Everolimus Pretreatment Protects the Lung From Ischemia-Reperfusion Injury

Everolimus Ön Tedavisi Akciğer İskemi-Reperfüzyon Hasarında Koruyucudur

Şevki Mustafa Demiroz[1], Ali Celik[1], Ilke Hacer Onen[2], Ece Konac[2], Erdem Vargol[3], Nalan Akyurek[4], Ozge Tugce Pasaoglu[5] Hatice Pasaoglu[6], Ismail Cuneyt Kurul[1]

[1] Department of Thoracic Surgery, Faculty of Medicine, Gazi University, Besevler, Ankara, Turkey
[2] Department of Medical Biology, Faculty of Medicine, Gazi University, Besevler, Ankara, Turkey
[3] Department of Pathology, Sakarya University Training and Research Hospital, Sakarya, Turkey
[4] Department of Pathology, Faculty of Medicine, Gazi University, Besevler, Ankara, Turkey
[5] Department of Nutrition and Dietetics, Faculty of Health Sciences, Gazi University, Besevler, Ankara, Turkey
[6] Department of Medical Biochemistry, Faculty of Medicine, Gazi University, Besevler, Ankara, Turkey

ABSTRACT

Purposes: A preventive strategy against lung ischemia reperfusion injury (LIRI) is pretreatment with certain drugs to make them more resistant to LIRI. The aim of this study was to investigate the preventive effects of everolimus (RAD001) on LIRI in a rat-ventilated ischemia-reperfusion model.
Methods: Rats were divided into 8 groups;1 control(I), 6 LIRI (IIa,b,c;IIIa,b,c) and 1 everolimus (IV). LIRI groups underwent 90 min of ischemia and 30 min (IIa-IIIa), 120 min (IIb-IIIb) and 240min (IIc-IIIc) of reperfusion, respectively, and were randomized into two, one without (II) and one with everolimus (III) pretreatment. Groups III received 1mg/kg/day everolimus via oral gavage for 1 week before IR. After reperfusion, bronchoalveolar lavage and lung tissue samples were collected. The mRNA expressions of the samples' pro-inflammatory genes were determined via qPCR. TNF-α and macrophage inflammatory protein 2 (MIP2) concentrations and caspase-3 activity were measured using ELISA kits. Also, myeloperoxidase (MPO) activity was measured spectrophotometrically.
Results: Everolimus significantly decreased the expression of all study mRNA pro-inflammatory genes for groups IIIb and IIIc($p<0.05$). It also decreased MIP2 protein levels in groups IIIb and IIIc, whereas TNF-α protein levels decreased only in group IIIc's lung tissue samples($p<0.05$). A significant downregulation of caspase-3 enzyme activity was detected only in group IIIa($p<0.05$).
Conclusion: This study shows that everolimus pretreatment, effectively prevents LIRI by decreasing pro-inflammatory genes' mRNA expressions, the protein expressions of TNF-α and MIP2, and the activity of caspase 3 and myeloperoxidase.

Keywords: lung ischemi-reperfusion; everolimus; inflammation; experimental model; qPCR

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ÖZET

Amaç: Akciğer iskemi reperfüzyon hasarını (LIRI) önleyici stratejilerden biri, belirli ilaçlar ile ön tedavi uygulayarak akciğeri LIRI'ya karşı daha dirençli hale getirmektir. Bu çalışmanın amacı, everolimusun (RAD001) LIRI üzerindeki önleyici etkilerini ratlarda ventile iskemi-reperfüzyon modelinde araştırmaktır.
Yöntem: Ratlar bir kontrol(I), 6 LIRI (IIa,b,c;IIIa,b,c) ve bir everolimus grubu (IV) olmak üzere 8 gruba ayrıldı. LIRI grubundaki ratlar everolimus verilmeyen (II) ve everolimus verilenler (III) olarak iki gruba randomize edildi, bu gruptaki ratlara 90 dakika iskemi sonrasında sırasıyla 30 (IIa-IIIa), 120 (IIb-IIIb) ve 240 (IIc-IIIc) dakika reperfüzyon uygulandı. Grup III'deki ratlara IR öncesinde bir hafta boyunca oral gavaj yoluyla 1mg/kg/gün everolimus verildi. Reperfüzyon süresi bitiminde bronkoalveolar lavaj ve akciğer doku örnekleri alındı. Örneklerdeki pro-inflamatuar genlerin mRNA ifadeleri qPCR ile belirlendi. TNF-α ve makrofaj inflamatuar protein 2 (MIP2) düzeyleri ile kaspaz-3 aktivitesi ELISA kitleri ile ölçüldü. Ayrıca, miyeloperoksidaz (MPO) aktivitesi spektrofotometrik yöntemle ölçüldü.
Bulgular: Everolimus, Grup IIIb ve IIIc'de, çalışılan tüm pro-inflamatuar genlerin mRNA ifadelerinde anlamlı azalmaya neden oldu ($p<0.05$). Ayrıca, grup IIIb ve IIIc'de MIP2 protein düzeyi azalırken, TNF-α protein düzeylerinde anlamlı düşüş sadece grup IIIc'de akciğer doku örneklerinde gerçekleşti. Kaspaz-3 enzim aktivitesinde anlamlı düşüş ise yalnızca grup IIIa'da belirlendi ($p<0.05$).
Sonuç: Çalışmamız, everolimus ile ön tedavinin, pro-inflamatuar genlerin mRNA düzeyindeki ifadelerini, TNF-α ve MIP2'nin protein düzeyindeki ifadelerini ve kaspaz-3 ile miyeloperoksidaz aktivitelerini azaltarak etkin şekilde LIRI'ya karşı koruyucu olduğunu göstermiştir.

