

# Amelioration of lung ischemia-reperfusion injury by JNK and p38 small interfering RNAs in rat pulmonary microvascular endothelial cells in an ischemia-reperfusion injury lung transplantation model

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**Abstract.** The inhibition of mitogen-activated protein kinases (MAPKs), including c-Jun NH<sub>2</sub>-terminal protein kinase (JNK), p38 MAPK (p38) and extracellular signal-regulated protein kinase 1/2 (ERK1/2), have an important effect on lung ischemia-reperfusion injury (IRI) during lung transplantation (LT). However, the way in which combined MAPK inhibition exerts optimal protective effects on lung IRI remains to be elucidated. Therefore, the present study evaluated the therapeutic efficacy of the inhibition of MAPKs in rat pulmonary microvascular endothelial cells (PMVECs) in an IRI model of LT. The rat PMVECs were transfected with small interfering RNAs (siRNAs) against JNK, p38 or ERK1/2. Cotransfection was performed with siRNAs against JNK and p38 in the J+p group, JNK and ERK1/2 in the J+E group, p38 and ERK1/2 in the p+E group, or all three in the J+p+E group. Non-targeting (NT) siRNA was used as a control. The PMVECs were then treated to induce IRI, and the levels of inflammation, apoptosis and oxidative stress were detected. Differences between compared groups were determined using Tukey's honest significant difference test. In all groups, silencing of the MAPKs was shown to attenuate inflammation, apoptosis and oxidative stress to differing extents, compared with the NT group. The J+p and J+p+E groups showed lower levels of interleukin (IL)-1 $\beta$ , IL-6 and malondialdehyde, a lower percentage of early-apoptotic cells, and higher superoxide dismutase (SOD) activity, compared with the other groups. No significant

differences were observed in the inflammatory response, SOD activity or early apoptosis between the J+p and J+p+E groups. These findings suggested that the dual inhibition of JNK and p38 led to maximal amelioration of lung IRI in the PMVECs of the IRI model of LT, which occurred through anti-inflammatory, anti-oxidative and anti-apoptotic mechanisms.

## Introduction

Lung transplantation (LT) is performed as a life-saving treatment for patients with end-stage lung disease (1). Ischemia-reperfusion injury (IRI) remains a significant contributor to morbidity and mortality rates following LT (2). The release of inflammatory mediators and reactive oxygen species (ROS) promotes IRI, causing cellular injury, pneumocyte necrosis and apoptosis (3).

Mitogen-activated protein kinases (MAPKs) are a family of serine-threonine protein kinases, which are activated in response to a variety of extracellular and intracellular stimuli, including cytokines, oxidative stress and growth factors (4). Three major MAPK signaling pathways, c-Jun NH<sub>2</sub>-terminal protein kinase (JNK), p38 MAPK (p38) and extracellular signal-regulated protein kinase 1/2 (ERK 1/2), regulate a variety of cellular activities, including proliferation, differentiation, survival and death (5). MAPK activation by IRI in the heart, liver and lungs has been reported *in vitro* and *in vivo* (6-8). In addition, the inhibition of MAPKs was shown to be pivotal in mediating the lung inflammatory response and cell death induced by IRI in our previous studies (9,10). Considering the significant cross-talk among these three signaling pathways, how to obtain the optimal protective effects on lung IRI by combined MAPK inhibition requires clarification.

Pulmonary microvascular endothelial cells (PMVECs) provide a dynamic and semi-permeable barrier, which is critical for lung gas exchange, regulation of fluids and solutes, passage of macromolecules between the blood and interstitial compartments, and adherence of circulating neutrophils during lung IRI (10-12). PMVECs are also primary targets of reactive oxygen species (ROS) and inflammatory mediators, for example, tumor necrosis factor- $\alpha$ , which can stimulate PMVECs to express adhesion molecules, including intercellular

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cell adhesion molecule-1 (10,13), suggesting that PMVECs are appropriate for investigating IRI in the pulmonary microcirculation endothelium. Therefore, the aim of the present study was to evaluate the possible effects of the combined inhibition of MAPKs in PMVECs of an IRI model of LT.

