances levels (assay kits was supplied by Nanjing Jiancheng Corp., Nanjing, China) as previously described.[12] The results were calculated as nmol·100 mg^{-1}. Superoxide dismutase activity in the supernatants was evaluated by inhibition of nitroblue tetrazolium reduction by O^2- generated by the xanthine/xanthine oxidase system (Nanjing Jiancheng Corp.) in accordance with the previous method.[12] The results were expressed by U·100 mg-1 protein.

Assessment of apoptosis in donor grafts

Apoptosis in the donor graft was evaluated using terminal deoxynucleotidyl transferase-mediated deoxyuridine-biofin nick end labeling enzyme

Table 1. Ischemic Time (min) and PaO_2 (mmHg)

Group	WIT (min)	TIT (min)	PaO_2-1	PaO_2-2	PaO_2-3	PaO_2-4
C (n=10)	36.3 6.5.42	153.6 5310.29	86.4 6.8.09	79.6 9.10.84	65.6 5.10.77	53.5 3.12.63
IPo (n=10)	37.5 7.5.18	160.4 608.98	85.5 5.7.10	81.8 1.12.78	75.4 5.9.92*	70.6 0.8.85*

Abbreviations: C, control; IPo, ischemic postconditioning; TIT, total ischemic time; WIT, warm ischemic time
Data expressed as means ± SD.
PaO_2-1, 2, 3, 4: PaO_2 at 10 min, 1 h, 2 h, and 4 h after reperfusion.
*P values < .05.

Table 2. Wet/Dry Ratio, Malondialdehyde (nmol·100mg^{-1}), Superoxide Dismutase (U·100mg^{-1}), and the Number and Density of Apoptotic Cells

Group	W/D	MDA	SOD	Average Number	Average Density
C (n=10)	7.64 ± 0.419	1.218 ± 0.055	12.805 ± 1.897	275.3 ± 60.52	157.25 ± 23.32
IPo (n=10)	5.94 ± 0.255*	1.103 ± 0.076†	16.177 ± 1.956†	145.2 ± 67.02†	101.97 ± 16.96†

Abbreviations: C, control; IPo, ischemic postconditioning; MDA, malondialdehyde; SOD, superoxide dismutase; W/D, wet/dry weight ratio
*P < .05 †P < .01

method, which identifies apoptotic cells in situ by using terminal deoxynucleotidyl transferase to transfer biotin-dUTP to the free 3'-OH of cleaved DNA. The biotin-labeled cleavage sites are then detected by a reaction with horseradish peroxidase conjugated streptavidin and visualized by 3,3'-diaminobenzidine showing a brown color. The detailed technique was described by Micoud and coworkers.[13]

Measurement of ICAM-1 mRNA

ICAM-1 mRNA level was measured by semiquantitative reverse transcriptase-polymerase chain reaction method. Total cellular RNA was isolated using TRIzol reagent (Sangon, Shanghai, China) in accordance with the manufacturer's instructions. After assessing purity of the final products by A value ratios at 260/280 nm, RNA samples were reverse transcribed into cDNA by a reverse-transcription kit (Promega Corporation, Madison, WI, USA). The ICAM-1 primers were 5′ TGTCAAACGGGAGATGAATGG 3′ (sense) and 3′ GGTAAATGTGGATAATGGCGGT 5′ (antisense). After amplification, polymerase chain reaction products were electrophoresed through a 2% agarose gel with ethidium bromide (0.5 mg/L) to visualize the DNA bands (ICAM-1/β-actin). The expected lengths of polymerase chain reaction products were 186 bp. The results were analyzed by a BF-300 image system and its software provided by Sixing Biotech (Shanghai, China).

Statistical analyses

All data are represented as means ± standard deviation (SD). The data of arterial blood gas were analyzed by repeated measurement ANOVA. All other data were analyzed by a 2-sample t test to identify significant differences between the experimental and control groups. Values for $P < .05$ were considered statistically significant.

Results

In each group, 10 recipient rats underwent a single left lung transplants in situ with non–heart-beating donor lungs whose warm ischemic time was 36.7 ± 5.62 minute. No mortality was observed at the end of the experiment. Table 1 shows that that non–heart-beating donor and total ischemic time of the grafts were not significantly different between the 2 groups ($P > .05$).