Anahtar Sözcükler: akciğer iskemi-reperfüzyonu; everolimus; inflamasyon; deneysel model; qPCR

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ORCID IDs: Ş.M.D. 0000-0003-2471-6970, A.C.0000-0001-5385-6492,I.H.O.0000-0002-7220-0066,E.K.0000-0001-5129-2515,E.V.0000-0003-1000-1050, N.A.0000-0002-2644-4609,O.T.P.0000-0002-5221-9034,H.P. 0000-0001-8343-7432,I.C.K.0000-0002-9480-010X

Address for Correspondence / Yazışma Adresi: Şevki Mustafa Demiröz, MD Department of Thoracic Surgery, Faculty of Medicine, Gazi University, 06500, Besevler, Ankara, Turkiye E-mail: demirozsm@gmail.com

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INTRODUCTION

Apart from ischemia in other organs, lung ischemia causes complicated pathophysiological injury due to ventilation (1). Two types of ischemia are mentioned in the clinic: 1) ventilated ischemia (e.g., pulmonary thromboembolism, primary pulmonary hypertension, acute chest syndrome in sickle-cell anemia patients), where blood flow is interrupted and ventilation and oxygen diffusion continue in the alveoli, and 2) total lung ischemia (e.g., lung transplantation), where both blood flow and ventilation are interrupted (2). It has been proven in the literature that aggressive mechanical ventilation alone may cause an inflammatory response in the lung tissue, but no such response occurs in mild mechanical ventilation (3).

Similarly, *in vivo* studies using animal lung IR models can be classified mainly as either ventilated or total lung ischemia. In our study, we aimed both to eliminate the lungs' inflammatory response to IR due to hypoxia and to minimize the effect of mechanical ventilation by applying a mild mechanical ventilator strategy. Therefore, we intubated rats via tracheostomy, maintained ventilation in an animal-type mechanical ventilator with mild ventilator settings and created a ventilated IR model by clamping only the pulmonary artery after thoracotomy and hilus dissection. Numerous transcription factors and signaling pathways regulate IR injury. It is outstanding that genes upregulated after reperfusion injury, such as cytokines, adhesion molecules, matrix metalloproteinases and chemokines were involved in proinflammatory responses (4).

Because of the complex LIRI phenomenon, numerous potential treatments are still being prevented or diminish this injury's results (1). One of these approaches includes donor/graft pre-treatment (pharmacological pretreatment) with immunosuppressive, anti-inflammatory, and chemotherapy drugs (5), as it has been shown that, pulmonary IR injury results in acute pro-inflammatory responses (5,6).

Everolimus (RAD001), a derivative of rapamycin (sirolimus), is a suppressor of mammalian targets of the rapamycin (mTOR) protein. This immunosuppressive macrolide arrests the cell cycle in the late G1 phase, thereby preventing cells from entering the S phase (7,8).

This study aimed to evaluate the potential shielding role of the mTOR inhibitor everolimus against ventilated IR injury. To our knowledge, this is the first study to evaluate the effects of everolimus pre-treatment to prevent LIRI in a rat-ventilated IR model, including pro-inflammatory gene and protein expression levels of selected cytokines', in rat lung tissue and BAL samples.

