The potential therapeutic effect of lipid-lowering drug, ezetimibe, on ischemia-reperfusion injury in a rat model of lung transplantation

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ABSTRACT

The potential therapeutic effect of lipid-lowering drug, ezetimibe, on ischemia-reperfusion injury in a rat model of lung transplantation

Ischemia-reperfusion injury (IRI), wherein inflammatory metabolites such as cytokines and reactive oxygen species accumulate, in solid organ transplantation can lead to the deterioration of the transplanted organ, resulting in early and late comorbidities posttransplantation . Thus, reducing IRI is critical for successful transplantation Ezetimibe (Eze) is a well-known lipid-redusing agent that also exerts an antioxidant effect by modulating the cascade of the adenosine 5'-monophosphate-activated protein kinase/nuclear factor erythroid-2-related factor 2, one of the critical pathways in IRI . The objective of this study was to analyze and evaluate the impact of Eze on IRI in a rat model of lung transplantation.

Lewis rats were assigned into four groups for the experiment: transplantation only, no treatment (LT); treatment of donors (LT-D); treatment of recipient (LT-R); and treatment of both donor and recipient (LT-DR). The treatment groups received Eze (1 mg/kg) 3 times per week for 14 days. Donor left lungs were stored for 6 hours at 4℃ and transplanted to the recipient. Following 2 hours of reperfusion, the transplanted lung's gas exchange function was assesed, and specimens were prepared for histological, infalmmatory, and apoptosis studies

The treatment groups showed significantly better gas exchange function of the graft (arterial partial pressure of oxygen in recipients' aorta: 265.1, 321.9, and 412.6 mmHg in LT, LT-D, LT-R, and LT-DR groups, respectively, $p = 0.015$) and lower grade of lung injury (lung injury score: 17.0, 12.0, 11.0, 10.5 in LT, LT-D, LT-R, and LT-DR groups, respectively, $p < 0.001$). The plasma levels of inflammatory markers such as chemokine (C-X-C motif) ligand 2, interleukin-1 beta, interleukin-6, interferon-gamma, and tumor necrosis factor-alpha were markedly decreased in the treatment groups. Moreover, myeloperoxidase levels in the lung parenchyma and apoptotic cell ratio demonstrated markedly lower levels in the treatment groups.

In conclusion, Eze exerts significant protective effects against IRI with a rat model of lung transplantation. Therefore, it has a potential to function as a protective agent against IRI in lung

transplantation.

Key words : lung transplantation, ischemic-reperfusion injury, ezetimibe

1. INTRODUCTION

During transplantation, all solid organs, including the lungs, are exposed to ischemia during organ procurement and reperfusion after anastomosis. During the ischemia-reperfusion process, organs are affected by the deleterious effects of ischemia-reperfusion injury (IRI) that causes a build-up of toxic metabolites, including cytokines, reactive oxygen species (ROS), and oxygen free radicals.[1] Given that IRI can result in both early and late deterioration of graft function owing to severe morbidity and mortality, strategies for reducing IRI are critical in transplantation.[1]

Ezetimibe (Eze) is a well-established agent for reducing lipid levels, functioning primarily by inhibiting cholesterol transportation into cells via Niemann-Pick disease type C1-like 1-(NPC1L1).[2,3] However, prior studies have revealed that Eze also has a pleiotropic effect independent of NPC1L1.[3-5] The antioxidative properties of Eze are mediated through modulation of glutathione and glutathione peroxidase levels, as well as activation of adenosine 5'-monophosphate-activated protein kinase (AMPK)/nuclear factor erythroid-2-related factor 2 (Nrf2) pathway.[5,6] AMPK phosphorylation mechanistically activates Nrf2, the key regulator of antioxidation, and reduces ROS by elevating downstream factors, which decreases the levels of proinflammatory cytokines (Figure 1).[2,7,8]

Although many potential agents targeting several pathways involved in IRI have shown good therapeutic effects in animal models, clinically effective and safe drugs remain to be identified. Therefore, this study sought to examine the effect of Eze, particularly its antioxidant effect, on IRI during lung transplantation, using a rat lung transplantation model. We hypothesized that pre-transplant administration of Eze would attenuate IRI-induced oxidative stress.

