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Protective effect of clotrimazole on lung injury in an experimental model of ruptured abdominal aortic aneurysm

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2025

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Funding sources
None declared

Conflict of interest
None declared

Acknowledgements
The authors thank Ercüment Beyhun from the Department of Public Health, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey, for providing statistical analysis and graphical representation.

Received on July 1, 2023
Reviewed on October 8, 2023
Accepted on January 18, 2024

Published online on February 27, 2024

Cite as
Pulathan Z, Ergene SM, Altun, Yuluğ E, Menteşe A. Protective effect of clotrimazole on lung injury in an experimental model of ruptured abdominal aortic aneurysm [published online as ahead of print on February 27, 2024]. *Adv Clin Exp Med*. 2025. doi:10.17219/acem/182821

DOI
10.17219/acem/182821

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Abstract

Background. Lungs are the target organs most affected by ischemia/reperfusion (I/R) injury, which is exacerbated when hemorrhagic shock occurs. Suppressing various proinflammatory cytokines, inflammation and oxidation that initiate and aggravate lung damage with various drugs or methods provides significant benefits in preventing lung damage.

Objectives. This study aims to evaluate the protective effect of clotrimazole (CLT), an antimycotic drug, on lung injury and systemic inflammatory response in rats by creating an experimental model of a ruptured abdominal aortic aneurysm (RAAA).

Materials and methods. Thirty-six male Sprague Dawley rats were randomly divided into 5 groups: sham, sham+CLT, sham+polyethylene glycol (PEG), shock+ischemia/reperfusion (SIR), and SIR+CLT. Saline, CLT and PEG were administered in the sham groups without shock and I/R. The hemorrhagic shock was developed in SIR groups by drawing blood for 1 h to keep the mean arterial pressure at 50 mm Hg. After 60 min, the SIR+CLT group was given 20 mg/kg CLT; then, the aortic clamps were opened, and rats were left for 120 min of reperfusion. The blood taken to create hemorrhagic shock was returned in a controlled manner during this time. At the end of the reperfusion procedure, samples were taken for cytokine levels in serum and lung tissue and for other biochemical analyses. Blood gas, histopathological examination and wet/dry weight measurements were performed to assess lung injury.

Results. An increase was observed in all parameters in the SIR group compared to the sham group. In the SIR+CLT group, the serum myeloperoxidase (MPO), tumor necrosis factor alpha (TNF-α), lung MPO values, histologically lung injury scores, and lung tissue wet/dry ratio were decreased significantly when compared to the SIR group ($p < 0.05$).

Conclusions. These results indicate that CLT may reduce the systemic inflammatory response and lung injury due to shock and I/R in an experimental model of RAAA.

Key words: IL-6, TNF-α, hemorrhagic shock, clotrimazole, aortic ischemia/reperfusion

Background

A ruptured abdominal aortic aneurysm (RAAA) is an emergency of cardiovascular surgery today, with high mortality and morbidity rates. Although endovascular repair has reduced the mean mortality to 26.8% in the treatment of RAAA, the mortality of open surgical repair is still around 40%.[1] One of the important reasons that increase mortality after ruptured aortic aneurysm treatment is pulmonary complications such as respiratory failure, pneumonia and acute respiratory distress syndrome (ARDS).[2] Pulmonary complications, seen at a 36–41% rate, are related to blood and fluid transfusions, cross-clamping duration and systemic inflammatory response.[3] Mortality ranges from 27% to 45% in cases with ARDS, depending on the severity of the clinical picture. Unlike other ischemia/reperfusion (I/R) injuries, 2 critical clinical events occur in RAAA. One of these is hypovolemic shock, which causes systemic hypoxia in all tissues due to bleeding. The other is lower body ischemia due to cross-clamping during treatment and reperfusion injury seen afterward. After RAAA surgery, high levels of cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), which increase inflammation, are high.[4] This has been associated with ARDS and increased mortality.[5,6] Activated neutrophils accumulate in the subendothelial space, releasing reactive oxygen species (ROS) and oxidative damage.[7] Ischemia causes an increase in calcium permeability by promoting its entry into cells. The intracellular accumulation of calcium ions (Ca^{++}) because of changes in the permeability of the plasma membrane and the decrease in its active ATP-depended transport results in the activation of phospholipases and proteases.[8]

