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Research Article

Brain damage from lung ischemia-reperfusion injury, its potential link to Alzheimer's disease, and the protective role of riociguat

Özlem Özmen[1], Adem Milletsever[1], Halil Aşcı[2]

[1]Department of Pathology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur, Türkiye
[2]Department of Pharmacology, Faculty of Medicine, Suleyman Demirel University, Isparta, Türkiye

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Correspondence:
Ö. ÖZMEN
(ozlemozmen@mehmetakif.edu.tr)

ORCID
Ö. ÖZMEN : 0000-0002-1835-1082
H. AŞCI : 0000-0002-1545-035X
A. MİLLETSEVER : 0000-0002-3614-7798

ABSTRACT

Lung ischemia-reperfusion (LIR) injury can cause widespread systemic and neurological damage, potentially increasing susceptibility to Alzheimer's disease (AD). Riociguat (RIO) has shown potential in reducing damage from ischemia-reperfusion injuries. This study examines whether RIO could help slow AD progression by decreasing brain injury, neuroinflammation, and beta-amyloid (Aβ) accumulation resulting from LIR. In this experiment, forty male Wistar Albino rats were randomly divided into four groups: sham, LIR, LIR+RIO, and RIO. LIR was induced by clamping the hilus for 60 minutes, followed by 60 minutes of reperfusion. RIO was administered 30 minutes before ischemia. Brain tissues were then analyzed through histopathological and immunohistochemical techniques. Histopathology in the LIR group revealed significant hyperemia, degenerative changes, neuronal loss, gliosis, and inflammatory cell infiltration. Immunohistochemical analysis showed elevated levels of Aβ, Caspase-3, and TNF-α. RIO treatment effectively reversed these changes, indicating its potential to reduce brain damage, neuroinflammation, and Aβ accumulation. These findings suggest that lung ischemia-reperfusion injury in this rat model may increase vulnerability to Alzheimer's disease, but that RIO could play a protective role.

INTRODUCTION

In ischemia-reperfusion events, blood flow to organs is temporarily reduced and then restored during reperfusion (Zhang et al., 2024; Kalogeris et al., 2016). Arteriosclerosis, which is often aggravated by chronic conditions such as diabetes and hypertension, as well as by environmental factors like stress and smoking, can lead to endothelial damage and aneurysmal ruptures, making it one of the most significant causes of ischemia (Gusev and Sarapultsev, 2023; Singh et al., 2020). Similar to atherosclerosis, the main contributor to hypoxia-induced damage in distal tissues is the inability to produce nitric oxide, which normally promotes vasodilation. This issue is compounded by lipid accumulation in the subendothelial space and platelet plug formation, which narrows arterial diameter and impairs tissue repair (Badimon et al., 2016; Kubota et al., 2016). Ischemic conditions or other causes that compromise the lung—the primary organ responsible for oxygenation—can lead to similar dysfunctions (Sakar et al., 2017; Ferrari et al., 2015). Research suggests that lung disease, reduced lung function, or poor lung health may be associated with an increased risk of dementia or cognitive decline (Lutsey et al., 2019; Dodd, 2015; Vidal et al., 2013; Rusanen et al., 2013; Pathan et al., 2011; Hozawa et al., 2006).

Prooxidant and proinflammatory molecules formed in hypoxic conditions, such as in lung ischemia-reperfusion (LIR), bind to receptors in distal tissues, triggering cellular damage and activating several intracellular damage pathways (Ferrari et al., 2015; Lahousse et al., 2015). Inflammation and apoptosis are key mechanisms underlying this cellular damage. These prooxidant and proinflammatory molecules can also disseminate through the bloodstream, potentially causing injury to distant organs (Kalogeris et al., 2012). In particular, damage to the blood-brain barrier (BBB) increases its permeability, leading to brain tissue injury and various neurological disorders, depending on the affected regions. Studies indicate that some of these injuries may contribute to the development of neurodegenerative diseases, such as Alzheimer's and Parkinson's (Archie et al., 2021).

In Alzheimer's disease, β-amyloid peptide (Aβ) accumulation, often accompanied by inflammation in specific brain regions, serves as a diagnostic hallmark. Impaired clearance of Aβ due to a compromised BBB and microglial dysfunction from neuroinflammation are contributing factors. According to recent literature, studies focused on reducing inflammation and apoptosis, both of which are critical to AD progression, have also reported reduced Aβ accumulation (Chen et al., 2023).