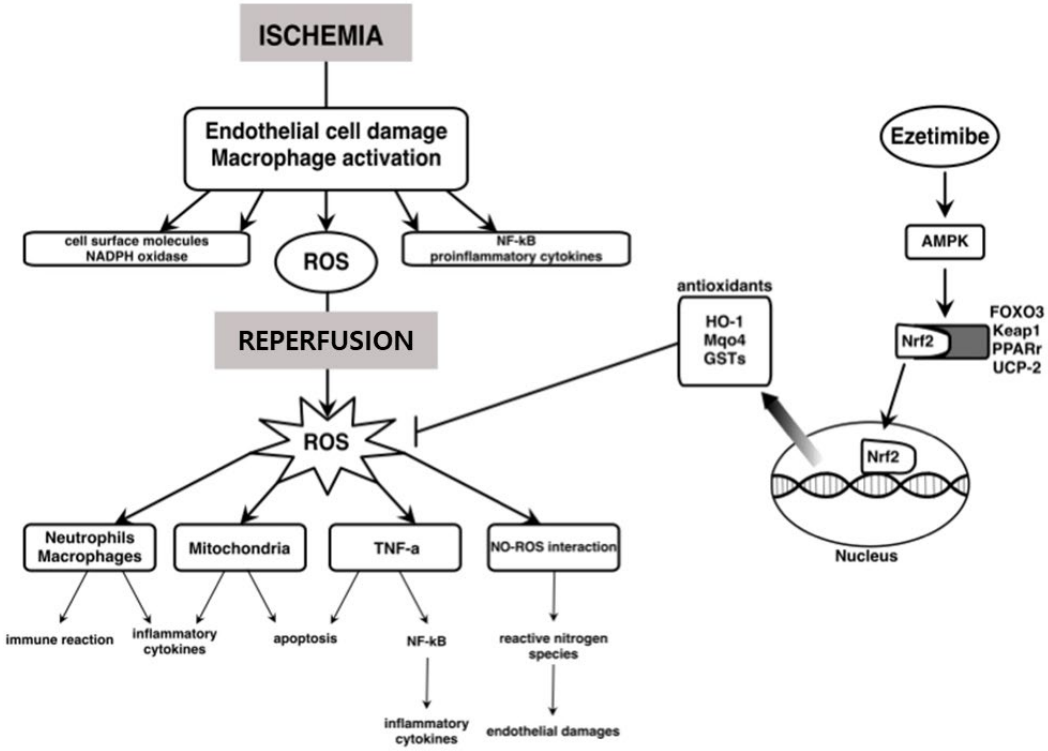


Fig 1. Summary of pathways contributing to lung injury in lung transplantation and signaling transduction pathway of ezetimibe in ischemia-reperfusion injury during lung transplantation.

NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; NO, nitric oxide

2. MATERIALS AND METHODS

2.1. Animal model

This research was approved by the Animal Care and Use Committee of Yonsei University College of Medicine (IACUC number: 2019-0198). Animal care and usage of this research adhered to all relevant international, national, and institutional guidelines.

Six-week-old male inbred Lewis rats weighing 270–330 g were used. The animals were assigned to one of the four experimental groups in a blinded and randomized manner as follows (n = 8 per group): (1) transplantation without pretreatment (LT, control), (2) pretreatment with Eze in donor rats (LT-D), (3) pretreatment with Eze in recipient rats (LT-R), and (4) pretreatment with Eze in both donor and recipient rats (LT-DR). Eze was provided as a pretreatment via oral administration at 1 mg per kg (0.2 cc per kg), three times a week for 14 days. The 1 mg/kg dosage was selected based on the available literature concerning the efficacy of ezetimibe and a preliminary study at our institute[9,10] (Figure 2).

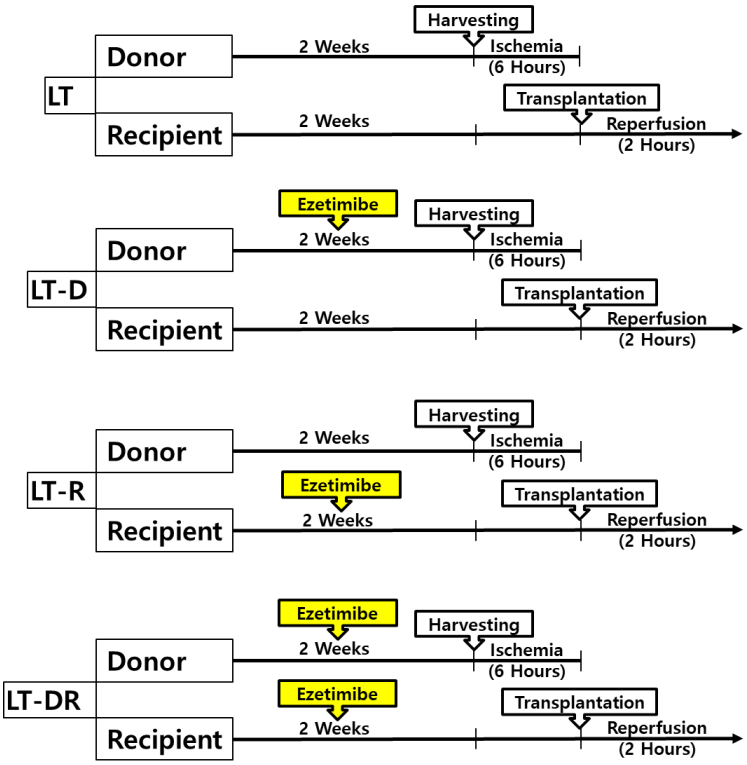


Fig 2. Study flow chart

2.2. Rat lung transplantation procedure

After 2 weeks of drug administration, donor animals were anesthetized using 2% isoflurane inhalation, and subsequently intubated using a 16-gauge venous catheter and connected to a mechanical ventilator. After laparotomy and median sternotomy, heparin sodium (300 U) was injected through the inferior vena cava. Blood was drawn from the abdominal aorta for gas analysis 5minuetes post-heparin administration. The inferior vena cava and left atrial appendage were transected to flush the perfusate. Twenty milliliters of Perfadex™, a standard lung preservation solution supplemented with 10mg of prostaglandin E1, was delivered via the main pulmonary artery to flush the graft. The trachea was ligated at the end-inspiratory phase, and the heart-lung blocks were harvested. The extracted lung blocks were stored in 20 ml Perfadex™ at 4℃ for 6 hours. The

