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Early Protection by Resveratrol in Rat Lung Transplantation

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Background: Resveratrol is a multifunctional bioactive substance that has effects in anti-inflammation and prevention of ischemia-reperfusion injury. This study compared the inflammation and expression of related proteins during the early stages after transplantation to explore the effects and mechanisms of resveratrol on transplanted lung.

Material/Methods: Sprague-Dawley rats were randomized to receive pretreatment of resveratrol suspension (60 mg/kg; RES group), dexamethasone (1 mg/kg; DEM group), or normal saline solution (2 mL/kg; control group) 1 h before lung transplantation. The cytokine concentration in the serum and bronchoalveolar lavage fluid (BALF) of the recipients was determined 24 h after transplantation. Histopathologic evaluation, including lung injury score, and the expression of necroptosis-associated proteins was assessed.

Results: Histopathologic evaluation showed pneumocyte damage and endothelialitis associated with hemorrhage in the alveoli in the control group, the severity of which was greater than that in the other 2 groups. The levels of interleukin-6 and tumor necrosis factor-α in the serum and BALF of the RES and DEM groups were lower than those in the control group. The expression of necroptosis-associated proteins in the RES group was lower than that in the control group, and was inversely proportional to lung injury.

Conclusions: Pretreatment with resveratrol protected rat lung in the early stages after transplantation. We determined a relationship between necroptosis-associated proteins and transplanted lung injury, which suggests that the mechanism of lung transplantation-associated ischemia-reperfusion injury may be related to necroptosis.

MeSH Keywords: **Bronchoalveolar Lavage Fluid • Lung Transplantation • Receptor-Interacting Protein Serine-Threonine Kinases • Reperfusion Injury**

Abbreviations: **EGF** – early graft failure; **IRI** – ischemia-reperfusion injury; **RIPK** – receptor-interacting protein kinase; **SD** – Sprague-Dawley; **NS** – normal saline solution; **DEM** – dexamethasone; **RES** – resveratrol; **BALF** – bronchoalveolar lavage fluid; **LI** – lung injury; **IHC** – Immunohistochemistry; **iNOS** – inducible nitric oxide synthase; **NF-κB** – nuclear factor-κB; **MLKL** – mixed-lineage kinase domain-like; **ELISA** – enzyme-linked immunosorbent assay; **IL** – interleukin; **TNF** – tumor necrosis factor

Units: **Weight** – kilogram, gram, milligram, microgram; **Volume** – milliliter, microliter

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Background

Lung transplantation is the most effective treatment for many end-stage lung diseases, but donor shortages and severe postoperative complications greatly limit its use. According to the most recent global data on adult lung and heart-lung transplantation from the International Society for Heart and Lung Transplantation, up to June 30, 2014, the registrations of adult lung transplant recipients reached 51 440 cases, in which the incidence of early graft failure (EGF) was 24.3%, and mortality rates ranged from 9% to 22% in patients who developed complications within 30 days after lung transplantation [1,2]. Such high proportions raise concern about EGF, which is mainly caused by transplantation-associated ischemia-reperfusion injury (IRI).

IRI refers to reperfusion that not only fails to restore organ function after ischemia, but also aggravates organ dysfunction and tissue damage. Transplanted lung tissue injury caused by ischemia-reperfusion has main 3 causes: hypoxic-ischemic injury of the donor lung, endothelial cell injury induced by oxygen free radicals, and alveolar pneumocyte damage due to the disturbance of the microcirculation [3,4]. The histopathologic features of transplanted lung with severe IRI are often similar to those of severe acute rejection, which appears as prominent alveolar cell damage and endothelialitis with intra-alveolar necrotic epithelial cells, macrophages, neutrophils, and hemorrhage and may be associated with parenchymal necrosis or necrotizing vasculitis [5]. Recent studies have discovered a special type of programmed cell death termed necroptosis, which is mediated by the receptor-interacting protein kinase family of proteins (RIPK1 and RIPK3) [6,7]. However, very little research has been done on whether necroptosis is involved in lung transplantation-associated IRI.

Resveratrol (trans-3,4,5-trihydroxystilbene) is a natural polyphenolic compound with anti-inflammatory, anti-fibrotic, and antioxidant effects that is found in red grape skin, peanuts, and other sources [8]. Currently, the application of resveratrol pretreatment in lung transplantation has not been reported. In this study, we explored the effects of resveratrol on lung transplantation and IRI in a rat model of orthotopic left lung transplantation and investigated whether the mechanism of lung transplantation-associated IRI is related to necroptosis.

Material and Methods

Animal care

Sprague-Dawley (SD) rats (male, 8 weeks old, 250 to 300 g) were purchased from the Experimental Animal Center at Zhejiang Academy of Medical Sciences and reared in the SPF Laboratory of the Key Laboratory of Multiorgan Transplantation at the Chinese Ministry of Health (the First Affiliated Hospital, Zhejiang University, Hangzhou). The animal study protocols complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committees of the First Affiliated Hospital and the Experimental Animal Center, Zhejiang University, China.