Riociguat (RIO), a soluble guanylate cyclase (sGC) stimulant,

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is approved for treating chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension in adults. It promotes vasodilation either independently or by enhancing the effects of nitric oxide (NO) (Mihalek et al., 2022; Lian et al., 2017). A recent study suggests that RIO protects neonatal rats from pulmonary hypertension and hyperoxia-induced lung damage without hindering long bone growth (Donda et al., 2018). Although RIO has limited penetration into the central nervous system, it may still help reduce hypoxia-induced damage through its effects on lung tissue (Frey et al., 2018).

Several pathways, particularly those involving systemic inflammation, oxidative stress, physiological stress, and damage to small vessels from prolonged hypoxemia, are thought to increase the risk of dementia and cognitive impairment (Dodd, 2015; Maclay & MacNee, 2013). However, little is known about how lung ischemia affects the brain, and the mechanisms remain unclear.

This study aims to examine brain pathology resulting from LIR and assess whether RIO, known for its antiproliferative, antihypertensive, antifibrotic, and anti-inflammatory properties, can reduce brain damage and Aβ accumulation associated with LIR.

MATERIALS and METHODS

Animals and ethical approval

All animal experiments in this study adhered to the Animal Research: Reporting in Vivo Experiments (ARRIVE) 2.0 guidelines. The experimental protocol was approved by the Suleyman Demirel University local animal ethics committee on June 6, 2024, under permission number 304. This research was funded by the Scientific Research Projects Coordination Unit of Suleyman Demirel University, with the project code TSG-2023-9010.

Forty male Wistar Albino rats, each weighing between 250 and 350 g, were obtained from the Suleyman Demirel University Experimental Animals Laboratory. Based on relevant parameters (α=0.05, 1-β=0.90, effect size=0.40) and calculated using GPower 3.1.9.7 software, the study was designed with four groups of 8 rats (total n=32). The rats were kept in a controlled environment with 12-hour light-dark cycles, at a constant temperature (21–22 °C) and humidity (55%). They were fed ad libitum and housed in Euro type-2 cages with wood shavings as bedding.

Experimental Procedure

To minimize potential confounding factors, all experimental procedures were conducted in a standardized manner within the same group and sequence. The rats were randomly assigned to four groups, each consisting of eight rats:

Sham Group: The hilus was visualized, and a thoracotomy was performed without inducing ischemia.

LIR Group: Following a left thoracotomy, a nontraumatic vascular clamp was placed on the hilus, and ischemia was induced for 60 minutes, followed by 60 minutes of reperfusion (Abu-Amara et al., 2010).

LIR+RIO Group: This group underwent the same procedure as the LIR group, but 10 mg/kg of Riociguat (RIO) was administered orally 30 minutes prior to the induction of ischemia (Seker et al., 2022).

RIO Group: Rats in this group received only an oral dose of 10 mg/kg RIO.

After a 12-hour fasting period, the thoracic area of each rat was shaved, and a left thoracotomy was performed under intraperitoneal anesthesia using 90 mg/kg Ketamine (Bioveta, Czech Republic) and 10 mg/kg Xylazine (Doğa İlaç, Türkiye). The left lung hilus was identified through visualization of the trachea, and a nontraumatic vascular clamp was applied for 60 minutes (in the LIR and LIR+RIO groups), followed by 60 minutes of reperfusion. Once blood flow was restored, the animals were sacrificed.

Blood was collected from the inferior vena cava through an abdominal incision to perform surgical exsanguination. After sacrifice, brain tissues were carefully extracted and preserved in a 10% formaldehyde solution for subsequent immunohistochemical and histological analyses.

Histopathological analysis

During necropsy, tissues from the brain, cerebellum, and hippocampal regions were carefully collected and preserved in 10% buffered formalin for histological analysis. The tissues were processed using an automated tissue processor according to standard protocols. A rotary microtome was utilized to section the paraffin blocks into 5 µm thick slices.

Following sectioning, the tissue samples underwent deparaffinization and were cleaned with xylene. They were then stained using hematoxylin-eosin (HE) and rehydrated with ethanol. After staining, the sections were mounted with coverslips for examination.

Histological alterations were evaluated under a light microscope. Various brain regions were semiquantitatively assessed for histopathological lesions, including the degree of gliosis, hemorrhage, hyperemia, and neuronal damage. Each type of damage was graded on a severity scale from 0 to 3, as outlined in Table 1 (Unlu et al., 2024).

Table 1. Histopathological scoring criterion for brain.