## Materials and methods

**Ethical approval.** The protocol for the present study was approved by Institutional Committee on Animal Care and Use of Harbin Medical University (Harbin, China).

**Isolation of rat PMVECS.** Pathogen-free male Wistar rats, 4-5 weeks old and weighing 60-80 g, were obtained from the Animal Experiment Center of Harbin Medical University. Rats had free access to rodent chow and water, and were maintained at  $24\pm 2^{\circ}\text{C}$  with a 12 h light-dark cycle. The rats were anesthetized with an intraperitoneal injection of 350 mg/kg chloral hydrate (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), and heparin (3,000 units) was injected intraperitoneally. Subsequently, a tracheotomy was performed using a sterile technique, and the lungs were perfused with medium 199 (M199; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and harvested. The rats were then sacrificed by exsanguination.

The rat PMVECs were isolated according to the protocols described in our previous study by Tan *et al* (10). Briefly, following flushing of the lungs with phosphate-buffered saline (PBS) at  $4^{\circ}\text{C}$ , the visceral pleura were stripped from the lung parenchyma to preclude mesothelial cells. The peripheral lung tissue was finely minced into small sections ( $\sim 1\text{ mm}^3$ ) and placed onto 25-mm<sup>2</sup> culture flasks coated with 1% gelatin (Sigma-Aldrich; Merck Millipore) containing M199 supplemented with 20% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 50  $\mu\text{g}/\text{ml}$  endothelial cell growth supplement (BD Biosciences, Franklin Lakes, NJ, USA) in a humidified atmosphere of 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . Subsequently, 100 U/ml penicillin-streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) and 8  $\mu\text{g}/\text{ml}$  tylosin (Sigma-Aldrich; Merck Millipore) were added to the culture medium. Following incubation at  $37^{\circ}\text{C}$  for 60 h, the tissues were removed, and the M199 was replaced to remove unattached cells. Contaminating cells were removed by scraping and aspiration. The rat PMVECs were used for subsequent experiments at passages 2-5.

**Study design.** The rat PMVECs were seeded at  $1\times 10^5$  cells/ml in 35-mm-diameter culture dishes 24 h prior to transfection. The transfection was performed with 100 pmol of small interfering RNAs (siRNAs) against JNK, p38 and ERK1/2 in the JNK group, p38 group and ERK1/2 group, respectively. The PMVECs were also cotransfected with 100 pmol each of the following siRNAs: JNK siRNA and p38 siRNA in the J+p group, JNK siRNA and ERK1/2 siRNA in the J+E group, p38 siRNA and ERK1/2 siRNA in the p+E group, and all three in the J+p+E group. Non-targeting (NT) siRNA was used as a control in the NT group. All siRNAs were prepared in Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) and all transfection procedures were performed using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) for

48 h. The target sequences of siRNAs were synthesized by Invitrogen; Thermo Fisher Scientific, Inc., and were as follows: JNK, 5'-UCAAGGAAUAGUGUGUGCAGCUAAU-3', p38, 5'-GGACCUCCUUAUAGACGAAUU-3', and ERK1/2, 5'-GACCGGAUGUUAACCUUUAUU-3'.

**Simulated IR.** Following transfection with the siRNAs, the PMVECs were exposed in a sealed container to simulate the rapid environmental changes during LT and were pre-ventilated with 95%  $\text{O}_2/5\%$   $\text{CO}_2$  at 1 l/min for 2 h, as described previously (10).

**Simulated cold storage.** The container was placed in a refrigerator ( $4^{\circ}\text{C}$ ), and M199 was immediately replaced with low-potassium dextran solution (Vitrolife, Kungsbacka, Sweden) with gas insufflation stoppage for 6 h.

**Simulated implantation.** The simulated implantation was performed by removing the container from the refrigerator and allowing it to return room temperature for 1 h.

**Simulated reperfusion.** Following the replacement of low-potassium dextran solution immediately with M199 pre-heated to  $37^{\circ}\text{C}$ , the container was ventilated with 50%  $\text{O}_2/5\%$   $\text{CO}_2/45\%$   $\text{N}_2$  for 2 h. The culture dishes were then removed from the container for detection. Gas concentrations in the container were monitored with a gas analyzer (S/N 32590; Datex Ohmeda, Helsinki, Finland).