same approach with the donor animals was used to anesthetize recipient rats. Through a lateral thoracotomy on the left side, the recipient lung was visualized and manipulated, and bronchus, pulmonary artery, and pulmonary vein were carefully dissected and clamped. Before the recipient procedure, donor lungs were prepared to apply a cuff technique.[11] The pulmonary vein, artery, and bronchus of donor graft was anastomosed respectively using a cuff technique to the recipient pulmonary hilum. The bronchus clamp was released, initiating the reinflation and reperfusion of the transplanted lung after the pulmonary vein and artery clamps were released. After 2 hours of ventilation with 100% oxygen and isoflurane, each recipient rat underwent a median sternotomy. The right pulmonary hilum was clamped, and the only transplanted lung was ventilated for 5 min. To assess graft oxygenation, blood was sampled from the aorta and pulmonary vein with a 27-gauge. After blood sample collection, the animals were euthanized. After ligating the right main bronchus, the trachea was cannulated using a 23-gauge flexible catheter for bronchoalveolar lavage fluid (BALF) analysis. The transplanted lungs were excised and divided into two portions for further analysis (Figure 2).

2.3. Measurement of ischemia-reperfusion injury

2.3.1. Histological assessment and lung injury scoring

The upper parts of the lung blocks were preserved in 10% formalin, embedded in paraffin, sectioned, and stained with standard hematoxylin and eosin techniques for histological analysis. The slides were assessed using bright-field microscopy, and the extent of lung injury was quantified through scoring criteria by a pathologist without prior knowledge of the experimental conditions. The scoring criteria assessed lung injury encompass alveolar hemorrhage (red blood cells present in alveolar spaces), vascular congestion (over 75% of alveolar septum filled by red blood cells), alveolar fibrin formation, and leukocyte infiltration. A 4-point scale (0-3) was used to rate each criterion: 0 (normal, 0%); 1 (mild, less than 10%); 2 (moderate, 10–50%); and 3 (severe, more than 50%).[12,13]

2.3.2. Cytokines

To analyze inflammatory markers, 2 ml of blood and BALF were collected and processed by centrifugation at 6,000 rpm for 5 minutes, following the manufacturer’s instructions for the Luminex

assay (R&D systems, Minneapolis, MN, USA). Plasma and BALF samples were obtained and preserved at -80℃ until further analysis. The levels of cytokines and chemokines in the plasma and BALF sample were determined using the Luminex assay following the manufacturer's protocol.

2.3.3. Myeloperoxidase

Myeloperoxidase (MPO) levels were assessed to analyze neutrophil infiltration into the lung parenchyma. MPO levels in plasma and tissue lysates were quantified using an MPO-specific enzyme-linked immunosorbent assay (ELISA) kit (USCN Life Science Inc., Wuhan, China) as per the provider's guidelines.

2.3.4. TUNEL staining and apoptotic cell count

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using an in-situ cell death assay kit (AP) (Roche Applied Science, Mannheim, Germany) following the company's protocols. Apoptotic cells were identified using a light microscope at 200× magnification in 10 randomly chosen fields per section, and the proportion of TUNEL-positive cells was assessed.[14)]

2.4. Statistical analysis

All results are presented as the mean and standard deviation (SD). Student's t-test or one-way analysis of variance (ANOVA) was used to assess the statistical differences in parametric data among the groups followed by Turkey's multiple comparison test. Statistical analyses were performed using the R software and the SPSS 25 software (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as a p value less than 0.05.

3. RESULTS

3.1. Lung function

Baseline blood gas analysis from donor aorta didn't show statistically significant differences between the groups (Figure 3A). Post-reperfusion arterial blood gas analysis in recipient animals showed remarkable differences in arterial oxygen partial pressure to inspired oxygen fraction ratio (PaO_2/FiO_2) between each treatment group and the control group ($p = 0.015$; Figure 3B).

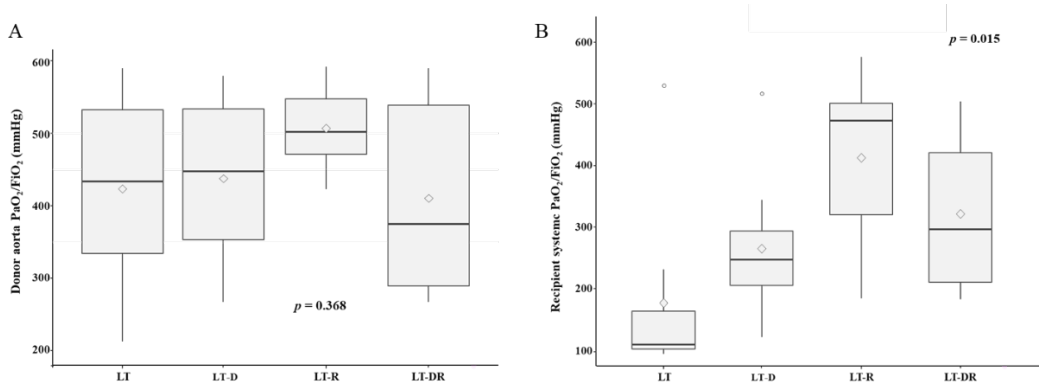


Fig 3. Comparison of gas exchange among the four groups. (A) Baseline blood gas analysis from donor aorta (B) Post-reperfusion (2 hours) arterial blood gas analysis of recipient, Data are expressed as the mean ± SD (n = 8 animals/group). *, $p < 0.05$
LT, lung transplantation only (without pretreatment); LT-D, donor-only pretreatment; LT-R, recipient -only pretreatment; LT-DR, both donor and recipient pretreatment