Grouping and orthotopic lung transplantation

All 108 SD rats were randomly assigned into pairs (one as a donor and the other as a recipient) and were then divided into 3 treatment groups of 36 rats each: normal saline solution (NS group or control group), dexamethasone (DEM group), and resveratrol (RES group). Due to physiological pulmonary characteristics, the groups were divided into left and right subgroups if necessary. Each recipient in the corresponding groups received an intraperitoneal injection of resveratrol suspension (60 mg/kg body weight), dexamethasone (1 mg/kg body weight), or normal saline solution (2 mL/kg body weight) 1 h before surgery. Generally, anesthesia should be given 20–30 min before orotracheal intubation, so that sufficient depth of anesthesia can be reached in all the recipient rats. Orthotopic left lung transplantation was performed in the SD-to-SD rat strain combination. As described in previous studies [9], the left lungs were transplanted orthotopically using the cuff technique. Recipient operation time is from cutting the recipient rat skin to the end of the skin suturing. The time of cold ischemia and warm ischemia for each donor lung and the operation time for each recipient were recorded. Cold ischemia time is the transition time between the first and the second part of the warm ischemia time. Warm ischemia time is composed of 2 parts: the first part is from donor rat's superior vena cava disconnection to lung lavage completion and the second part starts with anastomosis and ends with opening the recipient pulmonary artery. The recipients were sacrificed 24 h after transplantation. Serum and plasma samples were taken from all recipients and preserved at –80°C for later use.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed on the first 9 recipients that underwent successful transplantation in each group. First, the lungs of the recipients were taken from the chest cavity; the bronchoalveolar lavage fluid (BALF) was then collected by inserting a cannula into the left lung via the respiratory tract, with the right main bronchus clamped, and pouring in phosphate buffer solution (5 mL each), which was extracted after about 1 min. The above procedures were repeated 3 times to obtain the total BALF of the lung. The average recovery of BALF was not lower than 70%. The right lung was then lavaged in the same manner. Finally, the BALF was centrifuged (4°C, 3000 rpm, 10 min) to separate the supernatant, which was preserved at –80°C.

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Table 1. Characteristics of lung transplantation in rats.

Group	Successful rate (%)	Recipient operation time (min)	Warm ischemia time (min)	Cold ischemia time (min)
NS group	94.44	27.21±2.34	12.64±2.07	10.04±0.56
DEM group	88.89	27.33±2.37	12.69±2.28	10.05±0.53
RES group	94.44	27.32±2.25	12.73±2.21	10.04±0.60

There were 36 rats in each group, equally split between donors and recipients, so there were 18 lung transplantations in each group. Because of the hemorrhagic shock caused by donor pulmonary vein laceration during anastomosis, the surgery failed and the success rate of each group is shown in the table. There was no significant difference in operation time and ischemia time between the 3 groups, p>0.05. NS group – normal saline group; DEM group – dexamethasone group; RES group – resveratrol group.

Histology

The lungs of the tenth successful recipient and the remaining rats in each group were extracted for blinded histopathologic analysis, fixed *in situ* by intratracheal instillation of 10% formalin for 24 h, and embedded in paraffin. The tissue sections were prepared and stained with hematoxylin-eosin to evaluate lung injury (LI) by assessing the range of inflammatory infiltration and the thickness of the alveolar septa [10]. Each assessment was graded as follows: 3 for injury of 50% or greater, 2 for injury ranging from 20% to 50%, 1 for injury of 20% or less, and 0 for no injury. Five high-magnification fields from each slide were analyzed, and the average was calculated. The resulting 2 scores were added and presented as the LI score of a given section. The average value of all slices on the same side was taken as the LI score of the subgroup.

Immunohistochemistry (IHC)

IHC staining was performed using the streptavidin peroxidase complex method to detect inducible nitric oxide synthase (iNOS), nuclear factor-κB (NF-κB) P65, RIPK3, mixed-lineage kinase domain-like (MLKL) protein (1: 100, Affinity Biosciences, Cincinnati, OH, USA), and tumor necrosis factor (TNF)-α (1: 200, Abcam, Shanghai, China). The IHC score was the product of the staining intensity and the percentage of positive cells. The staining intensity was graded on the following scale: 3 for saddle-brown, 2 for brown-yellow, 1 for pale yellow, and 0 for colorless. The score for the percentage of positive cells was from 0 to 4 (4 for at least 75% of positive cells, 3 for 51% to 75% of positive cells, 2 for 10% to 50% of positive cells, 1 for fewer than 10% of positive cells, and 0 for negative cells). An IHC score of at least 3 was considered to indicate a positive immune response.

Enzyme-linked immunosorbent assay (ELISA)

Circulatory and BALF interleukin (IL)-6, IL-10, and TNF-α production was analyzed by the Rat IL-6, IL-10, and TNF-alpha Platinum enzyme-linked immunosorbent assay (Neobioscience Technology Co., Shenzhen, China) according to the manufacturer's instructions. Absorbance was measured using Bio-Rad iMark (Bio-Rad Laboratories, Inc., Kyoto, Japan), and the results are expressed in picograms per milliliter.

Statistical analysis

All numerical data are presented as means ± standard deviations, and categorical data are presented as count and percentage. The statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA), including Fisher's exact test for count data and t test and *q* test for measurement data, and ANOVA was used for comparisons of more than 2 groups. All P values were two-tailed, and differences were considered to be statistically significant for Pvalue <0.05.

Results

Animal model of lung transplantation

We successfully completed a total of 50 rat lung transplantations, with an average operation time for the recipient of about 27 min and a 92.6% success rate. Four surgical procedures failed among the groups (1 in the RES group, 2 in the DEM group, and 1 in the control group) because the pulmonary vein was torn during hilar anastomosis, which eventually led to death by hemorrhagic shock. No significant difference was found between the groups in operation time and ischemia time (Table 1). All of the successful recipients survived to 24 h after transplantation.