**Western blot analysis.** The rat PMVECs in culture dishes were washed twice with ice-cold PBS, and trypsinized and lysed in RIPA buffer (Beyotime Institute of Biotechnology) supplemented with 1% phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology). The PMVECs lysates were centrifuged at  $13,201\times g$  at  $4^{\circ}\text{C}$  for 20 min and the supernatants were collected. Protein concentrations were determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology) and proteins were boiled with loading buffer at  $95^{\circ}\text{C}$  for 5 min. Protein samples ( $\sim 20\text{ }\mu\text{g}$ ) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. These membranes were blocked in 5% non-fat dried milk for 2 h, and then incubated with primary polyclonal rabbit anti-rat antibodies (1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) against JNK (cat. no. 9252), p38 (cat. no. 9218), ERK1 (cat. no. 4372) or ERK2 (cat. no. 9108) overnight at  $4^{\circ}\text{C}$ . Following conjugation with a diluted horseradish peroxidase-labeled secondary antibody (1:5,000; goat anti-rabbit IgG; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 1 h. The membranes were exposed to enhanced chemiluminescence (cat. no. GIS2010; Tanon Science and Technology Co., Ltd., Shanghai, China), and quantified using ImageJ 1.48v software (National Institutes of Health, Bethesda, MD, USA). The expression levels of the measured proteins were determined as the ratio of target proteins to that of  $\beta$ -actin (1:1,000; cat. no. TA-09; Zhongshan Golden Bridge Biotechnology, Beijing, China).

**Measurements of inflammatory cytokines and oxidation-reduction markers.** Culture medium was collected and

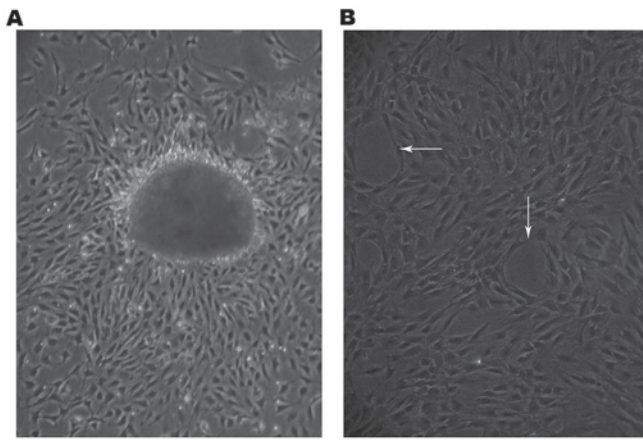


Figure 1. Characteristics of primary rat PMVECs. (A) Rat PMVECs migrated from small lung tissues. (B) PMVECs revealed capillary-like structures on the gelatin (white arrows; original magnification, x200). PMVECs, pulmonary microvascular endothelial cells.

centrifuged at 573 x g at 4°C for 20 min. The culture medium concentrations of IL-1 $\beta$  and IL-6 were assessed using an enzyme-linked immunosorbent assay according to the manufacturer's protocol (R&D Systems, Inc., Minneapolis, MN, USA). The cells were homogenized in 100  $\mu$ l of ice-cold PBS and centrifuged at 13,000 x g at 4°C for 10 min. The supernatants were collected and used to measure cellular levels of malondialdehyde (MDA) and activity of superoxide dismutase (SOD) using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**Flow cytometry.** An Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences) was used to detect apoptosis. The cells were collected by trypsinization, washed twice with ice-cold PBS and centrifuged at 297 x g at 4°C for 5 min. Subsequently, the cells were resuspended in binding buffer and adjusted to a density of 10<sup>6</sup> cells/ml. Equivalent quantities of Annexin V-FITC and propidium iodide (PI) were added to the cell suspension, followed by incubation in the dark at room temperature for 10 min. Cell apoptosis was then determined using flow cytometry (FACSsort; BD Biosciences) according to the manufacturer's protocol.