3.2. Histological characteristics and lung injury score

Characteristic sections of recipient lung histology, 2 hours following reperfusion, are shown in Figures 4A–4D. The alveolar structure of the treatment groups showed more intact features. The tendency of histologic integrity is more remarkable in the LT-DR group (Figure 4D). The treatment groups exhibited significantly lower total lung injury scores compared to the control group ($p < 0.001$; Figure 4E).

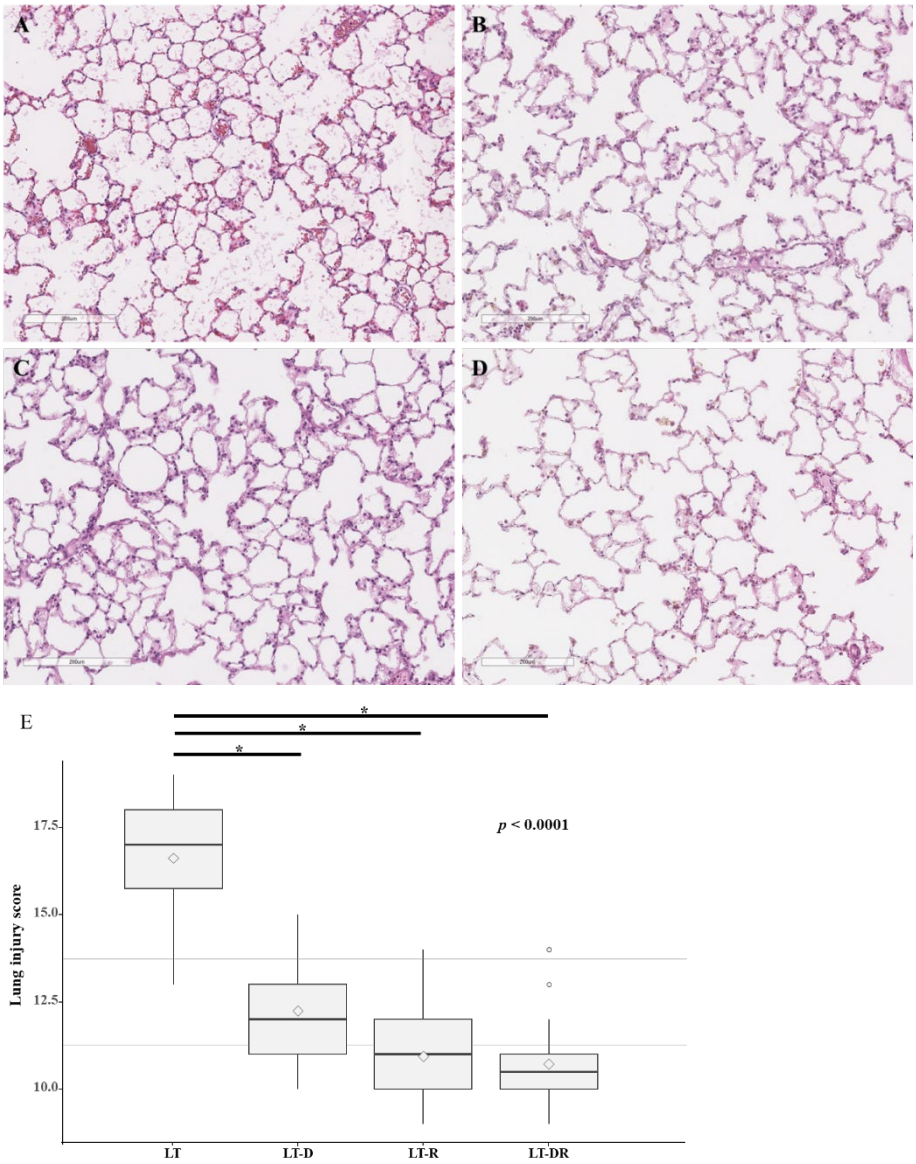
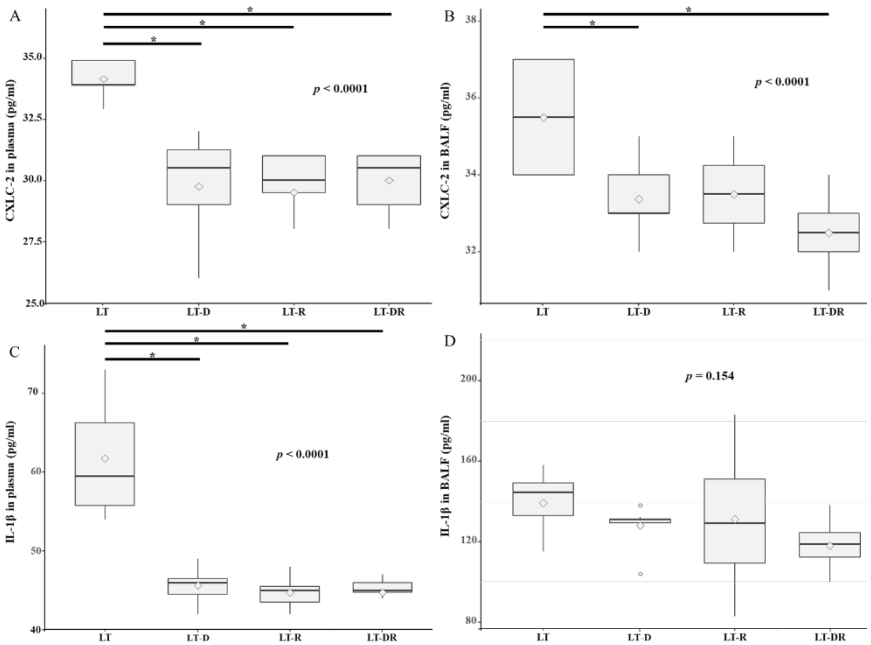