MATERIALS and METHODS

Animals

Experiments were performed using male Wistar-Albino rats, weighing 200–350 g. All animals received humane care in compliance with the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and The Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institutes of Health. The experimental protocol was approved by Gazi University Local Ethics Committee for Animal Experiments (G.U.E.T.-09.087, Ankara). Rats were randomized into eighth groups (n=6 each):

- Group I- Control (recevied no medical treatment or surgical intervention)
- Group IIa- Underwent 90 min ventilated-ischemia, 30 min reperfusion without everolimus pretreatment
- Group IIb-Underwent 90 min ventilated-ischemia, 120 min reperfusion without everolimus pretreatment
- Group IIc-Underwent 90 min ventilated-ischemia, 240 min reperfusion without everolimus pretreatment
- Group IIIa- Received 1.0 mg/kg/day per oral (PO) everolimus for 7 days, then underwent 90 min ventilated-ischemia, 30 min reperfusion
- Group IIIb- Received 1.0 mg/kg/day PO everolimus for 7 days, then underwent 90 min ventilated-ischemia, 120 min reperfusion
- Group IIIc- Received 1.0 mg/kg/day PO everolimus for 7 days, then underwent 90 min ventilated-ischemia, 240 min reperfusion
- Group IV- received 1.0 mg/kg/day peroral (PO) everolimus for 7 days but not underwent ischemia-reperfusion

Thus, different proinflammatory cytokines are important in the early stage and later period of the LIRI process we aimed to study three different reperfusion periods to show the difference of the both mRNA expression and its reflection to the end product formation.

Lung Ventilated Ischemia Model and Surgical Procedure

A warm in situ ventilated ischemia-reperfusion model was used. Anesthesia was induced with atropine (0.25 mg/kg) intramuscular followed by ketamine/xylazine intraperitoneal and the animals were ventilated through tracheostomy in a pressure-controlled mode at a fractional inspired oxygen concentration of 60% and inspiratory/expiratory ratio of 1:2. Peak inspiratory pressure was at 10 cm H_2O and positive end-expiratory pressure (PEEP) was set at 3 cm H_2O and frequency was set at 40 breaths/min (Harvard Apparatus Inc Mouse Ventilator Model 687).

Anesthesia was maintained with inhaled 2% isoflurane. All animals underwent laparotomy and received 500 U of intravenous heparin through the inferior caval vein. The left lung was mobilized atraumatically through anterolateral thoracotomy and the left pulmonary artery occluded witha noncrushing microvascular clamp. At the end of the 90-minute ischemic period, the clamp was removed and the lung was ventilated and reperfused for periods up to 30, 120, and 240 min. For 30'reperfusion group (IIIa) procedure was concluded under mechanical ventilation. For 120'group (IIIb) and 240'group (IIIc) reperfusion groups, after the ischemic period, thoracotomy was closed, rats were extubated and the reperfusion period was concluded in the animal intensive care unit. At the end of the reperfusion period, euthanasia was performed by intracardiac puncture method. Bronchoalveolar lavage (BAL) samples were obtained through 1 mL normal saline solution which was given and retrieved from tracheostomy. Later, sternotomy was performed and the lung tissue was completely removed. Tissue samples obtained were snap-frozen and all samples were stored at −80°C until the further assays were performed. Unmanipulated, untreated animals were put to death for comparative purposes (Group I). Another group was consisted from the animals, which received Everolimus 1.0 mg/kg/day but not underwent ischemia-reperfusion (Group IV). Animals pretreated by everolimus received 1.0 mg/kg/day of PO everolimus for 7 days before injury (Groups IIIa, b, c). In contrast, we did not pretreat the animals in the other groups by everolimus, but they underwent 90'of ischemia followed by 30', 120', and 240' of reperfusion, respectively (Groups IIa, b, c). Each group included 6 animals.

Histopathological evaluation

Lung tissue specimens were fixed in formalin and dehydrated, cleared, and embedded in paraffin. Specimens were cut into serial sections and stained with hematoxylin-eosin. Tissue samples were evaluated histopathologically and graded as mild, moderate, or severe for the level of inflammation as described previously in the literature (9).

RNA Isolation and cDNA Synthesis

Total cellular RNA was isolated using peqGOLD TriFast™ reagent (Peqlab, Erlangen, Germany) according to the manufacturer's guideline with minor modification. Approximately 0.1 g rat lung tissue was homogenized in TRIzol Reagent using the Ultra-Turrax T10 homogenizer (IKA® Werke GmbH & Co. KG, Staufen, Germany). To eliminate potential DNA contamination, the RNA samples were DNase treated (DNaseI, Roche Diagnostics, GmbH, Mannheim, Germany) at 37 ^{0}C for 30 min before washing with 75% ethanol. Then, RNA was quantified on a NanoDrop Spectrophotometer (NanoDrop ND-1000, Montchanin, DE, USA). RNA samples were stored at −80 °C until further analysis. One microgram of total RNA was reverse-transcribed into first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) and random hexamer primer, following the supplier's recommendations. cDNA reactions were performed in an Eppendorf Mastercycler ep Gradient S thermal cycler (Eppendorf, Hamburg, Germany). After the reaction, the samples were kept at −20 °C until used for quantitative real-time PCR (qPCR) analysis.