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard deviation, and all experiments were repeated at least three times. Differences between compared groups were determined using Tukey's honest significant difference test. Statistical analysis was performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Rat PMVEC characteristics.** The cells had migrated from the edge of the small lung tissues 60 h following tissue plating (Fig. 1A) and grew as capillary-like structures on the gelatin (white arrows, Fig. 1B).

**Protein expression of MAPKs.** The protein expression levels of JNK, p38, ERK1 and ERK2 in the PMVECs were reduced

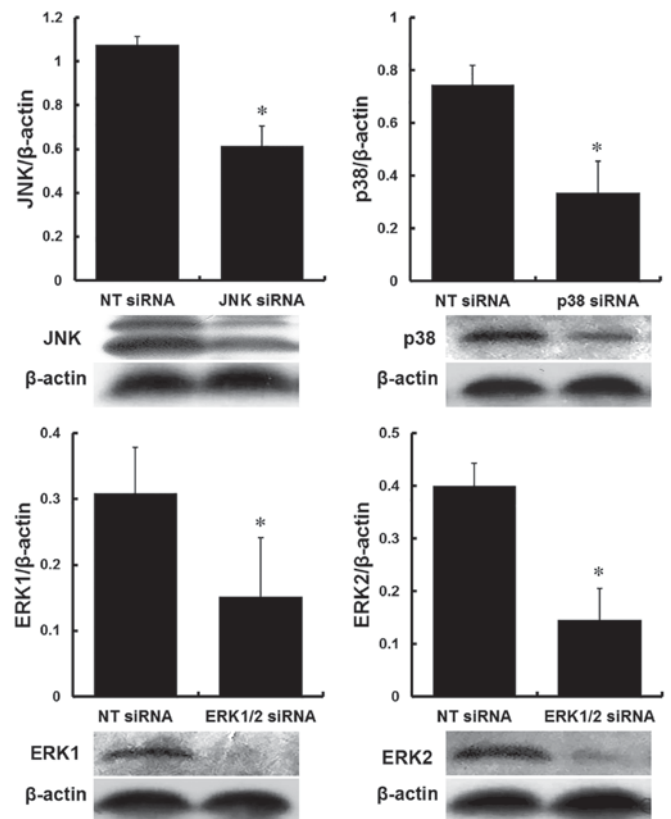


Figure 2. Protein expression of MAPKs. \* $P < 0.05$ , vs. NT siRNA transfection. MAPKs, mitogen-activated protein kinases; siRNA, small interfering RNA; JNK, c-Jun NH2-terminal protein kinase; ERK, extracellular signal-regulated kinase; NT, non-targeting.

by >35% following transfection with siRNAs against JNK, p38 and ERK1/2, compared with those in cells transfected with NT siRNA. The protein inhibition ratio to JNK was  $42.37 \pm 9.80\%$  following transfection with JNK siRNA ( $P < 0.05$ ); inhibition to p38 was  $55.70 \pm 12.90\%$  following transfection with p38 siRNA ( $P < 0.05$ ); inhibition ratios to ERK1 and ERK2 were  $53.01 \pm 20.78$  and  $63.16 \pm 17.28\%$ , respectively, following transfection with ERK1/2 siRNA ( $P < 0.05$ ; Fig. 2).

**Inflammatory cytokines and oxidation-reduction markers.** Compared with the NT group, significant decreases in the levels of IL-1 $\beta$  were observed in all other groups following simulation of IRI ( $P < 0.05$ ). In addition, there were significant reductions in the J+p and J+p+E groups, compared with the rest of the groups ( $P < 0.05$ ). No significant difference was observed between the J+p and J+p+E groups ( $P > 0.05$ ; Fig. 3A).

The levels of IL-6 were decreased in all groups, compared with that in the NT group ( $P < 0.05$ ). In addition, the levels of IL-6 were decreased in the J+p and J+p+E groups, compared with the other groups ( $P < 0.05$ ). No significant difference was observed between the J+p and J+p+E groups ( $P > 0.05$ , Fig. 3B).