Resveratrol pretreatment had a protective effect on transplanted lung

In hematoxylin-eosin-stained sections of transplanted lung from the control group, pneumocyte damage and endothelialitis, which may be associated with necrotic epithelial cells,

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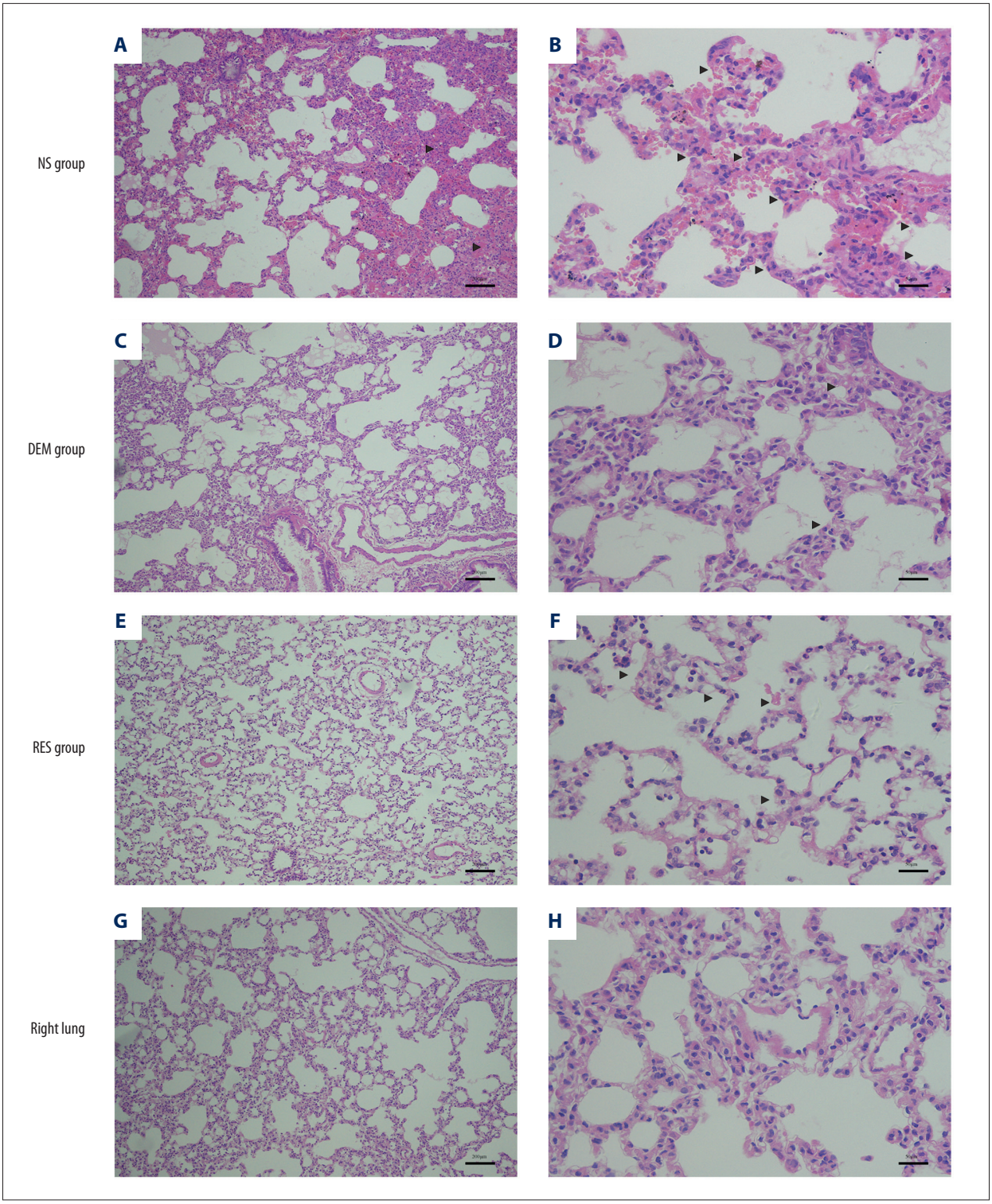

Figure 1. Lung tissues from different pretreatment groups stained with hematoxylin-eosin. The magnification of pictures is 100× on left side and 400× on right side. (**A**) Alveolar cell edema, hemorrhage, and local pulmonary atelectasis were observed, as shown in the triangle; (**B**) There were many red blood cells and neutrophils in the lung, and the nuclei of some cells became pyknotic (shrunken and dark) and broken. (**C–F**) A little bleeding and fewer inflammatory cells were shown in the lung tissue, and the alveolar structure was relatively complete. (**G, H**) Normal right lung tissues were used as control. NS group – normal saline group; DEM group – dexamethasone group; RES group – resveratrol group.

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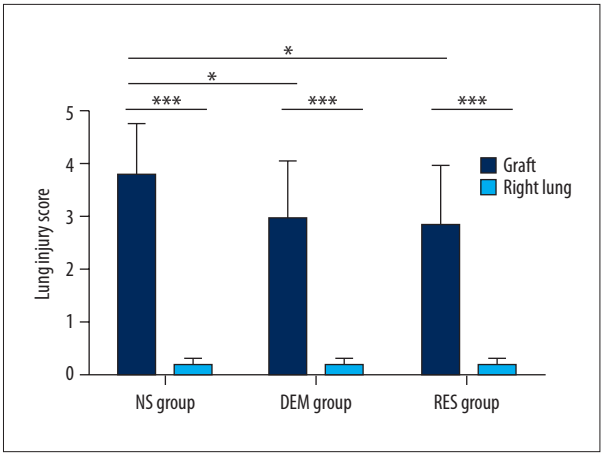

Figure 2. Lung injury scores in different groups (NS group: n=8; DEM group: n=7; RES group: n=8). All data shown are the means ± standard deviations. * p<0.05 and *** p<0.01. NS group – normal saline group; DEM group – dexamethasone group; RES group – resveratrol group.

inflammatory cells, or hemorrhage in pulmonary alveoli (in almost every transplanted lung, including the hilar and peripheral lung tissue), and atelectasis, necrosis, or infarction in the parenchyma were observed. The histology of the transplanted lungs from the RES group was similar to that of the DEM group and showed better alveolar structure, fewer necrotic cells, and milder inflammation and exudation. All right lungs showed normal pulmonary tissue with complete alveolar structure, no necrotic cells, and no hemorrhage or neutrophils in the alveoli (Figure 1).