Quantitative Real-Time PCR (qPCR)

Two pairs of intron spanning primers and probe combinations were designed using the Universal Probe Library (UPL) Assay Design Center (https://lifescience.roche.com/en_tr/brands/universal-probe-library.html#assay-design-center; Roche Applied Science, GmbH, Mannheim, Germany). The sequences of the primers and UPL probe numbers are indicated in Table 1.

qPCR reactions were run on a LightCycler 480 Real-Time PCR System and the program included a 10 min pre-incubation at 95 °C, followed by 50 cycles of 10 s at 95 °C, 20 s at 60 °C and then cooling to 40°C for 10 sec. β-actin (ACTB) gene was used to normalize expression levels of genes of interest (GOI). qPCR data were expressed as crossing points (Cp). Each sample was measured in duplicate (except for ACTB) and mean values were used for further calculation. PCR efficiency for each gene was determined by the serial dilution method.

Table 1. Primer sets sequences and probe numbers for UPL assay

Gene	Forward primer	Reverse primer	UPL probe number
IL1β	5′-TGTGATGAAAGACGGCACAC-3′	5′-CTTCTTCTTTGGGTATTGTTTGG-3′	78
IL6	5′-CCCTTCAGGAACAGCTATGAA-3′	5′-ACAACATCAGTCCCAAGAAGG-3′	20
NFκβ	5′-'CTGGCAGCTCTTCTCAAAGC-3′	5′- TCCAGCTTTCTTCAGGACTTG-3′	68
iNOS	5′-ACCATGGAGCATCCCAAGTA-3′	5′-CAGCGCATACCACTTCAGC-3′	128
eNOS	5′-CGGCATCACCAGGAAGAAGA-3′	5′-CATGAGCGAGGCGGAGAT-3′	5
TNF-α	5′-GTAGCCCACGTCGTAGCAA-3′	5′-GGTTGTCTTTGAGATCCATGC-3′	68
CXCL2	5′-GAGGATCGTCCAAAAGATACTGA-3′	5′-CTTTGATTCTGCCCGTTGAG-3′	92
PTGS2	5′-TCCAACCTCTCCTACTACACCAG-3′	5′-TCCAGAACTTCTTTTGAATCAGG-3′	69
ACTB	5′-CCCGCGAGTACAACCTTCT-3′	5′-GCCCAATACGACCAAATCC-3′	17

IL1β, interleukin 1 beta; IL6, interleukin 6; NFκβ, nuclear factor kappa beta; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; TNF-α, tumor necrosis factor alpha; CXCL2, C-X-C motif chemokine ligand 2; PTGS2, prostaglandin-endoperoxide synthase 2; ACTB, beta actin.

Protein expression levels of MIP2and TNF-α protein in lung tissue and BAL samples

Protein expression levels of MIP2 and TNF-α in both lung tissue and BAL samples were determined using commercial ELISA kits specific for rat MIP-2 (catalog no. KRC 1022) and TNF-α (catalog no. KRC 3012) (Biosource International, Camarillo, CA, USA) according to the supplier's guidelines.

Total protein extraction and determination of caspase 3 activity in tissues

Frozen tissue samples were homogenized with the help of an Ultra Turrax T10 homogenizer (IKA® Werke GmbH & Co. KG, Staufen, Germany) in an ice-cold extraction buffer (provided in ApoTarget Caspase-3 Protease Assay Kit). After centrifugation of whole-tissue extracts in a microcentrifuge (MIKRO 220R, Hettich, Germany) at 10.000 rpm for 10min at 4 °C, supernatants were collected. The total protein content of tissues was assayed by BCA kit protocol. (Pierce-Thermo Scientific, Rockford, IL, USA). To determine apoptosis occurrence, Caspase 3 activity was determined using the ApoTarget Caspase-3 Protease Assay Kit (BioSource International, Inc., Camarillo, CA, USA) according to the manufacturer's protocol.

Measurement of Lung Tissue and Bronchoalveolar Lavage Myeloperoxidase (MPO) Activity

Frozen lung tissue were thawed and MPO was extracted by homogenization followed by sonication as describedby Krawisz et al. (10). Briefly, tissues were homogenized and the pellets were sonicated three times for 30s in potassium phosphate buffer containing 0.5% hexadecyl trimethylamonnium bromide (HETAB). An aliquot of supernatant, bronchoalveolar lavage, was mixed with potassium phosphate buffer 0.01 mol/L, containing 0,0006% hydrogen peroxide and 2 mmol/L ethylenediamine tetraaceticacid (EDTA), which was followed by the addition of o-dianisidine dihydrochloride (Sigma, St. Louis, MO, USA) as a hydrogen donor.

MPO activity was measured spectrophotometrically at 15s intervals as the change in absorbance at 460 nm, resulting from the decomposition of H_2O_2 in the presence of o-dianisidine. Results are expressed as U/g wet tissue or U/ml.