The levels of MDA were substantially decreased in all groups transfected or cotransfected with target siRNAs, compared with that in the NT group ( $P < 0.05$ ). In addition, the level of MDA in the J+p group was significantly decreased, compared with the levels in the other groups ( $P < 0.05$ ; Fig. 3C).

Compared with the NT group, increased SOD activity was observed in all other groups ( $P < 0.05$ ). The activities of SOD

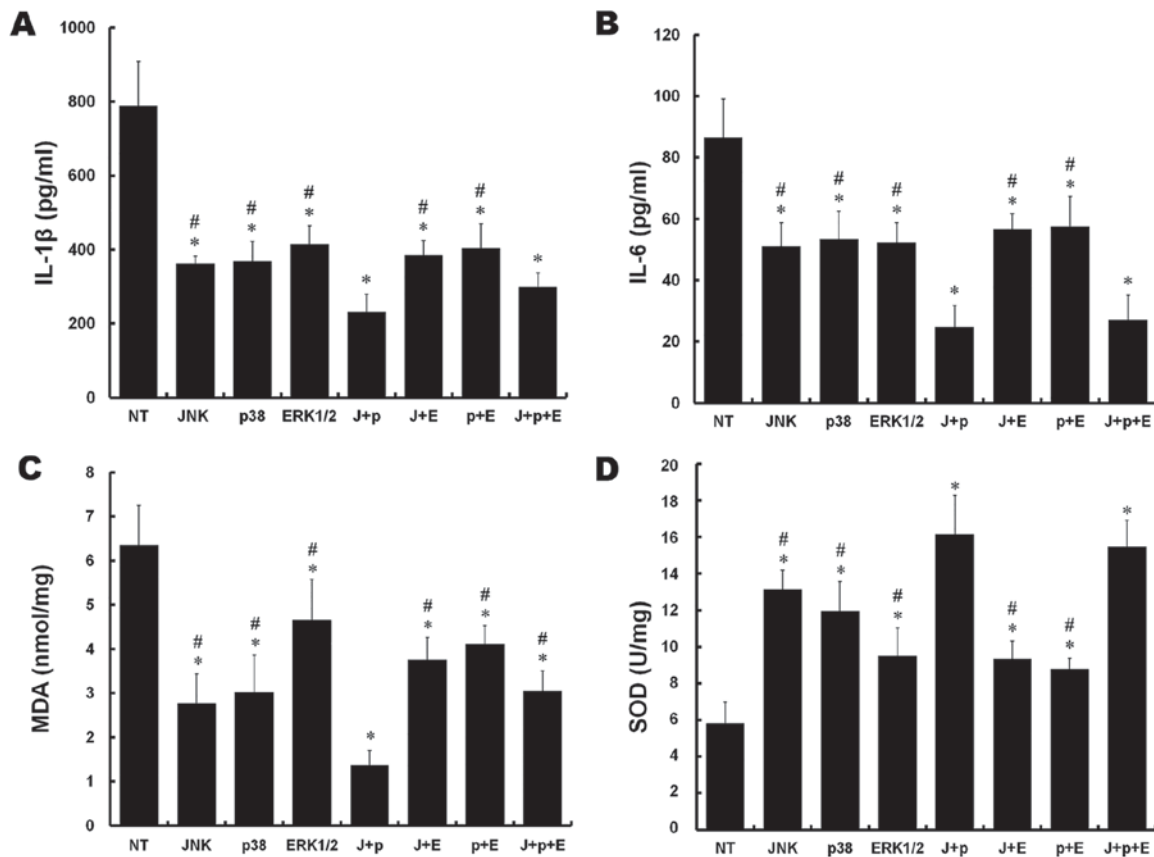


Figure 3. Effect of silencing of MAPKs on pro-inflammatory cytokines and oxidation-reduction markers in rat PMVECs. (A) Culture medium concentrations of IL-1 $\beta$ . (B) Culture medium concentrations of IL-6. (C) MDA levels. (D) SOD activity. \* $P < 0.05$ , vs. NT group; # $P < 0.05$ , vs. J+p group. J+p, JNK and p38; J+E, JNK and ERK1/2; p+E, p38 and ERK1/2; and J+p+E, JNK, p38 and ERK1/2. PMVECs, pulmonary microvascular endothelial cells; MAPKs, mitogen-activated protein kinases; IL, interleukin; MDA, malondialdehyde; SOD, superoxide dismutase; JNK, c-Jun NH2-terminal protein kinase; ERK, extracellular signal-regulated kinase; NT, non-targeting.

in the J+p and J+p+E groups increased significantly, compared with those in the other groups ( $P < 0.05$ ), however, no significant differences were observed between the J+p and J+p+E groups ( $P > 0.05$ ; Fig. 3D).