0	No lesions
1	Lesions in fewer than 20% of the fields
2	Lesions in 20% to 60% of the fields
3	Lesions in every field

Immunohistochemical analysis

For the immunohistochemical analysis, antibodies against beta-amyloid [Beta amyloid Recombinant Antibody [EPR16630] (ab205529)], Caspase-3 [Recombinant Anti-Caspase-3 p12 antibody [EPR16888] (ab179517)], and TNF-α [Anti-TNF alpha recombinant antibody [RM1005] (ab307164)] were utilized, all

sourced from Abcam, Cambridge, UK. The tissue slices were mounted on poly-L-lysine slides for the immunohistochemical staining process, which employed the streptavidin-biotin-peroxidase technique.

Each primary antibody was diluted to 1:100 and applied to the sections, which were then incubated overnight. Following this incubation, sections were stained using biotinylated secondary antibodies and a streptavidin-alkaline phosphatase conjugate. A ready-to-use commercial kit, the EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436) from Abcam, provided the necessary secondary antibody and chromogen. For negative controls, the primary antiserum was replaced with the antibody dilution solution.

Blinded samples were employed to eliminate bias, and each analysis was conducted by a trained histopathologist from a different university. The percentage of cells positively immunostained for each marker was calculated in ten distinct fields on each slide for each group, using an objective magnification of X40. The images were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, version 1.48), and microphotographs were captured using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan).

Statistical Analysis

Statistical analysis of the histopathological scores and the number of immunohistochemically positive cells was performed using GraphPad Prism software. The Shapiro-Wilk test was initially applied to assess the normality of data distribution. Since the data demonstrated a normal distribution ($P > 0.05$), ANOVA was utilized to compare the groups. Pairwise differences between the groups were determined using the post hoc Duncan test. A significance threshold of $P < 0.05$ was established, and results are presented as means ± standard deviation.

RESULTS

No significant macroscopic findings were observed in the brains of any group, except for the LIR group, which exhibited marked hyperemia and edema.

During histopathological evaluation, both the control group and the RIO group displayed normal findings without abnormalities. In contrast, the LIR group showed significant hyperemia, edema, mild degeneration, neuronal death, and slight gliosis within the brain. Additionally, edema and infiltration of inflammatory cells were noted in the meninges of this group. Following treatment with RIO in the RIO+LIR group, notable improvements in these lesions were observed, as depicted in Figure 1.

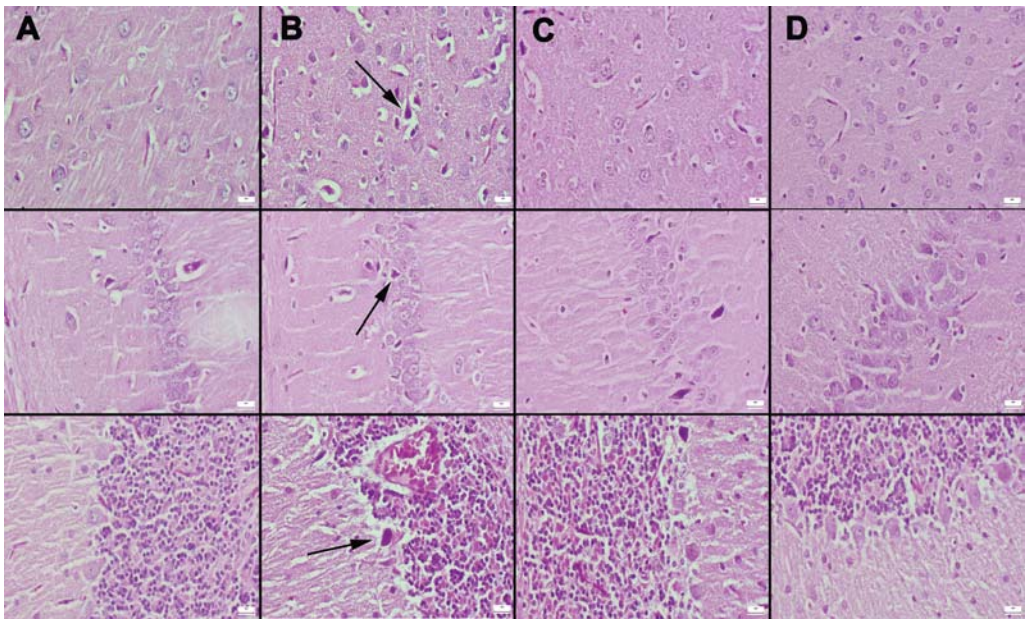

Figure 1. Histopathological differences between groups in the brain (top row), hippocampus (middle row), and cerebellum (bottom row) tissues. (A) Normal tissue histology in the control group, (B) Significant hyperemia and edema (arrows) in the LIR group, (C) Reduced histopathological lesions in the LIR+RIO group, (D) Normal tissue histology in the RIO group. HE staining, scale bars = 20 μm.