Statistical Analysis

The significance of the fold change in the mRNA expression was tested using two-sided Pair-wise fixed Reallocation Randomisation Test, as provided in the REST 2009 software (11)**.** Statistical analysis of caspase-3 in lung tissue, MPO enzyme activities, MIP2 and TNF-α protein levels in both lung tissue and BAL was performedby using SigmaStat 4.0. Statistical comparisons between groups (n = 6 per group) of rats were carried out using Student's t test with $p < 0.05$ being considered significant. The results were expressed as mean ±standard deviation (SD).

RESULTS

Histopathological evaluation showed no significant difference in interstitial edema, perivascular edema, or leukocyte infiltration in the everolimus pretreatment groups compared to matched untreated groups with the same reperfusion periods (IIa-IIIa, IIb-IIIb, and IIc-IIIc), but the greatest change was in the 240min reperfusion group (IIc-IIIc). (Table 4)

We used a control group (Group I) that included animals that did not receive any therapy or intervention. To determine the effects of oral everolimus pretreatment (1mg/kg/day for 1 week), we formed another group that did not undergo the IR procedure (Group IV). Our findings showed that the mRNA expression of IL1β, IL6, NFκβ, iNOS, eNOS, TNF-α, CXCL2 (MIP2 gene) and PTGS2 (COX2 gene) significantly decreased in the everolimus groups compared with Group I ($p<0.05$). Additionally, when we compared the Group I and the LIRI groups that were not pretreated with everolimus (IIa, b, c), we found that the subject genes' mRNA levels significantly increased ($p<0.05$). (Figure 1)

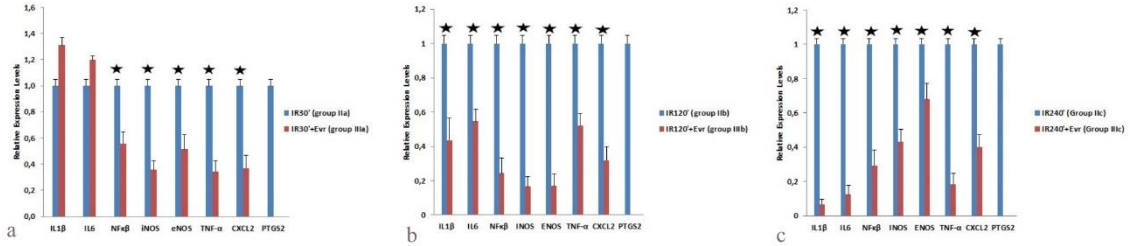

Figure 1. Comparison of mRNA expression levels of target genes in IR30'(IIa) and IR30'+Everolimus (IIIa) (a), in IR120'(IIb) and IR120'+Everolimus (IIIb) (b) and in IR240' (IIc) and IR240'+Everolimus (IIIc) (c) groups. Gene expression levels were normalized to ACTB mRNA expression. *; p<0.05.

The expression of the pro-inflammatory genes (except IL1β and IL6) decreased in the IR30 min +everolimus group (Group IIIa) but not in the IR30 min group (Group IIa) (p<0.05; Figure 1a). Moreover, in comparing the IR120 min group (Group IIb) with the IR120 min + everolimus group (Group IIIb) , all of the pro-inflammatory genes displayed statistically significant downregulation (p<0.05; Figure 1b).The pro-inflammatory genes' mRNA levels were also outstandingly diminished in the IR240min group (Group IIc) compared with the IR240min + everolimus group (Group IIIc; p<0.05; Figure 1c). Additionally, the complete inhibition of PTGS2 mRNA expression after everolimus treatment was found in all groups (Figure 1). The pro-inflammatory genes expression data are given in Table 2.

We also examined the MIP2and TNF-α protein levels in the rat lung and BAL samples. Our findings indicated that there was no difference between MIP2 andTNF-α protein expressions in the lung tissue of groups IIa and IIIa (p>0.05; Figure 2a). However, the MIP2 and TNF-α protein expression decreased in Group IIb compared with Group IIIb, and this down regulation showed statistical significance for both proteins in the lung tissue (p<0.05; Figure 2b). Compared with Group IIc, in Group IIIc, the MIP2 and TNF-α protein levels were lower in the lung tissue, though the TNF-α protein did not reach a statistical difference (p>0.05; Figure 2c). None of the MIP2 and TNF-α protein expression levels in the BAL samples save the MIP2 protein levels in groupB240, reached a statistical difference compared with Group IIIc (p>0.05). The p-values of the MIP2 and TNF-α proteins identified in the lung tissue and BAL samples are shown in Table 3.