**Early apoptosis.** Annexin V<sup>-</sup> and PI<sup>-</sup> cells were used as controls, and Annexin V<sup>+</sup> and PI<sup>+</sup> cells are representative of early-apoptotic cells. The presence of Annexin V<sup>+</sup> and PI<sup>+</sup> cells is considered as a sign of late apoptosis, whereas Annexin V<sup>-</sup> and PI<sup>+</sup> cells are considered necrotic. In the NT group, numerous early-apoptotic cells were observed. Compared with the NT group, the percentages of early-apoptotic cells were decreased in the JNK, p38, ERK, J+p, J+E, and J+p+E groups ( $P < 0.05$ ). In addition, the percentages of early-apoptotic cells in the J+p and J+p+E groups were decreased significantly, compared with those in the other groups ( $P < 0.05$ ). No significant differences were observed between the J+p and J+p+E groups ( $P > 0.05$ ; Fig. 4).

## Discussion

The major finding of the present study was that the dual inhibition of JNK and p38 decreased the levels of IL-1 $\beta$ , IL-6 and MDA, and percentage of early-apoptotic cells, and increased the activity of SOD in PMVECs in an IRI model of LT. In addition, the inhibition of ERK1/2 had a marginal effect on

the levels of IL-1 $\beta$ , IL-6 and SOD, and on early apoptosis, compared with the effects of the dual inhibition of JNK and p38.

Due to their ease of harvesting and being relatively inexpensive, several cell types have been used to investigate lung IRI previously, including pulmonary artery endothelial cells (PAECs) and human umbilical vein endothelial cells (HUVECs) (14,15). PMVECs were used as the cell model to mimic lung IRI in the present study for several reasons. PMVECs are the early target cells of IRI and important in the initiation and development of pulmonary inflammation (16). Significant differences between PMVECs and PAECs have also been confirmed, including in morphology, proliferation, endothelial function, and endothelial barrier integrity (17,18). Previously, the replacement of PMVECs with HUVECs to investigate lung IRI showed that they differ from PMVECs in terms of biological properties and immune recognition (19). These discrepancies suggest that PMVECs may be more appropriate for investigating IRI in the pulmonary microcirculation endothelium.

Activation of the immune system is important in lung IRI (20). When the immune response is activated, pattern recognition molecules, including Toll-like receptors, are activated, triggering the MAPK signaling pathways (21). Ultimately, MAPKs induce the production of pro-inflammatory cytokines and chemokines, which contribute significantly to