The application of LIR resulted in a significant increase in the expression levels of Aβ, Caspase-3 (Cas-3), and TNF-α within the neurons of the central nervous system. Immunohistochemical analysis revealed predominantly negative to very slight expressions of these three proteins in brain, hippocampal, and cerebellar tissues. However, following RIO therapy, the expressions of Aβ, Cas-3, and TNF-α were reduced in the brain, cerebellum, and hippocampal regions of the LIR+RIO group. Minimal to negligible expression of Aβ, Cas-3, and TNF-α was observed in both the RIO and control groups (Figures 2-8). The results of the statistical analysis are also presented in the figures. The possible pathogenetic mechanism of brain damage caused by LIR is illustrated in Figure 9.

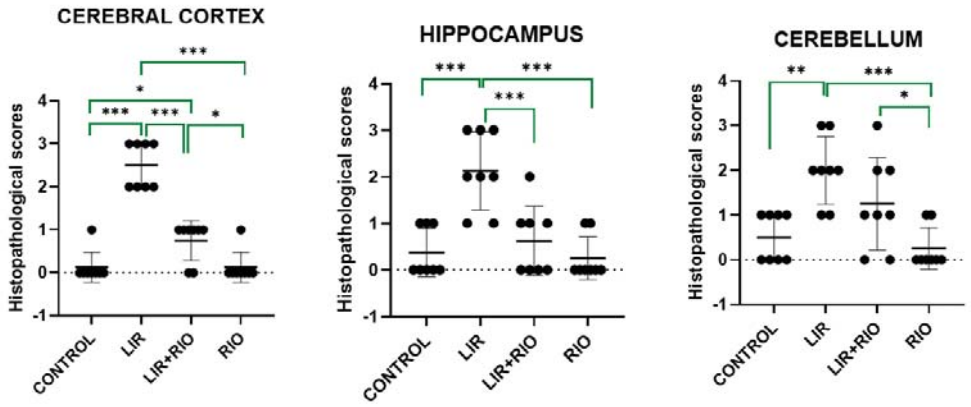

Figure 2. Statistical analysis results of histopathological scores.

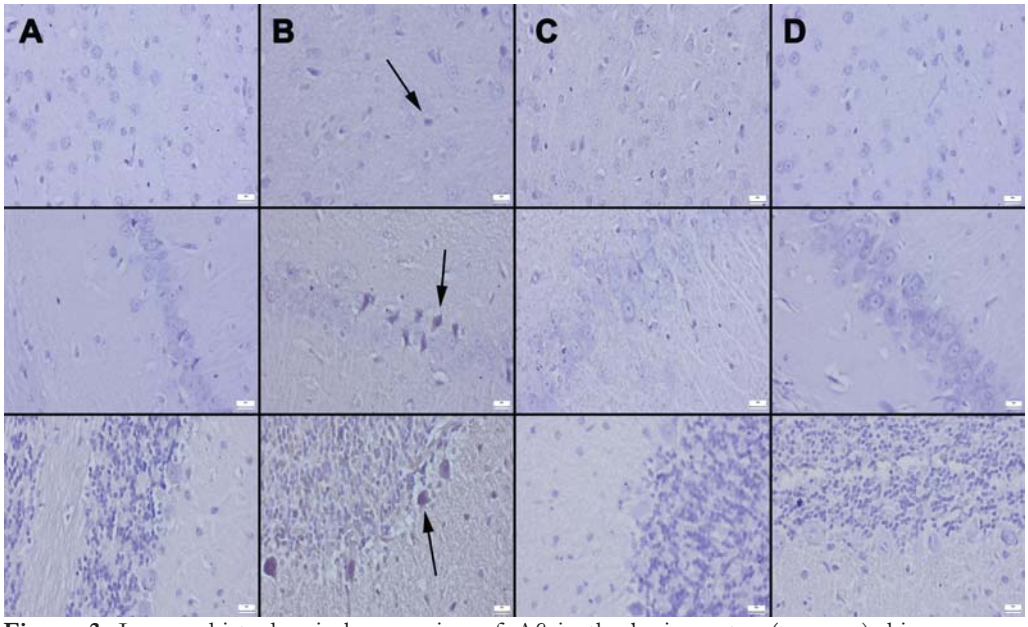

Figure 3. Immunohistochemical expression of Aβ in the brain cortex (top row), hippocampus (middle row), and cerebellum (bottom row) across groups. (A) No expression in the control group, (B) Increased expression (arrows) in the LIR group, (C) Decreased expression in the LIR+RIO group, (D) Negative expression in the RIO group. Streptavidin-biotin peroxidase method, scale bars = 20 μm.