The graft LI score of the control group was 3.80±0.96 and was noticeably higher than that in the DEM group and the RES group (2.80±1.17, $P=4.16\times10^{-2}$ and 2.94±1.11, $P=4.88\times10^{-2}$), between which there was no significant difference (P=0.47). The LI scores of the 3 right subgroups were 0.18±0.13, 0.19±0.17, and 0.15±0.18, respectively (no significant differences) and were significantly lower than those of the left subgroups in the same group (Figure 2).

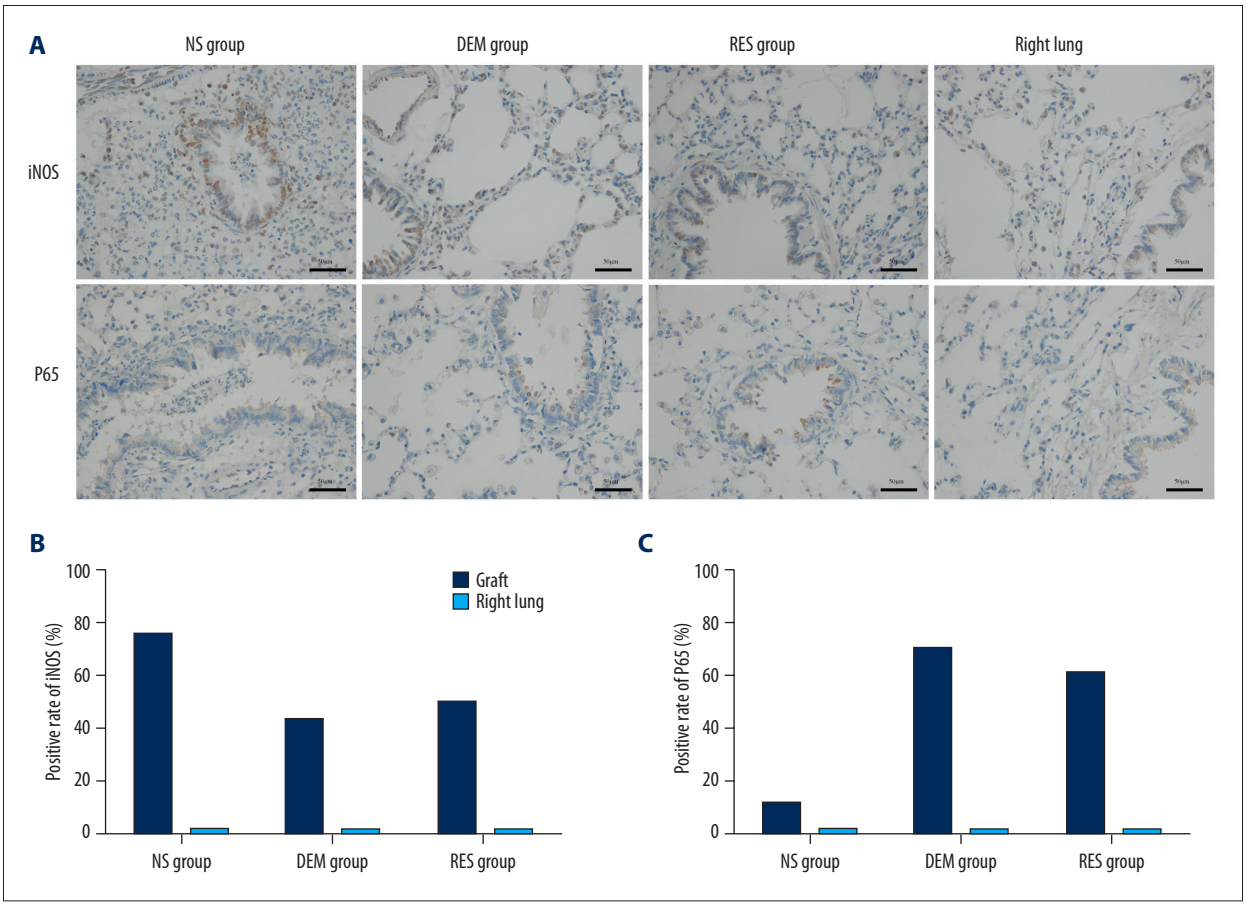

Figure 3. Immunohistochemistry of iNOS and P65 in lung grafts or *in situ*. The magnification of pictures is 400× (**A**). Protein expression was assessed as positive immune response or negative immune response depending on the immunohistochemistry score of different groups (NS group: n=8; DEM group: n=7; RES group: n=8). Positive immune response slices had more brown-staining cells in particular areas than the negative ones (**B, C**). NS group ,– normal saline group; DEM group – dexamethasone group; RES group – resveratrol group; iNOS – inducible nitric oxide synthase; P65 – nuclear factor-κB (NF-κB) P65.

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Table 2. Correlation between iNOS, P65, TNF-α, and LI score.