Clotrimazole (CLT), an azole antifungal drug (1-2 chlorophenyl-diphenyl methyl-1H- imidazole), is frequently used for prophylaxis and treatment of candidiosis following transplantation.[9] In addition, it is used in treating diseases such as malaria, sickle cell anemia, tuberculosis, and rheumatoid arthritis, and is effective as an antiapoptotic and immunosuppressive agent.[10] Also, it has been reported that CLT acts by inhibiting both cytochrome P450 (CYP2, CYP3A) and Ca^{2+}-linked K^+ channels.[11] These include the depleting intracellular calcium-dependent potassium channels and inhibiting voltage-dependent calcium channels in the sarcoplasmic reticulum Ca^{+2}-ATPase.[12] In this way, CLT has been shown to prevent the formation of free oxygen radicals in the organism for various reasons.[13] Some cytochrome P450 epoxygenases catalyze the formation of anti-inflammatory epoxyeicosatrienoic acids (EETs) from arachidonic acid. Epoxyeicosatrienoic acids inhibit inflammation by reducing nuclear factor-kappa B (NF-κB) activation. Thus, EETs play essential roles in various biological activities such as blood pressure regulation and cardioprotection.[14] Epoxyeicosatrienoic acids also show an anti-inflammatory effect on vascular remodeling by regulating TNF-α-induced VCAM-1 expression, therefore providing endothelial protection.[15] In addition, its anti-inflammatory and antioxidant efficacy has been shown by numerous studies. Clotrimazole also exerts anti-inflammatory effects by inhibiting NF-kB-dependent cytokines such as TNF-α and IL-8 in vivo and in vitro.[16] Clotrimazole also suppresses NOS-mediated ROS production while increasing adenosine triphosphate (ATP) production in mitochondria in I/R injury by inhibiting the entry of Ca^{2+} via transient receptor potential (TRPM2) channels.[17]

Objectives

The aim of this study was to investigate the potential anti-inflammatory and antioxidant effects of CLT in the systemic circulation and lung tissue in an experimental model of ruptured abdominal aortic aneurysm simulating hypovolemic shock and I/R.

Materials and methods

Chemicals

Clotrimazole (1-[2-Chlorophenyl] diphenyl methyl-1H-imidazole, Cas No. 23593-75-1) and PEG, the solvent of CLT (Polyethylene glycol 400, Cas No: 25322-68-3) were purchased from Sigma-Aldrich (St. Louis, USA). Ketamine hydrochloride (Ketalar, 500 mg/10 mL) was obtained from Pfizer (New York, USA), and xylazine hydrochloride (Rompun 2%) was obtained from Bayer (Leverkusen, Germany).

Animals

Thirty-six Sprague Dawley rats were used, weighing 420 ±56 g and aged 4–5 months. Before the experiment, the rats were kept in wire cages in a 12-hour light/dark cycle at 24–26°C and a relative humidity of 50 ±10%. Rats were acclimated to the environment and treated under ethical animal use and care principles. Twelve hours before the experiment, nutrition, but not water, was stopped. First, rats were randomly divided into 2 main groups: sham (Sh) and shock+ischemia/reperfusion (SIR). Sham groups were redivided into 3 groups as sh (n = 6), Sh+clotrimazole (Sh+CLT; n = 6) and Sh+polyethylene glycol (Sh+PEG; n = 6). Shock+ischemia/reperfusion groups were also divided into SIR (n = 9) and SIR+CLT (n = 9). The number of animals was determined using the power analysis method. A minimum sample size of 6 animals per group would provide the appropriate power ($1 - \beta = 0.8$) to identify significant ($\alpha = 0.05$) differences in malondialdehyde (MDA), considering large effect size d = 2.0, with a variance test. Sample size calculation was performed using inG*Power 3.1.9.2 (Kiel University, Kiel, Germany) for 2 individual comparisons between 2 groups (Sh-Sh+CLT-Sh+PEG and Sh-SIR-SIR+CLT). For SIR and

Table 1. Experimental groups and applied procedures

Groups	n	Shock 60 min	Drug 1 mL	Ischemia 60 min	Reperfusion 120 min
Sh	6	–	saline	–	–
Sh+CLT	6	–	CLT	–	–
Sh+PEG	6	–	PEG	–	–
SIR	9	+	saline	+	+
SIR+CLT	9	+	CLT	+	+

Sh – sham; CLT – clotrimazole; PEG – polyethylene glycol; SIR – shock+ischemia/reperfusion.