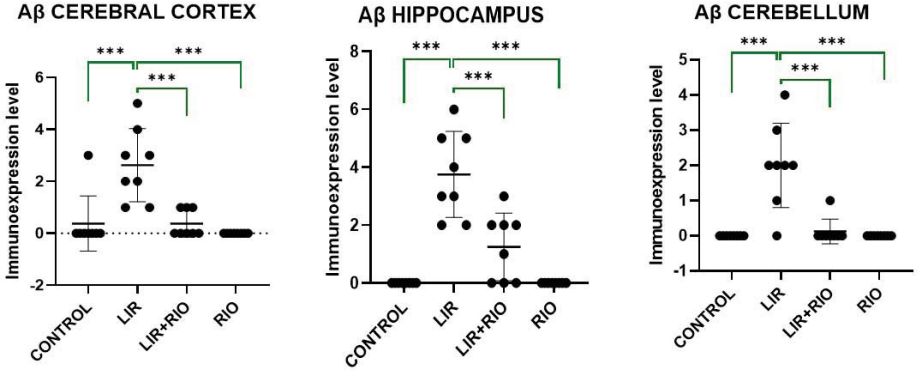

Figure 4. Statistical analysis results of Aβ expression scores

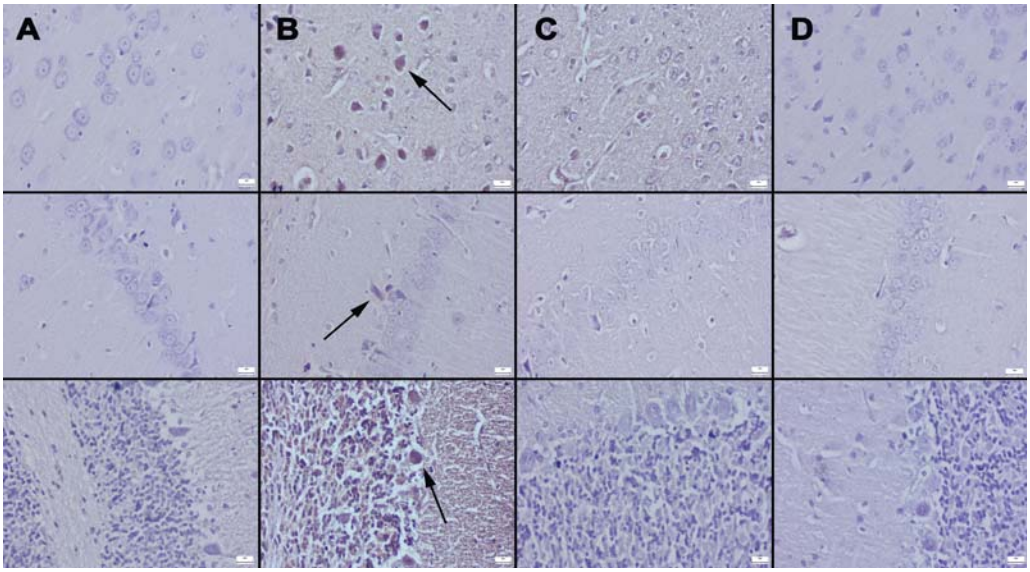

Figure 5. Cas-3 expression in the brain cortex (top row), hippocampus (middle row), and cerebellum (bottom row) across groups.(A) Negative expression in the control group, (B) Increased expression (arrows) in the LIR group, (C) Decreased expression in the LIR+RIO group, (D) Negligible to slight expression in the RIO group. Streptavidin-biotin peroxidase method, scale bars = 20 μm.