Fig 4. Lung injury score on hematoxylin and eosin staining. (A) LT group. (B) LT-D group. (C) LT-R group. (D) LT-DR group. (E) Comparison of lung injury score among the groups. Data are presented as the mean ± SD (n = 8 animals/group). *, $p < 0.05$
LT, lung transplantation only (without pretreatment); LT-D, donor-only pretreatment; LT-R, recipient -only pretreatment; LT-DR, both donor and recipient pretreatment

3.3. Inflammatory markers

The treatment groups demonstrated markedly reduced plasma concentrations of chemokine (C-X-C motif) ligand 2 (CXCL-2) compared to the non-treatment group ($p < 0.001$; Figure 5A). The LT-D and LT-DR groups exhibited significantly reduced BALF levels of CXCL-2 compared to the LT group (Figure 5B). The treatment groups exhibited significantly lower plasma levels of interleukin-1 beta (IL-1β) (Figure 5C) and interleukin-6 (IL-6) (Figure 5E) compared to the non-treatment group. Meanwhile, no significant differences were observed in BALF IL-1β levels among the groups (Figure 5D). The LT-R and LT-DR groups demonstrated markedly reduced BALF IL-6 levels compared to the LT group (Figure 5F). Plasma levels of interferon-gamma (IFN-γ) were significantly lower in the LT-DR group than in the LT group (Figure 5G). BALF IFN-γ levels were significantly lower in the treatment groups compared to the non-treatment group ($p < 0.001$; Figure 5H). Finally, plasma concentrations of tumor necrosis factor-alpha (TNF-α) were markedly lower in all treatment groups compared to the non-treatment group (Figure 5I). Additionally, BALF levels of TNF-α were also significantly lower in the LT-R and LT-DR groups compared to the LT group (Figure 5J).