Table 2. Proinflammatory genes' mRNA expression fold changes in groups

	IR30' vs IR30'+Everolimus (Group IIa vs Group IIIa)			IR120' vs IR120'+Everolimus (Group IIb vs Group IIIb)			IR240' vs IR240'+Everolimus (Group IIc vs Group IIIc)		
	Exp	P val	Fold Change	Exp	P val	Fold Change	Exp	P val	Fold Change
IL1β	1,309	0.374	1,3↑	0,435	**0.005***	2,3↓	0,068	**0.005***	14,7↓
IL6	1,197	0.599	1,2↑	0,547	**0.048***	1,8↓	0,125	**<0.001***	8↓
NFκβ	0,557	**0.034***	1,8↓	0,243	**0.004***	4,1↓	0,293	**0.041***	3,4↓
iNOS	0,357	**0.012***	2,8↓	0,165	**0.001***	6,1↓	0,434	**0.032***	2,3↓
eNOS	0,513	**0.008***	1,9↓	0,170	**0.002***	5,9↓	0,481	**0.026***	2,1↓
TNF-α	0,344	**0.022***	2,9↓	0,519	**0.042***	1,9↓	0,182	**0.022***	5,5↓
CXCL2	0,370	**0.021***	2,7↓	0,319	**0.006***	3,1↓	0,403	**0.015***	2,5↓
PTGS2	ND			ND			ND		

Exp, expression; P val, P value; Down Reg, down regulation; IL1β, interleukin 1 beta; IL6, interleukin 6; NFκβ, nuclear factor kappa beta; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; TNF-α, tumor necrosis factor alpha; CXCL2, C-X-C motif chemokine ligand 2; PTGS2, prostaglandin-endoperoxide synthase 2; ACTB, beta actin. ND, undetermined.
* Statistically significant.

Table 3.P-values of MIP2 and TNF-α proteins identified from lung tissues and bronchoalveolar lavage (BAL) samples

	MIP2 (P values)	TNF-α (P values)
Lung Tissues		
IR30' (Group IIa) vs IR30' +Everolimus (Group IIIa)	0,076	0,757
IR120' (Group IIb) vs IR120'+Everolimus (Group IIIb)	**0,009***	**0,002***
IR240' (Group IIc) vs IR240'+Everolimus (Group IIIc)	**0,040***	0,074
BAL		
IR30' (Group IIa) vs IR30' +Everolimus (Group IIIa)	0,354	0,351
IR120' (Group IIb) vs IR120'+Everolimus (Group IIIb)	0,191	0,180
IR240' (Group IIc) vs IR240'+Everolimus (Group IIIc)	**0,002***	0,589

* Statistically significant.

Table 4. Histologic evaluation of lung tissue showing interstitial edema, perivascular edema and perivascular leukocyte infiltration according the degree of change

Group	Interstititat edema	Perivascular edema	Perivascular leukocyte infiltration
I	Mild	Mild	-
IIa	Medium	Mild	-
IIb	Medium	Mild	-
IIc	Medium	Mild	-
IIIa	Mild	Medium	-
IIIb	Mild	Medium	+
IIIc	Mild	Medium	+
IV	Mild	Mild	-

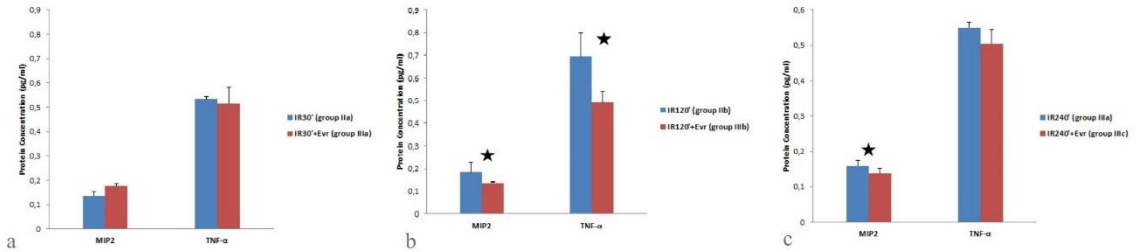

Figure 2. Comparison of MIP2 and TNF-α protein levels in IR30'(IIa) and IR30'+Everolimus (IIIa) (a) , IR120'(IIb) and IR120'+Everolimus (IIIb) (b) and in IR240'(IIc) and IR240'+Everolimus (IIIc) (c) groups. *; p<0.05.

To determine everolimus' apoptosis-prevention effects in rat lung tissue after LIRI induction, we measured caspase-3 enzyme activity. compared with Group IIa, there was a statistically significant downregulation of caspase-3 enzyme activity in Group IIIa (p=0.002). A regulation of caspase 3 enzyme activity was detected in Group IIIb compared with Group IIb. However, this increase was statistically unimportant (p>0.05). Although caspase-3 enzyme activity was lower in Group IIIc than in Group IIc, it did not reach a statistical difference (p>0.05; Figure 3).