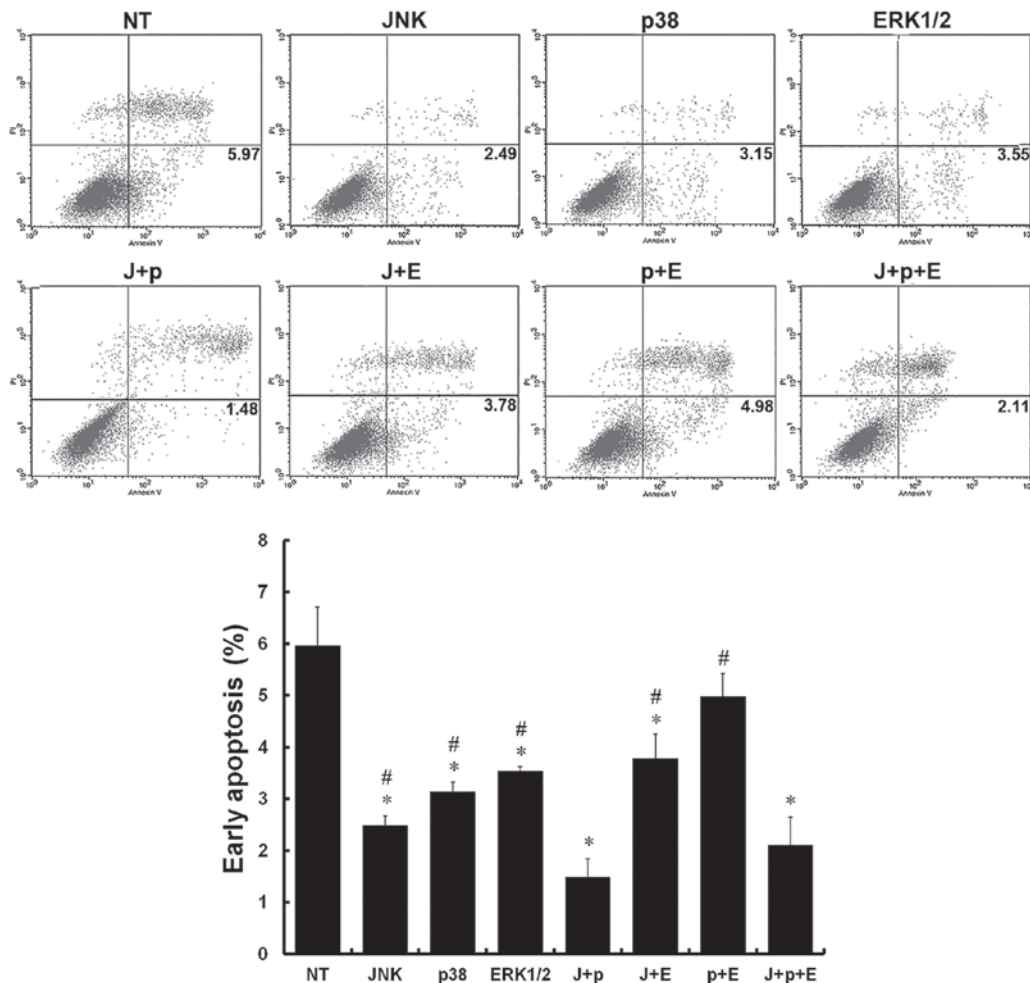


Figure 4. Effect of silencing MAPKs on early apoptosis. Stages of apoptosis were detected using flow cytometry in rat PMVECs. \* $P < 0.05$ , vs. NT group; # $P < 0.05$ , vs. J+p group. J+p, JNK and p38; J+E, JNK and ERK1/2; p+E, p38 and ERK1/2; and J+p+E, JNK, p38 and ERK1/2. PMVECs, pulmonary micro-vascular endothelial cells; MAPKs, mitogen-activated protein kinases; IL, interleukin; MDA, malondialdehyde; SOD, superoxide dismutase; JNK, c-Jun NH2-terminal protein kinase; ERK, extracellular signal-regulated kinase; NT, non-targeting; PI, propidium iodide.

lung IRI (22,23). As MAPKs are important in aggravating lung IRI, inhibiting the MAPK pathways may be an effective way to ameliorate lung IRI. Therefore, the present study inhibited MAPKs to identify a method to alleviate lung IRI and investigate the role of MAPKs, and examine the effects on lung IRI.

siRNAs are a novel class of RNA inhibitors, which specifically degrade target RNAs via the RNA-induced silencing complex (24). They have become increasingly popular as a potential technique for silencing specific genes due to their higher specificity, higher effectiveness, lower dose and fewer side effects, compared with inhibitors used in previous studies (25,26). In the present study, due to the transfection with siRNAs, the protein expression levels of JNK, p38, ERK1 and ERK2 in the PMVECs were reduced by  $>40\%$ , which indicated that the MAPK pathways were inhibited significantly and provided a suitable system for investigating the role of MAPKs in lung IRI.