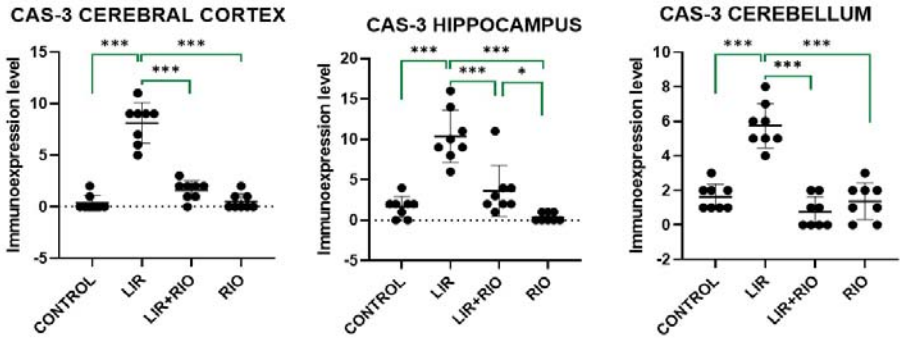

Figure 6. Statistical analysis results of Cas-3 expression scores

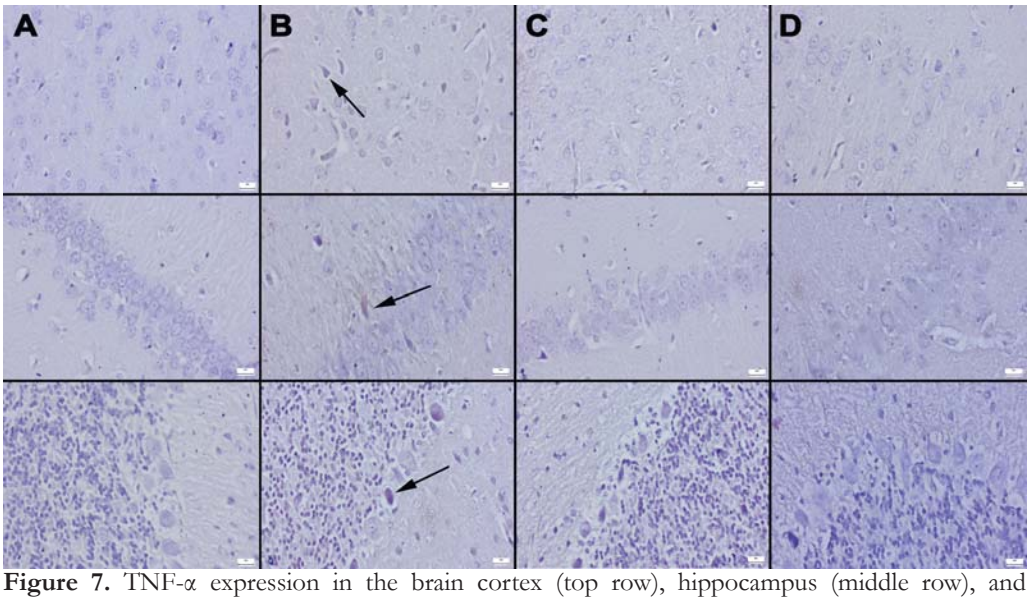

Figure 7. TNF-α expression in the brain cortex (top row), hippocampus (middle row), and cerebellum (bottom row) across groups. (A) No expression in the control group, (B) Increased expression (arrows) in the LIR group, (C) Decreased expression in the LIR+RIO group, (D) Negative expression in the RIO group. Streptavidin-biotin peroxidase method, scale bars = 20 μm.