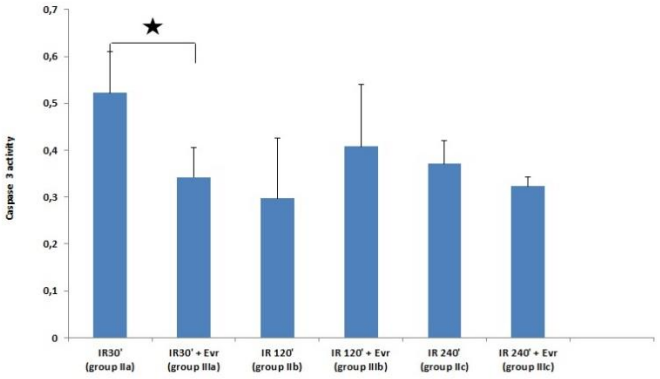

Figure 3. Comparison of caspase-3 enzyme activity at different reperfusion times (IR30',IR120', IR240') versus the Everolimus with IR30', IR120', IR240'reperfusion duration, respectively.

In our study, we also examined the MPO levels in the lung tissues and BAL samples. In the lung tissue samples, MPO decreased significantly in subjects who received everolimus in Group IIIa (p <0.05), while in groups IIb and IIc, there was an increase in MPO by prolonged reperfusion times (groups E and F). In the BAL samples, only in Group IIIc did MPO decrease significantly (p <0.05; Figure 4).

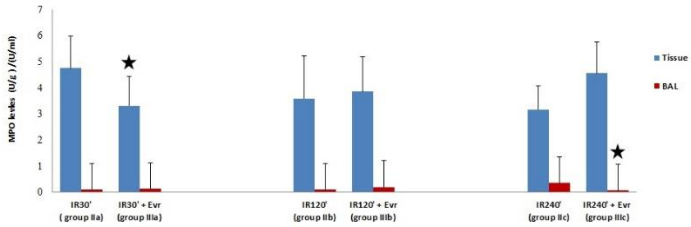

Figure 4. MPO levels (U/g) in tissue and BAL samples (U/ml) for IR and IR + Eve groups at each reperfusion time. *; p<0.05.

DISCUSSION

In this study, we used a rodent lung-ventilated IR model to evaluate the effects of everolimus pretreatment before LIRI. Our model was confirmed to be successful in terms of IR through both pathological examination and inflammatory mediator-level assessments. We showed that the mRNA expression of the selected pro-inflammatory genes (IL1β, IL6, NFκβ, iNOS, eNOS, TNF-α, CXCL2, and PTGS2) significantly decreased with everolimus pretreatment in lung tissue. The TNF-α and MIP2 protein levels studied in the BAL and lung tissue samples also decreased significantly with everolimus use.

The acute, sterile inflammation that frequently develops in lung tissue that inevitably reperfuses after the ischemic period is called "LIRI" (12). LIRI is characterized by increased microvascular permeability, pulmonary edema, increased pulmonary vascular resistance and impaired oxygenation (13). The release of inflammatory mediators such as chemokines, cytokines and oxygen radicals causes these symptoms (3). Severe IR injury leads to PGD, which is the most important cause of morbidity and mortality both in the short and long term after transplantation. LIRI, may also develop after extracorporeal circulation, pulmonary embolism, and pneumonectomy, leading to pulmonary dysfunction and cause serious lung damage (14).

IR injury causes a fast and complex inflammatory response. This immune response is primarily triggered by rapid and excessive reactive oxygen species (ROS) release, leading to cell and tissue damage, the activation of various cell types, the peroxidation of membrane lipids, and the release of inflammatory cytokines and damage-associated molecular patterns (DMAPs) (12).

Various strategies have been developed to limit LIRI damage. One of these preventive strategies is to make the lung tissue more resistant to LIRI before IR. Essentially, these pretreatments can be divided into three types: hyperthermic, ischemic, and chemical (1). Immunosuppressive, anti-inflammatory, and chemotherapeutic drugs, cytokines, vasoprotective agents, monoclonal antibodies, and antioxidants have all been used as chemical treatment (5).

Today, supportive treatments are given after LIRI development, but there is no specific therapeutic agent to prevent it (12). Since it is a clinical problem that has not yet been resolved, determining an effective and safe method or drugs to prevent LIRI remains a current research area (14).

A wide variety of transcription factors regulate the development of lung damage. Genes that are upregulated after reperfusion injury play an effective role in the formation of a pro-inflammatory response (4). The mTOR pathway also reshapes cellular metabolism in mammalian cells given its importance to the innate immune system, as it regulates translation, cytokine response, antigen presentation, macrophage polarization, and cell migration. The effect of mTOR inhibition differs among monocytes, macrophages, dendritic cells, neutrophils, mast cells, and innate-like natural killer cells (15). In our study, we focused on the anti-inflammatory effects of mTOR inhibition in LIRI.