Previous studies have investigated the functions of JNK, p38 and ERK1/2 alone and in combination; however, comparison of their therapeutic efficacies has not been evaluated. Due to the substantial cross-talk among MAPKs, circumscribed investigations or speculative hypotheses may ignore potential

possibilities. In the present study, a comprehensive method was used to investigate the functions of MAPKs in IRI. JNK, p38 and ERK1/2 were silenced, and the independent function of each pathway during IRI was confirmed. In addition, to observe the effect of multiple inhibitions on IRI, gene silencing of any two of the MAPKs or all three was performed. This enabled determination of a preferred therapeutic strategy through the inhibition of MAPKs to ameliorate IRI, using reliable data rather than theoretical possibilities.

Cargnello and Roux (4) and Murayama *et al* (27) found that JNK and p38 are important components involved in inflammation and apoptosis. The results of the present study showed that the inhibition of JNK or p38 decreased the levels of IL-1 $\beta$  and IL-6, and decreased the percentage of early-apoptotic cells. Therefore, the activation of JNK and p38 was confirmed to be pro-inflammatory and pro-apoptotic in the model, which was similar to the results reported Zhang *et al* and Liou *et al* (28,29). ERK1/2 has been suggested to be pro-inflammatory and pro-apoptotic (10,30), anti-apoptotic (31), or not involved in inflammation or apoptosis (32). However, the true effects of the ERK1/2 pathway differ depending on the stimuli and cell types (33). In the present study, the inhibition of ERK1/2 led to reductions in inflammation and apoptosis. The data

indicated that ERK1/2 was a pro-inflammatory and pro-apoptotic pathway in the IRI model. It has been reported that MAPK proteins can be activated by oxidative stress (34,35), whereas JNK mediates the increase of ROS production during stress (36), and the inhibition of p38 and ERK1/2 pathways has been shown to be involved in resistance to oxidative stress during renal IRI (37). The data obtained in the present study suggested that the silencing of JNK, p38 or ERK1/2 attenuated oxidative stress to different extents. Therefore, the inhibition of JNK, p38 or ERK1/2 may ameliorate lung IRI via anti-inflammatory, anti-apoptotic and anti-oxidative mechanisms.

The results of the present study showed that the inhibition of JNK or p38 alone decreased the levels of IL-1 $\beta$ , IL-6 and MDA, and the percentage of early-apoptotic cells, and increased the activity of SOD. The dual inhibition of these two kinases further increased this effect. In addition, the dual inhibition of JNK and p38 was the most effective technique for attenuating inflammation, apoptosis and oxidative stress in the IRI model of the present study. There are several possible reasons for these results. JNK interacts with and shares components with p38. These respond to common upstream activators and phosphorylate common downstream targets (28). JNK and p38 phosphorylate pro-apoptotic protein B-cell lymphoma 2-interacting modulator of cell death at the same site to initiate apoptosis and also activate effector caspases, including caspase 3, cooperatively (31,32). They can also regulate cytokine expression by modulating transcription factors, including nuclear factor- $\kappa$ B (4). Owing to the significant cross-talk, the silencing of either JNK or p38 alone is not sufficient to ameliorate apoptosis, inflammation or oxidative stress, therefore, the dual inhibition of the two kinases is important to obtain substantial amelioration. The gene silencing of JNK, p38 and ERK1/2 simultaneously had a notable effect on attenuating IRI. However, compared with the dual inhibition of JNK and p38, the additional inhibition of ERK1/2 had no increased positive effect on IRI, only resulting economic loss and waste of resources.

A limitation of the present study was that the mechanism underlying the interactions of MAPKs in IRI were not precisely determined. For example, the common downstream signaling pathways of JNK and p38, which can enhance the protective effect on IRI, remain to be elucidated. In addition, artificial cell model cannot completely mimic the actual physiological processes during LT, including alloimmunity and lung compliance.

In conclusion, the dual inhibition of JNK and p38 led to maximal amelioration of lung IRI via anti-inflammatory, anti-oxidative and anti-apoptotic mechanisms. These results demonstrated an optimal protective measure in MAPK pathways during lung IRI, and provide a therapeutic strategy against lung IRI induced by transplantation for further animal experiments and clinical applications.

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