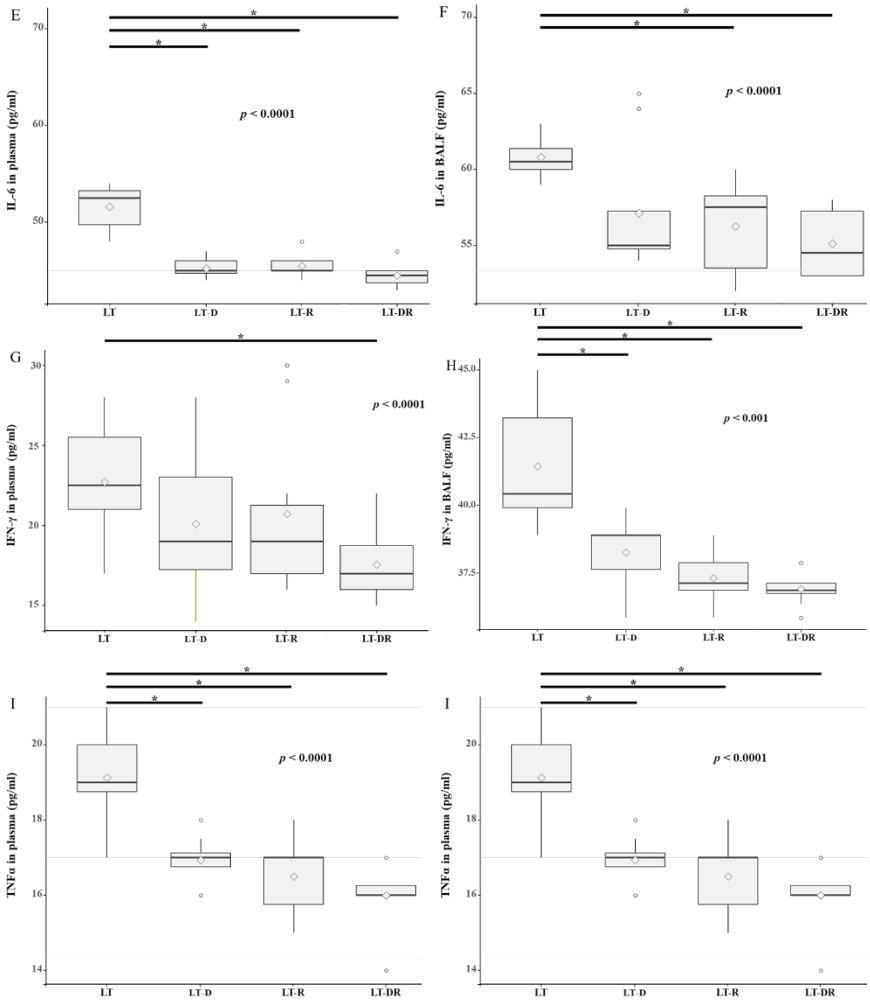


Fig 5. Plasma and bronchoalveolar lavage fluid (BALF) concentrations of pro-inflammatory cytokines. (A) Chemokine (C-X-C motif) ligand 2 (CXCL-2) levels in plasma. (B) CXCL-2 levels in BALF. (C) Interleukin-1 beta (IL-1β) levels in plasma. (D) IL-1β levels in BALF. (E) Interleukin-6 (IL-6) levels in plasma. (F) IL-6 levels in BALF. (G) interferon-gamma (IFN-γ) levels in plasma. (H) IFN-γ levels in BALF. (I) Tumor necrosis factor-alpha (TNF-α) levels in plasma. (J) TNF-α levels in BALF. Data are expressed as the mean ± SD (n = 8 animals/group). *, $p < 0.05$
LT, lung transplantation only (without pretreatment); LT-D, donor-only pretreatment; LT-R, recipient -only pretreatment; LT-DR, both donor and recipient pretreatment

3.4. MPO levels

There were no statistical differences in plasma MPO levels across the four groups (Figure 6A, p = 0.391). However, in the lung tissue lysates, MPO levels were significantly higher in the non-treatment groups (Figure 6B).

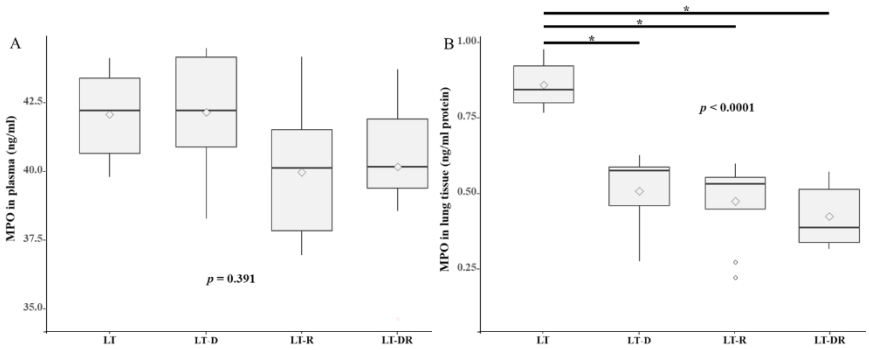


Fig 6. Myoperoxidase (MPO) levels in (A) plasma and (B) lung tissue lysate. Data are expressed as the mean ± SD (n = 8 animals/group). *, $p < 0.05$
LT, lung transplantation only (without pretreatment); LT-D, donor-only pretreatment; LT-R, recipient -only pretreatment; LT-DR, both donor and recipient pretreatment

3.5. Cell death markers on TUNEL staining

The proportion of TUNEL-positive cells relative to total cells was significantly lower in the treatment groups compared to the LT group (2.49% ± 1.00% in the LT group vs 1.66% ± 0.55% in the LT-D group, 2.21% ± 1.29% in the LT-R group, and 1.78% ± 0.73% in the LT-DR group, p = 0.005; Figure 7E). Representative sections of TUNEL staining for each group are shown in Figures 7A–7D.