Rapamycin and its derivatives are immunosuppressive agents that suppress the natural immune response by inhibiting mTOR (18). Everolimus (RAD001), a derivative of rapamycin (sirolimus), is a suppressor of mTOR protein. It stops the cell cycle in the late G1 phase, preventing cells from entering the S phase (7,8). In our study, we determined the effect of prophylactic everolimus treatment given before IR on the mRNA expression of the pro-inflammatory genes IL1β, IL6, NFκβ, iNOS, eNOS, TNF-α, CXCL2 (C-X-C Motif Chemokine Ligand 2), and PTGS2 in a rat-ventilated IR model.

In another ventilated IR rodent model, Parakash et al. (16) showed that IL-6 is rapidly released after IR (in 1–3 hours) and recovers within 12–24 hours in parallel with histology. The same report suggested that IL-1β force macrophages to produce an exaggerated endothelial inflammatory cytokine because of IR. In our study, everolimus's effect was not observed in the early stages of reperfusion (Group IIIa) in both IL-6 and IL-1 β mRNA expression levels, but in the advanced stages of reperfusion (Group IIIb and F) the expression of these genes significantly decreased in the everolimus group ($p<0.05$).

Naidu et al. (20) showed that the TNF-α release from alveolar macrophages was important in the early stage of the inflammatory cascade in LIRI (3). In our study, there was no significant difference in TNF-α levels between the Groups IIa and IIIa, but the fact that everolimus significantly reduced TNF-α levels in groups IIIb and IIIc suggested that while it could not prevent TNF-α's early effects on LIRI, it was effective in the later period of the process.

Krishnadasan et al. (18) showed that β-chemokines such as MIP-1β play a role in LIRI (3). In the study Lin et al. (19), another mTOR inhibitor, sirolimus (which everolimus is derived from) was shown to significantly reduce the lipopolysaccharide (LPS) induced expression of MIP-1α in THP-1 cells and the expression of both MIP-1α and MIP-1β was reduced in LPS-treated human primary monocytes in the human monocyte cell line. In our study, we found that MIP-2 protein levels did not change significantly in Group IIIa in parallel with TNF-α, whereas everolimus significantly decreased the MIP-2 protein levels in groups IIIb and IIIc.

COX2 is a cytokine inducible isoform of cyclooxygenase (COX) that is synthesized from many types of cells, including endothelial cells, macrophages, monocytes, and pulmonary epithelial cells. It appears to be the main isoform present under prolonged inflammatory conditions (20). To our knowledge, PTGS2 (the COX2 gene) expression levels have not been studied before in a rodent ventilated IR model. Therefore, although we could not compare our results with the literature, we determined that PTGS2 mRNA expression was completely inhibited in all groups pretreated with everolimus.

In ventilated lung ischemia, oxidative stress plays a crucial role in the injury process. IR increases pulmonary vascular resistance (PVR) leading to decreased alveolar perfusion and hypoxemia. As a result, epithelial and endothelial injury occurs. Nitric oxide (NO) plays a pivotal role in the regulation of pulmonary circulation. (21). Enzymes of the NO-synthase (NOS) family include constituent (eNOS and nNOS) and inducible (iNOS) isoforms (22). NOS activity is regulated at the transcriptional and post-transcriptional level. During inflammation caused by IR, iNOS is induced at the transcriptional level. Activating the transcription factors NFκB and AP-1 can cause NO overproduction resulting from iNOS isoforms. Recent studies showed an association between NO production and vascular injury during IR (21). According to our results, iNOS, eNOS and NFκB expressions increased significantly in mRNA levels in all three reperfusion groups (IIa, b, c) compared with groups pretreated with everolimus. Our results also showed that everolimus pretreatment is effective by suppressing NO synthesis and preventing LIRI.

MPO, a heme-containing peroxidase expressed mainly in neutrophils and proven to be an important local mediator of tissue damage, has become an important therapeutic target of inflammatory conditions (23). We also examined the MPO levels in lung tissue and BAL fluid of rats. There was only a significant MPO decrease in the BAL samples for group IIIc.

Everolimus's effectiveness on IR injury is still being investigated. Two studies presented that everolimus pre-treatment failed to reduce IR injury in rat kidneys (24,25). However, our results, showed that the prophylactic use of everolimus, an mTOR inhibitor, has a preventive effect on LIRI in rats by decreasing the expression of inflammatory genes.

This is the first study to determine the effects of everolimus pretreatment on LIRI in a rat lung-ventilated IR model and both the mRNA and protein expression of subject genes and selected cytokines. Although we could not clarify the effects of everolimus on LIRI by histopathological studies, its effects on both mRNA and protein expressions were clearly identified.

Our study's limitation is that we could not compare all of our findings with the literature because of the absence of one-to-one studies. We suggest further experimental trials to evaluate the effect of pretreatment with everolimus to prevent serious LIRI results.

Conflict of interest

No conflict of interest was declared by the authors.

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