Parameters	iNOS (–) (n=10)		iNOS (+) (n=13)		P value
LI score (–) (n=10)	7	(4.35)	3	(5.65)	3.97×10^{-2}
LI score (+) (n=13)	3	(5.65)	10	(7.35)	
Parameters	**P65 (–) (n=12)**		**P65 (+) (n=11)**		**P value**
LI score (–) (n=10)	4	(5.22)	6	(4.78)	0.41
LI score (+) (n=13)	8	(6.78)	5	(6.22)	
Parameters	**P65 (–) (n=12)**		**P65 (+) (n=11)**		**P value**
iNOS (–) (n=10)	4	(5.22)	6	(4.78)	0.41
iNOS (+) (n=13)	8	(6.78)	5	(6.22)	
Parameters	**TNF-α (–) (n=15)**		**TNF-α (+) (n=8)**		**P value**
LI score (–) (n=10)	9	(6.52)	1	(3.48)	3.93×10^{-2}
LI score (+) (n=13)	6	(8.48)	7	(4.52)	
Parameters	**TNF-α (–) (n=15)**		**TNF-α (+) (n=8)**		**P value**
iNOS (–) (n=10)	9	(6.52)	1	(3.48)	3.93×10^{-2}
iNOS (+) (n=13)	6	(8.48)	7	(4.52)	
Parameters	**TNF-α (–) (n=15)**		**TNF-α (+) (n=8)**		**P value**
P65 (–) (n=12)	5	(7.83)	7	(4.17)	9.42×10^{-3}
P65 (+) (n=11)	10	(7.17)	1	(3.83)	

All 23 samples are from transplanted lungs (normal saline group: 8; dexamethasone group: 7; resveratrol group: 8). iNOS – inducible nitric oxide synthase; P65 – nuclear factor-κB (NF-κB) P65; TNF-α – tumor necrosis factor-α; LI score – lung injury score.

Resveratrol pretreatment alleviated transplantation-associated IRI

The lung cells in which IRI-related protein expression was observed in IHC were small-airway epithelial cells and alveolar epithelial cells surrounding the small vessels [11,12]. We observed that the expression of iNOS in the RES left subgroup was about 50%, which was lower than that in the control group (75%) and similar to that in the DEM group (42.86%). However, the expression of P65 was the inverse of that of iNOS in all left subgroups. The positive rate of expression of P65 was highest in the DEM left subgroup (71.43%), followed by the RES left subgroup (62.5%), and was lowest in the control left subgroup (12.5%). Furthermore, none of the right lung slices highly expressed iNOS or P65, differing significantly from the status of the transplanted lung (Figure 3).

To determine the relationship between transplanted lung injury and the LI score, we refined the index of LI. We defined tissue damage as positive or negative for the specimens with LI scores above or below the mean and counted their numbers separately to make a four-by table (Table 2). Because the purpose of this experiment was to understand the intrinsic relationship between the 2 indicators, we classified the 3 left subgroups as a whole for the statistical analysis. We used Fisher's exact test to calculate the correlations between the LI score, iNOS, P65, and TNF-α, and found that the LI score, iNOS, and TNF-α were positively correlated (LI score and iNOS, $P=3.97\times10^{-2}$; LI score and TNF-α, $P=3.93\times10^{-2}$; TNF-α and iNOS, $P=3.93\times10^{-2}$), and TNF-α and P65 were positively correlated ($P=9.42\times10^{-3}$), but that P65 expression was not correlated with the LI score (P=0.41) or iNOS (P=0.41) (Table 2).

Resveratrol pretreatment reduced the level of IL-6 and TNF-α in serum and BALF

ELISA results showed that, in comparison with the control group, the levels of IL-6 and TNF-α in the serum and BALF of the RES left subgroup had different degrees of regression, the anti-inflammatory effect of which was similar to that in the DEM left subgroup. However, no significant difference was observed in the IL-10 levels in the serum or BALF of the 3 left subgroups or in the cytokine concentrations in BALF between the subgroups in the same group (Figure 4).

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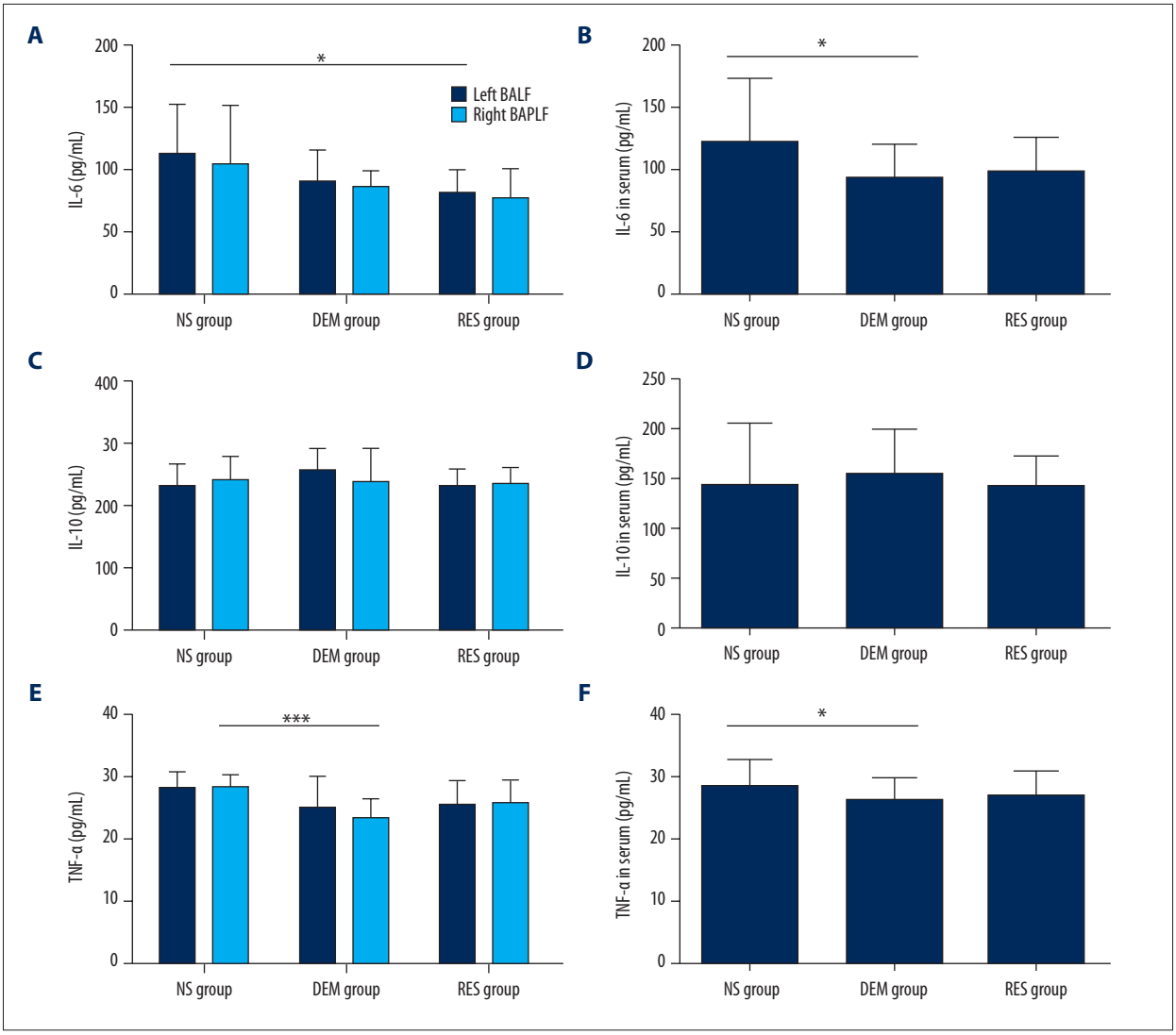