Changes of PaO_2 after reperfusion

Recipient arterial blood gases were measured at 10 minutes, 1 hour, 2 hours, and 4 hours from the onset of reperfusion after lung transplant. To eliminate the influence of the right lungs, PaO_2 was measured solely from the transplanted left lung by snaring the right hilum. As shown in Table 1, the mean PaO_2 decreased as time passed in both groups, and it was not significantly different between the 2 groups at 10 minutes and 1 hour. However, at 2 hours and 4 hours, the mean PaO_2 was much higher in the IPo group than in the control group ($P < .05$), suggesting that the lung functions of the grafts in the IPo group were preserved much better.

Changes in the wet/dry lung weight ratio

The wet-to-dry lung weight ratios were measured to evaluate fluid accumulation in the transplanted lung

specimens after reperfusion. The 20 left lung specimens removed from the recipient rats before the transplant had a mean wet-to-dry lung weight ratio of 3.86, which represents a control value of a normal lung. Compared with the value of normal lungs, the wet-to-dry lung weight ratios in both groups were increased, implying a significant increase in fluid accumulation after lung transplant. However, as shown in Table 2, the mean wet-to-dry lung weight ratio was significantly lower in the IPo group than it was in the control group ($P < .05$).

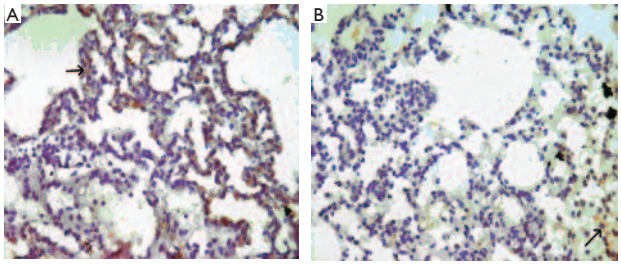
Changes of the level of malondialdehyde and the activity of superoxide dismutase

To detect the oxidative stress in the transplanted lungs, we measured the level of malondialdehyde and the activity of superoxide dismutase of the transplanted lungs. From Table 2, we found that compared with the control group, the mean malondialdehyde levels of the transplanted lung tissues in the IPo group were significantly lower ($P < .01$) and the mean superoxide dismutase activities were highly increased ($P < .01$).

Detection of apoptosis in donor grafts

We further tested apoptotic cells of the transplanted lungs using the terminal deoxynucleotidyl transferase-mediated deoxyuridine-biofin nick end labeling method by assessing DNA fragments of the apoptotic cells. The apoptotic cells were counted under fluorescence microscope. Determined by the average number and the average density of apoptotic cells, our results showed that apoptotic cells of the IPo group were dramatically fewer than those of the control group (Table 2, Figure 1; $P < .01$).

Figure 1. Apoptotic Cells of the Transplanted Lungs



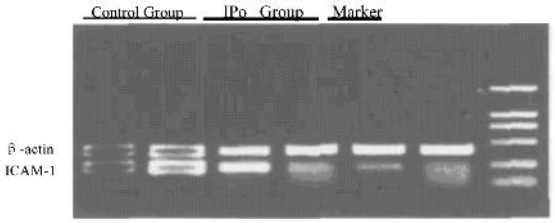
C group (×100) IPo group (×100)

The apoptotic cells, with brown and yellow staining in the nucleus (as labeled by an arrow) were markedly fewer in the lungs of the IPo group (B, ×100) than that of the C group (A, ×100). The nucleus of non-apoptotic cells were stained lightly blue.

Expression of ICAM-1 mRNA in donor grafts

Figure 2 illustrates that the ICAM-1 mRNA level was significantly lower in the IPo group that in the control group.

Figure 2. RT-PCR Analysis for Detecting ICAM-1 mRNA of Lung Grafts



Discussion

The latest data show that the long-term patient and graft survival rates of donation after cardiac death lung transplant are equivalent to those after brain death. However, non–heart-beating donors have been used infrequently for clinical lung transplant because unavoidable warm ischemia, with concomitant reperfusion injury, remains a limiting factor for transplanted lungs recovered from cardiac death donors. Some methods, such as topical cooling and nitroglycerin reperfusion or inhalation,[14-17] have been introduced to facilitate lung donation after cardiac death.