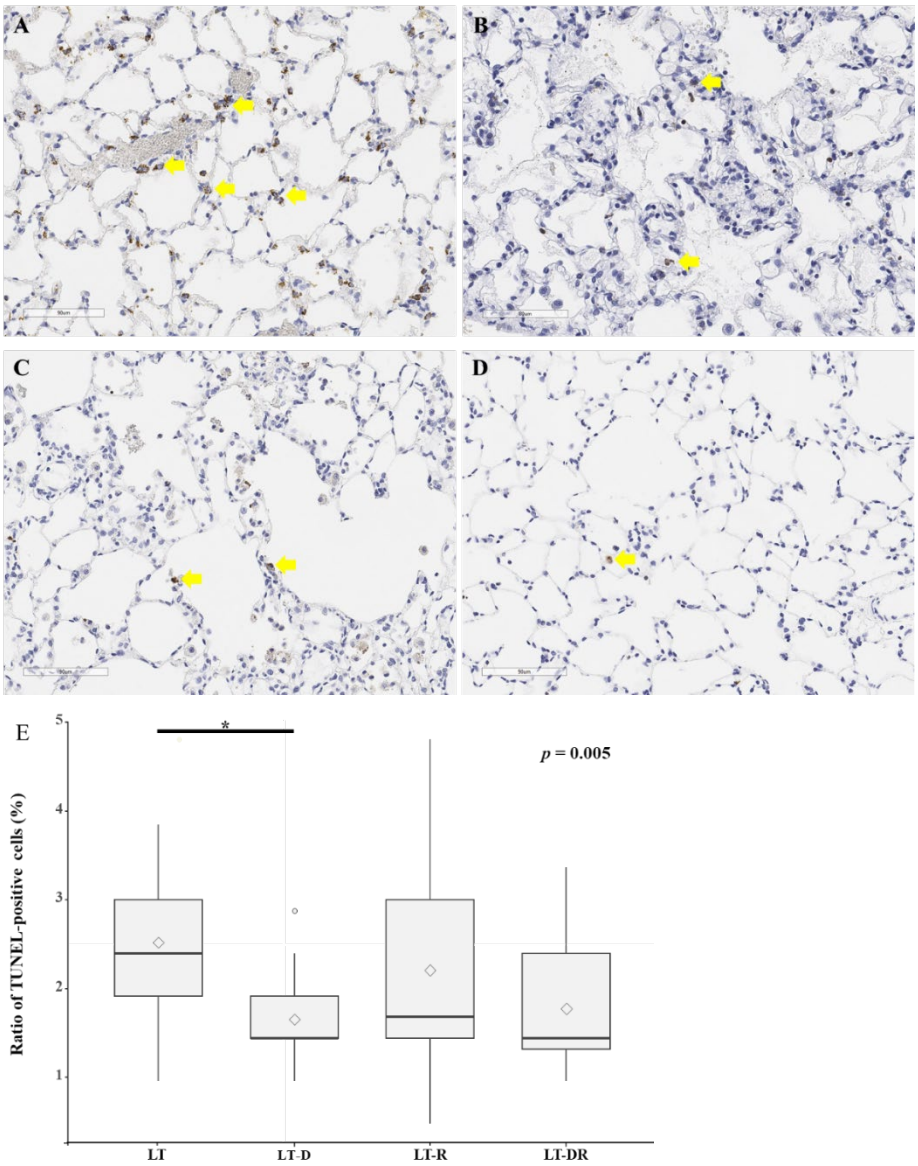


Fig 7. Results of TUNEL staining. Representative images in the (A) LT, (B) LT-D, (C) LT-R, and (D) LT-DR groups. Arrows in each image indicate apoptotic cells. (E) Comparison of the proportion of TUNEL-positive cells relative to total cells among the groups. Data are expressed as the mean ± SD (n = 8 animals/group). *, $p < 0.05$
LT, lung transplantation only (without pretreatment); LT-D, donor-only pretreatment; LT-R, recipient -only pretreatment; LT-DR, both donor and recipient pretreatment

4. DISCUSSION

This study highlights the protective role of Eze in mitigating IRI in a rodent lung transplant model. All treatment groups (LT-D, LT-R, and LT-DR) showed better oxygenation function of the graft, improvements in graft parenchymal changes, and lower plasma and BALF levels of inflammatory markers.

IRI is a multifaceted process that leads to neutrophil accumulation, inflammatory cytokine production and release, and the induction of apoptosis.[15] Investigations into IRI are essential because it is a significant cause of primary graft dysfunction, and it is associated with early and late post transplantation morbidity and mortality that can lead to a poor prognosis.[16-18] IRI involves neutrophil infiltration into lung tissue followed by the release of ROS and eventual activation of proteases that can damage the lung parenchyma.[19] Although the underlying molecular mechanisms of lung allograft dysfunction is still under investigation, excessive ROS accumulation induced by an inflammatory reaction plays a vital role in pulmonary parenchymal injuries. The transcription factor Nrf2 plays an vital role in the transcription cascade for eliminating ROS accumulated by IRI.[20-22] AMPK is located upstream of cellular antioxidant pathways and is significantly involved in anti-inflammatory activity.[20,21] Nrf2 and AMPK are functionally linked, exhibiting a cascade-like interaction.[20,23] Recent researches on oxidative stress and AMPK-Nrf2 antioxidative signaling in inflammation suggest the therapeutic potential of this pathway.[21,24-26] Nrf-knockout mice have exhibited worse lung inflammation and epithelial cell injury.[27] Moreover, the transcriptional activity of Nrf2 was found to be related to the occurrence of pulmonary disease in smoking-induced emphysema patients.[21,26] These findings motivate research on the potential of AMPK-Nrf2 activators.

Eze is a well-known agent for regulating dietary cholesterol absorption in the small intestine.[28] Moreover, it has been found to attenuate ischemia-related oxidative stress and inflammation by activating antioxidants through the AMPK-Nrf2 signaling pathway in animal models of nonalcoholic steatohepatitis, ischemic stroke, and oxidative stress-associated injuries in the lungs.[2-6,29] Eze also exerts the effect of lowering anti-inflammatory cytokines in inflammatory diseases, such as rheumatoid arthritis and atherosclerosis.[30]