Figure 4. (**A–F**) The level of IL-6, IL-10, and TNF-α by enzyme-linked immunosorbent assay in BALF (n=9 in each group) and serum (NS group: n=17; DEM group: n=16; RES group: n=17) with different pretreatments. All data are presented as the means ± standard deviations. * p<0.05 and *** p<0.01. NS group, normal saline group; DEM group – dexamethasone group; RES group – resveratrol group. IL – interleukin; TNF-α – tumor necrosis factor-α; BALF – bronchoalveolar lavage fluid.

Resveratrol pretreatment down-regulated necroptosis pathway protein expression in transplanted lungs

A wide range of pulmonary cells, including parenchyma and interstitial cells, may be able to express necroptosis pathway proteins [13,14]. In our experiments, we found that RIPK3 and MLKL protein were expressed in almost all sections (including grafts and naive lungs) but were differentially expressed in bronchiolar epithelial cells in different groups; we therefore mainly focused on small-airway epithelial cells to evaluate the IHC score of these 2 proteins. We found that the expression of RIPK3 and MLKL protein in the RES left subgroup was 50% and 37.5%, respectively, which was lower than that in control left subgroup (75% and 50%) and in the DEM left subgroup (71.43% and 57.14%) (Figure 5A–5C). Also, we observed that the expression of TNF-α in the RES left subgroup was 25%, which was lower than that in the control group (62.5%) and similar to that in the DEM group (14.29%), and none of the right lung slices highly expressed TNF-α (Figure 5A, 5D).

We also calculated the correlations between the LI score, RIPK3, MLKL, TNF-α, and the other 2 proteins mentioned above. We found that the LI score was correlated with RIPK3 ($P=3.93\times10^{-2}$) and MLKL ($P=3.61\times10^{-2}$), and TNF-α expression was correlated with RIPK3 ($P=6.24\times10^{-3}$) but not MLKL (P=0.40) (Table 3). We found a negative correlation trend between RIPK3 and P65 ($P=8.94\times10^{-2}$) (Table 4).