SIR+CLT groups, to minimize the effect of mortality, 9 rats have been included in the study. Eventually, the experiment was continued with a total of 5 groups (Table 1).

Ethical standards

This experimental study was approved by Karadeniz Technical University Animal Experiments Local Ethics Committee (approval No. 2019/33). All experiments were blinded and randomized according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines by a separate researcher who created the experimental protocol and performed the experiments at Karadeniz Technical University Surgical Practice and Research Center.[18]

Surgical procedures

In this study, we used the ruptured abdominal aortic aneurysm model in rats developed for the first time in 1995 by Lindsay et al.[19] Sham groups underwent no surgical procedure except for aortic exploration. Shock+ischemia/reperfusion groups experienced hypovolemic shock for 60 min, ischemia for 60 min and reperfusion for 120 min (Table 1). Rats were anesthetized without disrupting spontaneous breathing. For this purpose, xylazine hydrochloride was administered intramuscularly (im.) at 10 mg/kg, and ketamine hydrochloride was administered im. at a dose of 50 mg/kg and repeated when necessary. A catheter was inserted through the right carotid artery for arterial pressure monitoring. The left internal jugular vein was catheterized for blood and fluid replacement (Novacath; Medipro, Los Angeles, USA). During the experiment, the rats' blood pressure, heart rate, rectal temperature, and respiratory rate were monitored (Nikon Kohden BSM-4113; Nikon Corp., Tokyo, Japan). The rectal temperature was kept around 36.5°C using a heat lamp. For insensible fluid losses, saline (0.9% NaCl) was infused at 3 mL/kg/h (Perfusor Compact S; Brown, Melsungen, Germany). Blood was drawn from the carotid artery cannula using a heparinized plastic syringe until mean arterial pressure (MAP) decreased to 50 mm Hg to induce hypovolemic shock in the SIR groups. The amount of blood drawn was less than 30% of the total blood volume. At the 45 min of hemorrhagic shock and its equivalent duration, 1 mL of saline was given to the rats in the Sh and SIR groups, 1 mL of PEG to the Sh+PEG group, and 30 mg/kg of CLT to the Sh+CLT and SIR+CLT groups intraperitoneally (ip.), and laparotomy and aortic exploration was performed at the 60 min. After 60 min of hypovolemic shock, systemic heparinization was performed. The abdominal aorta was then clamped in 2 places, above the supramesenteric and iliac bifurcation, resulting in lower torso ischemia. Half of the blood drawn from the rats was reinfused into the jugular vein. Thus, surgical treatment and resuscitation were simulated. Once the aortic clamps were opened, the abdomen was closed using a 5/0 prolene suture, and rats were allowed 120 min of reperfusion. The remaining blood was reinfused, Ringer's lactate solution was given when needed, and MAP was maintained at 100 mm Hg.

Sample preparation

At the end of the reperfusion, all rats were euthanized by exsanguination. Arterial blood samples were taken for blood gas and biochemical analysis. Lung tissues were removed, part of the left lung was frozen at –80°C for biochemical analysis, and the remainder was fixed in formaldehyde for histopathological examinations. The right lung was reserved for wet/dry weight measurements.

Biochemical analysis

The amount of MDA in rat blood samples was measured using the thiobarbituric acid reactive substance (TBARS) method developed by Yagi in 1984.[20] As a result of the measurement, plasma MDA amounts were calculated as nanomoles per milliliter (nmol/mL). Serum MPO levels were determined using the enzyme-linked immunosorbent assay (ELISA) kit (cat. No. BMS622; Bender MedSystems, Vienna, Austria). Results are shown in nanograms per milliliter (ng/mL). Serum TNF-α levels were determined using the same ELISA kit. Results are given in picogram per milliliter (pg/mL). Serum IL-6 levels were also determined using the same ELISA kit. Results are given in picogram per milliliter (pg/mL). Ischemia-modified albumin (IMA) measurement was evaluated with the rapid and colorimetric determination method developed by Bar et al.[21] Results are reported in absorbance units (ABSU). At the end of the experiment, blood was taken from the heparinized syringe

via the carotid artery and transferred to the cartridge, and blood gases were measured (IRMA TruPoint Blood Analysis System, Guangzhou, China). A piece of lung tissue was homogenized, and MDA levels in tissues were studied with the thiobarbituric acid method developed by Mihara and Uchiyama in 1978.[22] Uchiyama Tissue MDA levels were expressed as nmol MDA/g wet tissue. Tissue MPO levels were determined using the ELISA kit (cat. No. HK105; Hycult Biotech, Uden, the Netherlands). The results are given in ng/mL per gram of wet tissue.