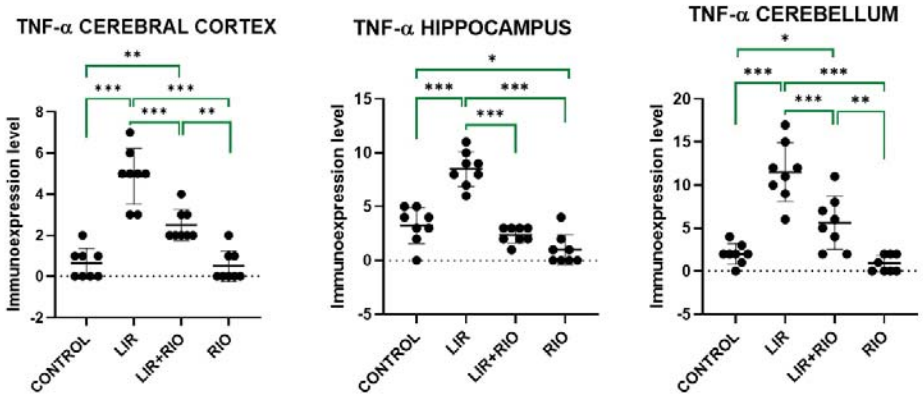

Figure 8. Statistical analysis results of TNF-α expression scores

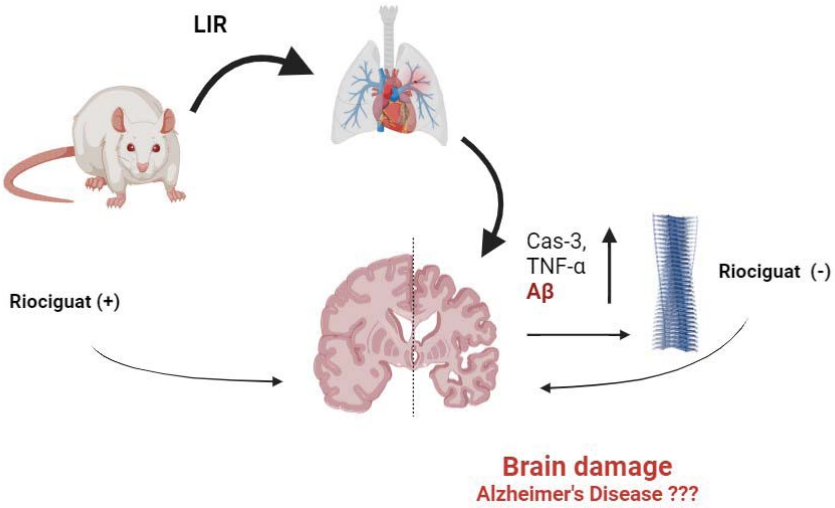

Figure 9. Possible mechanisms of RIO on LIR.

DISCUSSION

Lung ischemia-reperfusion injury is a significant clinical challenge, often leading to severe neurological and systemic side effects, including the exacerbation of neurodegenerative diseases such as Alzheimer's disease (AD). This study highlights the neuroinflammatory response in brain tissue following LIR, which contributes to the accumulation of amyloid beta (Aβ) plaques, a hallmark of AD. The findings suggest that Riociguat (RIO) could serve as a protective agent against the risk and progression of Alzheimer's disease by mitigating brain damage, reducing neuroinflammation, and decreasing Aβ accumulation as a consequence of LIR.

The lungs, being one of the most vascularized organs in the body, can be influenced by pathological events occurring elsewhere. Damage to lung tissue can have detrimental effects on multiple organ systems (Zhang et al., 2024; Mammoto and Mammoto, 2019; Kalogeris et al., 2016). Inflammatory responses triggered by conditions such as interstitial lung injury, trauma, or ischemic damage can lead to the release of inflammatory cytokines into the bloodstream due to increased vascular permeability in the lung. These cytokines can then affect distant organs, exacerbating the injury (Elma et al., 2022; Kalogeris et al., 2012). Notably, the disruption of the blood-brain barrier during inflammation allows these cytokines to reach brain tissue, initiating neuroinflammation and potentially leading to neuronal cell death through apoptotic mechanisms. Literature supports that protecting lung tissue, which plays a crucial role in these processes, could reverse the pathological changes occurring in brain tissue (Sun et al., 2022; Yang et al., 2022).

Histopathological findings from this study reveal marked hyperemia, edema, and gliosis in the cortex, along with edema and inflammatory cell infiltration in the meninges of brain tissues in the pulmonary ischemia group. These observations indicate an inflammatory response, while the documented neuronal cell death suggests apoptosis is also occurring. Furthermore, the presence of similar pathological changes in both hippocampal and cerebellar tissues indicates a more generalized brain injury. The application of RIO, with its potential vasodilatory effects as a soluble guanylate cyclase (sGC) activator, appears to reduce lung tissue damage, leading to a propor-

tional decrease in brain injury due to the reduced systemic release of inflammatory cytokines. This is further supported by the results demonstrating that the LIR+RIO group exhibited less severe pathological changes compared to the LIR group.

The protective effects of Riociguat (RIO) may indeed play a crucial role in preventing balance disorders associated with cerebellar dysfunction and mitigating cognitive impairments related to hippocampal damage. This study highlights the significance of maintaining lung health as a strategy to preserve neurological function and decrease the risk of neurodegenerative diseases following ischemic injuries.

Previous research indicates that Aβ plays a central role in synaptic damage via pathways activated by local caspases (Park et al., 2020). Elevated levels of the pro-inflammatory cytokine TNF-α have been observed in the brains of individuals with Alzheimer's disease. Given that TNF-α-converting enzyme (TACE) releases TNF-α from cell membranes, inhibiting TACE may reduce the deleterious effects of TNF-α in Alzheimer's patients (Kim et al., 2008). Notably, our study found that expressions of TNF-α and Aβ significantly increased 60 minutes post-LIR, suggesting a rapid neuroinflammatory response that could exacerbate neuronal damage.