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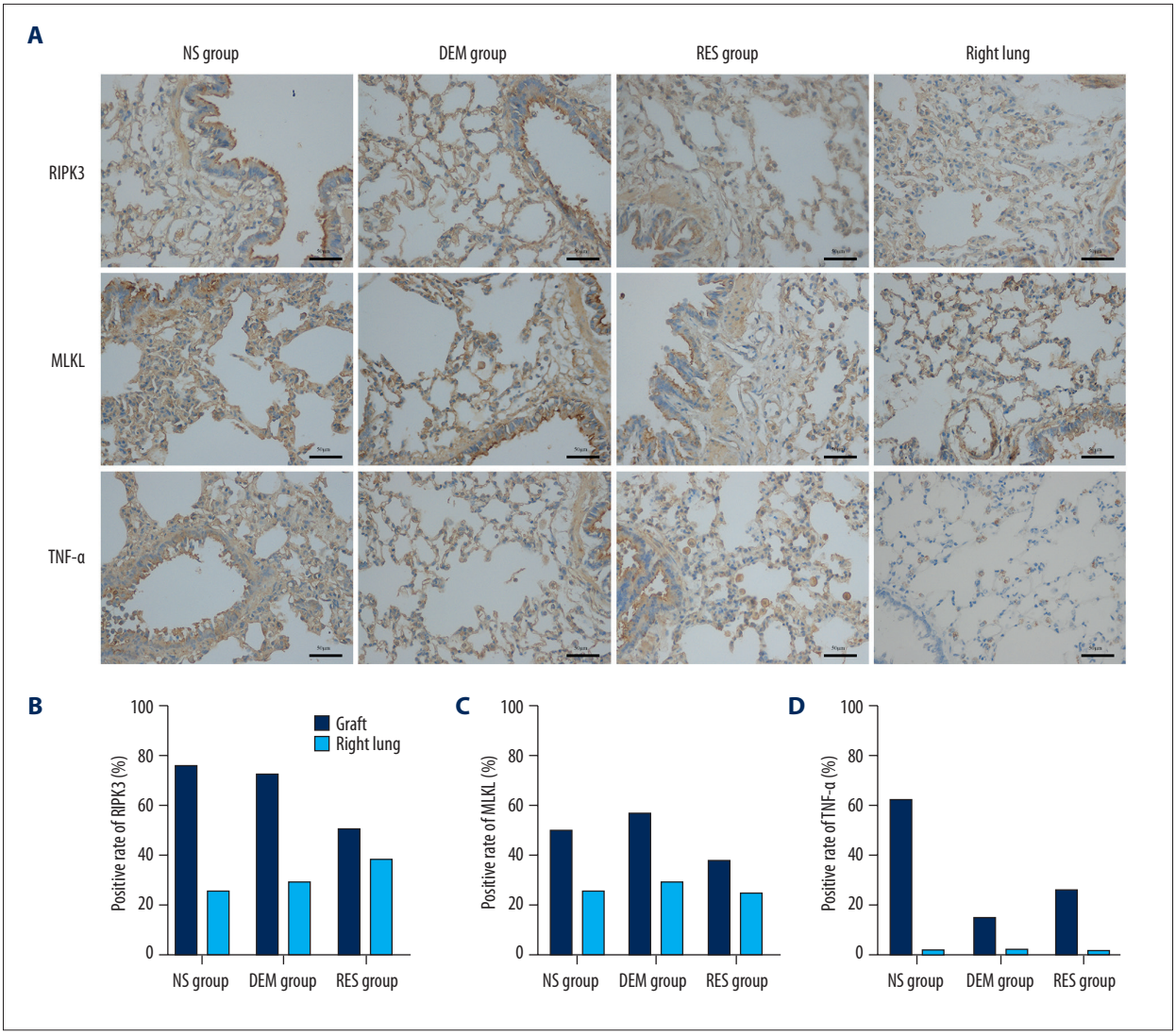

Figure 5. Immunohistochemistry of RIPK3, MLKL, and TNF-α in lung grafts or *in situ*. The magnification of pictures is 400× (**A**). Protein expression was assessed as positive immune response or negative immune response depending on the immunohistochemistry score of different groups (NS group: n=8; DEM group: n=7; RES group: n=8). Positive immune response slices had more brown-staining cells in particular areas than the negative ones (**B–D**). NS group – normal saline group; DEM group – dexamethasone group; RES group – resveratrol group; RIPK3 – receptor-interacting protein serine/threonine kinase 3; MLKL – mixed-lineage kinase domain-like; TNF-α – tumor necrosis factor-α.

Discussion

The main factors that influence the long-term survival of lung transplant patients are acute and chronic rejection. Some studies have indicated that patients with acute rejection after lung transplantation have a higher probability of chronic rejection than those who never had acute rejection, and that the degree of lung transplantation-associated IRI can affect the incidence of acute rejection [1,15]. Therefore, protecting transplanted lungs from IRI may help to improve the prognosis of lung transplant patients. Resveratrol is a chemical with anti-inflammatory and antioxidant properties that has been shown to relieve IRI in cardiac muscle cells, neural stem cells, and other cells [16,17]. A literature search was conducted in the PubMed database using the key words "resveratrol" and "lung transplant", and no relevant report was found as of December 2017. We therefore decided to explore whether resveratrol would help to reduce IRI in lung transplantation.

Numerous studies have found that, during reperfusion, cell membrane injury and intracellular oxidative phosphorylation disorders caused by oxygen radicals result in cell necrosis, local inflammation, and leukocyte chemotaxis; pro-inflammatory cytokine secretion was promoted, and the level of systemic

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Table 3. Correlation between RIPK3, MLKL, TNF-α and LI score.

Parameters	RIPK3 (–) (n = 8)		RIPK3 (+) (n = 15)		P value
LI score (–) (n=10)	6	(3.48)	4	(6.52)	3.93×10^{-2}
LI score (+) (n=13)	2	(4.52)	11	(8.48)	
Parameters	**MLKL (–) (n=12)**		**MLKL (+) (n=11)**		**P value**
LI score (–) (n=10)	8	(5.22)	2	(4.78)	3.61×10^{-2}
LI score (+) (n=13)	4	(6.78)	9	(6.22)	
Parameters	**MLKL (–) (n=12)**		**MLKL (+) (n=11)**		**P value**
RIPK3 (–) (n=8)	7	(4.17)	1	(3.83)	2.72×10^{-2}
RIPK3 (+) (n=15)	5	(7.83)	10	(7.17)	
Parameters	**TNF-a (–) (n=15)**		**TNF-a (+) (n=8)**		**P value**
RIPK3 (–) (n=8)	8	(5.22)	0	(2.78)	6.24×10^{-3}
RIPK3 (+) (n=15)	7	(9.78)	8	(5.22)	
Parameters	**TNF-a (–) (n=15)**		**TNF-a (+) (n=8)**		**P value**
MLKL (–) (n=12)	9	(7.83)	3	(4.17)	0.40
MLKL (+) (n=11)	6	(7.17)	5	(3.83)	

All 23 samples are from transplanted lungs (normal saline group: 8; dexamethasone group: 7; resveratrol group: 8). RIPK3 – receptor-interacting serine/threonine protein kinase 3; MLKL – mixed-lineage kinase domain-like; TNF-α – tumor necrosis factor-α; LI score – lung injury score.

Table 4. Correlation between RIPK3, MLKL, iNOS, and P65.