In this study, we hypothesized that IPo was another approach to effectively reduce ischemic reperfusion injury of lungs recovered from non–heart-beating donors. Recent research[18-20] has shown in that IPo is as effective as ischemic precondition in reducing ischemia/reperfusion-induced lung injury. However, whether IPo has such effects in non–heart-beating donor lungs and what are the optimal cycle numbers and duration of intermittent episodes, remain unknown. Zhang and associates,[21] after collecting many materials, conclude that the duration of intermittent episodes might be species dependent, which is empirically shorter in small animals and longer in large animals and human beings, and the duration is more important than cycle numbers. Because IPo on reperfusion-initiated lung injury has been scarcely reported, we took 5 cycles of 1 minutes' reperfusion and 1 minutes' reocclusion as the algorithm of IPo, because in our preliminary test we found it effective in reducing ischemic reperfusion injury of ischemic lungs.

Recipient rats underwent a left single lung transplant in situ with grafts having more than

30 minutes warm ischemia after cardiac death that were then ventilated for 4 hours. A serial measure of PaO_2 was used as the main variable when comparing lung function between groups. The results show that PaO_2 decreased gradually as time passed in accord with development of ischemic reperfusion injury of the donor grafts, and that mean values of PaO_2 were significantly higher at 2 hours and 4 hours in the IPo group than they were in the control group. All of which might suggest that IPo can preserve lung function by reducing ischemic reperfusion injury.

The injury experienced by transplanted lungs after reperfusion also could be reflected in the increase in wet-to-dry lung weight ratio. The wet-to-dry lung weight ratios were significantly higher in the transplanted lungs than they were in the normal lungs. However, compared with controls, the mean wet-to-dry lung weight ratio in the IPo group was lower. This indicates that IPo might attenuate fluid accumulation in the transplanted lung after reperfusion and improve lung function.

As reactive oxygen species have been thought to be an important contributor to reperfusion injury, we measured malondialdehyde and superoxide dismutase in this experiment. The results show that the level of malondialdehyde was reduced, and the activity of superoxide dismutase was higher, in transplanted lungs after 4 hours reperfusion in the IPo group compared with controls, indicating that reactive oxygen species generation during reperfusion was attenuated by IPo. At onset of reperfusion, there is a "respiratory burst" lasting several minutes, followed by moderately but persistently elevated production of superoxide anions. It is speculated that IPo may reduce reactive oxygen species generation by limiting delivery of oxygen during the rapidly repetitive occlusions. Conversely, limited blood flow at the onset of reperfusion may prevent the scavengers of reactive oxygen species, such as superoxide dismutase, from being massively flushed away.

Apoptosis is another factor resulting in reperfusion injury early after lung transplant.[22] Recent studies[23,24] have discovered that up-regulating the reperfusion injury salvage kinase pathway is one of the most important mechanisms in cardioprotection of IPo, including activation of phosphatidylinositol 3-kinase-Akt and/or extracellular signal-regulated kinase, which reduces apoptosis and necrosis by inhibiting the opening of mitochondrial permeability transition pore. The data in our study also show that apoptosis in the transplanted grafts was markedly reduced in the IPo group compared with the control group. However, the detailed mechanism needs further investigation.

Early after lung transplant, ICAM-1 expression in the transplanted lung is up-regulated, which is related to enhancement of ICAM-1 mRNA. As a ligand, ICAM-1 can bond with the receptor CD11/CD18 at the surface of neutrophil granulocytes, making neutrophil granulocytes migrate to tissue gaps and release inflammatory factors (eg, INF-α and IL-1β), which would further aggravate reperfusion injury of the transplanted graft. In our experiment, we noted that the ICAM-1 mRNA level was significantly lower in the IPo group than it was in the control group, which demonstrates that ischemic postconditioning could inhibit enhancement of ICAM-1 mRNA after transplant so as to down-regulate ICAM-1 expression, helping to reduce ischemic reperfusion injury.

In summary, the present study demonstrates that IPo has protective effects to non–heart-beating donor lungs in the rat lung transplant. It can preserve the structure and function of the transplanted lungs by reducing reactive oxygen species generation and inhibiting apoptosis and expression of ICAM-1 mRNA. Nonetheless, this hypothesis of IPo remains a purely experimental one and long-term observations are desired. Postconditioning is a promising means of facilitating lung transplant from non–heart-beating donors, because it is easy to manipulate and be combined with other protective strategies such as topical cooling and cold ventilation.

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