Alzheimer's disease is characterized by progressive neurodegeneration and inflammation (Archie et al., 2021). Memory impairments resulting from neuronal loss, particularly within the cholinergic system, severely impact patients' daily lives (Park et al., 2020). The increased synthesis of Aβ during neuroinflammatory states—often associated with inadequate microglial responses—can lead to its accumulation in various brain regions, which is critical for diagnosing AD. Studies suggest that the severity of clinical progression in Alzheimer's disease may correlate with the extent of Aβ aggregation (Chen et al., 2023).

In this context, the lack of Aβ accumulation in the RIO-treated groups aligns with the histopathological findings, suggesting that RIO may prevent significant neurodegenerative changes linked to Alzheimer's disease. The reduction of LIR-induced Aβ accumulation in the cerebral cortex, hippocampus, and cerebellum through RIO treatment may thus protect cognitive functions and alleviate neurological and balance disorders associated with ischemic brain injury. Overall, these findings emphasize the potential of RIO as a therapeutic intervention in mitigating neurodegenerative processes triggered by inflammatory responses following lung ischemia-reperfusion injury.

The correlation between TNF-α expressions—an acute phase reactant associated with inflammatory diseases—and the histopathological findings of inflammation underscores the extent of brain damage secondary to LIR. The observation that elevated TNF-α levels across all three examined tissues could be reversed by RIO suggests its potential effectiveness in protecting brain tissue from inflammatory damage. Although RIO is known to have limited permeability through the blood-brain barrier, it may exert protective effects by either utilizing the increased permeability associated with inflammation or by mitigating peripheral lung tissue damage, which subsequently reduces inflammatory signaling to the brain.

The relationship between inflammation and apoptosis is well-documented in physiopathological mechanisms. Inflammatory processes can activate various intracellular pathways leading to apoptosis, while apoptosis does not inherently trigger inflammation due to the preservation of cellular membranes (Zhang et al., 2018). The elevated expression of Caspase-3 observed in our immunostaining results aligns with TNF-α levels and the histopathological evidence of neuronal cell death. Notably, one of the most significant findings of this study is that RIO treatment downregulates Cas-3 expression across all examined tissues, indicating that neuronal protection may prevent the neuronal death associated with the pathogenesis of Alzheimer's disease.

Furthermore, it has been documented that anti-inflammatory mechanisms are activated during the clearance of apoptotic cells, which may contribute to a reduction in inflammation (Szondy et al., 2017; Wan et al., 2013). Our study demonstrated that, in a rat model, brain expressions of Aβ, Cas-3, and TNF-α significantly increased 60 minutes following lung ischemia and subsequent reperfusion. Given the challenges associated with examining brain tissue during this critical period in human patients—who are often under anesthesia—animal models serve as a valuable alternative to provide insights into these processes.

However, this study has notable limitations. Being a preliminary investigation, it did not allow for the examination of molecular changes in the brain during the ischemic and reperfusion phases. Future studies should aim to assess the molecular alterations occurring in the brain during this time frame and investigate whether these changes are reversible. Such research would enhance our understanding of the protective mechanisms of RIO and its potential therapeutic applications in the context of neurodegenerative diseases following ischemic events.

CONCLUSION

As a result, expressions of TNF-α and Caspase-3, which are indicators of damage in brain tissue, along with Aβ expressions that are crucial to the onset of Alzheimer's disease, were found to be elevated in the cerebral cortex, hippocampus, and cerebellum following lung ischemia-reperfusion (LIR) injury. Treatment with Riociguat (RIO) reduced both histopathological and immunohistochemical findings, thereby preserving brain tissue. These results highlight the need for further studies incorporating more detailed molecular investigations.

DECLARATIONS

Ethics Approval

The experimental protocol received approval from the local animal experiments ethics committee of Suleyman Demirel University date 06.06.2024 and approval number 304.

Conflict of Interest

The authors have no conflict of interest with any person, institution or organization.

Consent for Publication

Publication is appropriate

Author contribution

Idea, concept and design: OO, HA

Data collection and analysis: OO, AM, HA

Drafting of the manuscript: OO, HA

Critical review: OO, AM, HA

Data Availability

The data is available from the corresponding author on reasonable request.

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