Parameters	RIPK3 (–) (n = 8)		RIPK3 (+) (n=15)		P value
iNOS (–) (n=10)	3	(3.48)	7	(6.52)	1
iNOS (+) (n=13)	5	(4.52)	8	(8.48)	
Parameters	**MLKL (–) (n=12)**		**MLKL (+) (n=11)**		**P value**
iNOS (–) (n=10)	6	(5.22)	4	(4.78)	0.68
iNOS (+) (n=13)	6	(6.78)	7	(6.22)	
Parameters	**RIPK3 (–) (n=8)**		**RIPK3 (+) (n=15)**		**P value**
P65 (–) (n=12)	2	(4.17)	10	(7.83)	8.94×10^{-2}
P65 (+) (n=11)	6	(3.83)	5	(7.17)	
Parameters	**MLKL (–) (n=12)**		**MLKL (+) (n=11)**		**P value**
P65 (–) (n=12)	5	(6.26)	7	(5.74)	0.41
P65 (+) (n=11)	7	(5.74)	4	(5.26)	

All 23 samples are from transplanted lungs (normal saline group: 8; dexamethasone group: 7; resveratrol group: 8). RIPK3 – receptor-interacting serine/threonine protein kinase 3; MLKL – mixed-lineage kinase domain-like; iNOS – inducible nitric oxide synthase; P65 – nuclear factor-κB (NF-κB) P65.

inflammation was affected [18,19]. Our results show that, in the transplanted lung tissue of the control group, most of the alveolar and bronchial epithelial cells were swollen, some of which were necrotic, and neutrophils infiltrated in the peribronchovascular interstitium. This was not observed in any of the right lungs *in situ*. In contrast, we found that tissue edema, necrosis, and inflammation were milder in the RES left subgroup, suggesting that resveratrol protected the histological structure of the transplanted lung. In this study, we introduced a subjective index of lung injury score (which contains 2 indicators

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of infiltration of inflammatory cells and thickness of the respiratory membrane), which very accurately reflected the extent of IRI as an index of iNOS [10]. As expected, the right lungs in all groups were normal, with an average score close to 0, whereas the transplanted lungs in the control group, which had the most serious manifestations of IRI, had the highest score of all 3 groups, indicating that the drug pretreatment protected the lung tissue.

Within 24 h after lung transplantation, resveratrol significantly lowered the levels of IL-6 and TNF-α in rats. In addition, we observed an interesting phenomenon of no significant difference in the levels of IL-6, IL-10, or TNF-α in BALF between subgroups within the same group, and the trend in the level of these cytokines in serum was consistent with that in BALF. We considered that hypoxia and ischemia-reperfusion increased vascular permeability and the oxygen radical in the donor lung tissue, which enhanced the migration of inflammatory cells and the release of cytokines [3,20]. The inflammatory signal was also constantly amplified after the responding cells were influenced by various inflammatory factors in blood circulation, with the result that elevated plasma cytokines act on bilateral lung tissue. Therefore, the variation in the trend of cytokines in the serum was the same as that in BALF, and the cytokine concentrations in bilateral BALF did not differ. Therefore, oxygen radical and neutrophil infiltration might be the main cause of morphological differences between the transplanted lung and the right lung *in situ*, while cytokines might only have a supporting role.

Necroptosis is a special type of programmed cell death that was discovered in recent years. Although necroptosis has similar morphologic features to the previously well-known cell necrosis, it was found to be regulated by RIPK3, MLKL, TNF-α, and other proteins and to play an important role in myocardial IRI, acute lung injury, and other pathological processes [13,14,21]. We observed a number of signs of cell necrosis in transplanted lung tissue in preliminary experiments and questioned whether necroptosis was involved in the process of transplanted lung injury. The results showed that, in some of the small-airway epithelial cells that suffered IRI, the RIPK3 and MLKL proteins were expressed and were positively correlated with the LI score, suggesting that the injury to the transplanted lung was related to necroptosis. As RIPK3 was found to be the effector protein for TNF-α, in line with expectations, we found a strong correlation between them ($P=6.24\times10^{-3}$). Moreover, the effect of drug preconditioning on the expression of TNF-α in lung tissue was consistent with that in BALF. We also found that the expression of TNF-α has a positive correlation with that of iNOS. Some studies have reported that a large amount of reactive oxygen species was produced in necroptosis [22,23], which is consistent with our findings. Some researchers found that cells might have a stress reaction, such as apoptosis or necroptosis, to ischemia-reperfusion or endogenous pyrogen under gene regulation and that P65 played an important role in the pathway selection in this process. We know that P65 is a key protein in the NF-κB pathway, which functions completely differently in various situations [24–26]. TNF-α, as an important regulatory protein of the NF-κB pathway, is one of the cytokines that make up the acute-phase reaction. In our study, we observed that the high expression rate of P65 was inversely correlated with that of TNF-α and RIPK3 in different groups. We suspected that when cells were subjected to intense stimulation, p65 may have a protective effect by downregulating TNF-α and then reducing the expression of RIPK3 to avoid necroptosis. However, the p value did not reach 0.05, which may be caused by other mechanisms involved or the insufficient number of experimental samples.

Conclusions

We found that resveratrol has a strong anti-inflammatory effect and that resveratrol pretreatment protected lung tissue in the early stages after transplantation in the rat orthotopic lung transplantation model. We also discovered that the mechanism of lung transplantation-associated IRI may be related to necroptosis. In the future, the bioactive substance resveratrol could be used in the early treatment of lung transplantation recipients to improve their quality of life after surgery, and large-scale animal experiments could further explore the intrinsic link between transplantation-associated IRI and necroptosis.

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