Using a rat left single-transplantation model, this study demonstrated that administering Eze to donors or recipients consistently mitigated IRI following lung transplantation. Among many

potential mechanisms, we hypothesized that Eze reduced ROS by activating the AMPK-Nrf2 pathway. Therefore, we investigated the expression of multiple oxidative damage markers, including oxygenation, inflammatory cytokines, neutrophil infiltration, and cell death. The results showed that pretransplant administration of ezetimibe reduced the lung parenchymal injury and helped to maintain the integrity of alveolar structures. The levels of proinflammatory cytokines (CXCL-2, IL-1β, IL-6, INF-γ, TNF-α) were markedly reduced in the treatment groups in both plasma and BLAF analysis. MPO levels in the tissue lysates were also significantly lower int the treatment groups. The TUNEL staining showed lower apoptotic cells in the ezetimibe treatment groups. Collectively, these results suggest that Eze could potentially alleviate IRI in lung transplantation

IRI is the primary cause of early and permanent allograft dysfunction following transplantation, and thus, finding an effective treatment target for IRI prevention in patients undergoing lung transplantation is essential. Although many potential agents targeting several pathways involved in IRI have shown good therapeutic effects in animal models, clinically effective and safe drugs remain to be identified. The practical application of drugs in humans requires additional simultaneous investigations of patient safety, pharmacokinetics, pharmacodynamics, and multiple pathways involved in IRI in the clinical setting.

This study has some limitations. Despite the several significant results obtained, the sample size was small. Moreover, donor grafts obtained from rats do not develop from brain death-related inflammation, which occurs in almost all allografts used for human lung transplantation. Moreover, the distinction between pre- or postoperative administration of ezetimibe is unclear with this study, although we can find tendencies that both donor and recipient treatment group showed better outcomes than the other treatment groups. Additional studies are needed to determine the appropriate timing of administration and dosage of Eze to reduce IRI in other animals, considering species specificity, and in humans for real-world applications.

5. CONCLUSION

In this study using a rat lung transplant model, Eze exerted protective effects against IRI during lung transplantation by improving oxygenation, preserving lung structure, and reducing

inflammatory reactions. Further studies using large animal models and clinical lung transplantations should be conducted to confirm the clinical application of Eze for enhancing graft survival after lung transplantation.

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Abstract in Korean

항지질제인 Ezetimibe 의 허혈-재관류 손상에 대한 잠재적 치료 효과 연구: 랫트 폐이식 모델 연구

고형 장기 이식에서 허혈-재관류 손상은 cytokines, reactive oxygen species 등의 염증 관련 인자들의 축적을 유발하고, 그 결과 이식편 손상을 야기하는 것이 잘 알려져 있다. 이로 인해 발생한 이식편 손상은 이식 직후, 또한 이식 후 장기적인 불량한 예후의 원인이 된다. 그러므로 고형 장기 이식에서 허혈-재관류 손상을 해결하는 방안에 대한 연구는 여러 장기에서 중요한 화두이다. Ezetimibe는 잘 알려진 항지질제이며, 항지질제로서의 효과 이외에도 AMPK/Nrf2 pathway를 통한 항산화, 항염증 효과가 밝혀져 있다. AMPK/Nrf2 pathway는 허혈-재관류 손상에서 염증 관련 인자들의 축적에 관여하는 주요 기전으로, 본 연구에서는 ezetimibe의 폐이식에서 허혈-재관류 손상을 낮추는 잠재적인 치료제로서의 가능성을 탐구하였다.

Cuff-technique를 이용한 루이스 래트 일측 폐이식 모델을 활용하였으며 루이스 랫트를 네 그룹; LT group (이식만 시행), LT-D group(기증자에 ezetimibe 투여), LT-R group (수혜자에 ezetimibe 투여), LT-DR (기증자와 수혜자에 ezetimibe 투여)에 각각 여덟 마리씩 배정하여 치료 그룹에 kg 당 1mg의 ezetimibe를 14일 동안 1주일에 3회 투여하였다. 기증 장기 적출 후 기증 동물의 왼쪽폐는 허혈을 유도하기 위해 4℃에서 6시간 동안 보관한 후 수혜 동물에 이식하였다. 2시간의 재관류 후, 이식편의 기체 교환능, 조직학적인 변화, 염증 및 세포 사멸에 대한 인자들을 분석하기 위한 샘플들을 수집하였다.

Ezetimibe 를 투여한 그룹에서 이식편의 산소화가 우수한 것을 확인할 수 있었으며 (partial arterial gas pressure of oxygen in recipients' aorta: 176.8 mmHg in LT, 265.1 mmHg in LT-D, 321.9 mmHg in LT-R, and 412.6 in LT-DR, p = 0.015), 폐 손상 점수도 치료 군에서 유의미하게 낮음을 확인할 수 있었다 (p <

0.001). 이식편의 plasma 샘플에서 분석한 CXCL-2, IL-1β, IL-6, IFN-γ, TNF-α 와 같은 염증 관련 인자들이 ezetimibe 투여군에서 유의미하게 낮음을 확인할 수 있었다. 또한, 치료군에서 MPO 농도와 사멸된 세포의 숫자도 더 낮음을 확인할 수 있었다.

본 연구에서는 ezetimibe 가 폐이식에서 발생하는 허혈-재관류 손상 항산화 및 항염증 효과가 있음을 래트 폐이식 모델을 통해 확인할 수 있었다. 본 연구 결과를 근거로 ezetimibe의 폐이식에서 허혈-재관류 손상에 대한 치료제로서의 잠재적 유용성을 제시하고자 한다.

핵심 되는 말: 폐이식, 허혈-재관류 손상, ezetimibe