For lung wet/dry weight measurements, the right lung was separated from other tissues and weighed on a microbalance. After 48 h at 70°C, it was weighed again. The wet/dry ratio was calculated, and the increase was interpreted in favor of pulmonary edema.

Histopathology

For histopathological examination, the tissues were processed employing a standard paraffin-embedded technique. Then, the sections prepared this way were stained with hematoxylin and eosin (H&E). An experienced histologist, unaware of the study groups, performed a histopathological evaluation. In the damage scoring of lung tissues, 5 different areas at high magnification (×400) (Olympus Bx51; Olympus Corp., Tokyo, Japan) in the lung preparation of each group were evaluated semi-quantitatively according to the criteria described below. Microscopic damage scoring was performed on a 4-grade scale: Grade 0. Normal lung morphology: Grade 1. Mild intra-alveolar edema and mild inflammatory cell infiltration: Grade 2. Moderate alveolar edema and moderate inflammatory cell infiltration: Grade 3. Severe alveolar edema, severe inflammatory cell infiltration, and focal hemorrhage: Grade 4. Diffuse inflammatory cell infiltration and alveolar damage.

Statistical analyses

Statistical analysis was performed using the IBM SPSS v. 23.0 software (IBM Corp., Armonk, USA). The levels of serum biomarkers, arterial blood and lung tissue findings have been presented as median and interquartile ranges (IQRs). The statistical significance of the levels of serum biomarkers, arterial blood, and lung tissue findings has been tested within Sham – sham+CLT – sham+PEG and sham – SIR – SIR+CLT with the Kruskal–Wallis test. Post hoc analysis within subgroups was tested with the Dunn's test and Bonferroni adjustment. Due to the small number of samples, nonparametric methods were chosen. Test statistics, degrees of freedom (df) values, p-values and adjusted p-values have been given in the results section. The levels of parameters among groups have also been shown via boxplot graphics with data points. A p-value under 0.05 indicates statistical significance.

Results

Biochemical results in blood serum are shown in Table 2 and Fig. 1. One of the reactions caused by free oxygen radicals is lipid peroxidation, and one of the end products of lipid peroxidation is MDA.

There was no statistically significant differences between levels of serum MDA across groups of Sh-Sh+PEG and Sh+CLT ($v = 0.495$, $df = 2$, $p = 0.781$), and Sh, SIR and SIR+CLT (Kruskal-Wallis test H-statistic = 4.43, $df = 2$, $p = 0.109$).

Measuring serum MPO values gives information about neutrophil activation. There was a significant difference across groups of Sh, Sh+PEG and Sh+CLT (Kruskal–Wallis test H-statistic = 9.579, $df = 2$, $p < 0.01$), and Sh, SIR and SIR+CLT (Kruskal–Wallis test H-statistic = 20.250, $df = 2$, $p < 0.001$). Serum MPO levels were significantly higher in the Sh+CLT group than in the Sh+PEG group (Dunn's test statistic = 9.500, adj. $p = 0.006$). Serum MPO levels were also significantly higher in the SIR group compared to Sh (Dunn's test statistic = –16.500, adj. $p < 0.001$) and SIR+CLT groups (Dunn's test statistic = 9.500, $p < 0.03$).

There were no statistically significant differences between the levels of serum TNF-α across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 4.643, $df = 2$; $p = 0.098$), but there was a significant difference between Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 13.302, $df = 2$; $p = 0.001$). The TNF-α

Table 2. Serum biochemical parameters in all groups

Parameters median (IQR)	Sh (n = 6)	Sh+CLT (n = 6)	Sh+PEG (n = 6)	SIR (n = 9)	SIR+CLT (n = 9)
MDA [nmol/mL]	1.54 (0.26)	1.41 (0.60)	1.57 (0.51)	2.11 (2.97)	1.76 (0.87)
MPO [ng/mL]	83.72 (6.52)	87.30 (6.91)[a]	75.13 (11.66)[a]	397.63 (40.81)[b]	179.65 (50.74)[b]
TNF-α [pg/mL]	154.28 (13.08)	155.16 (24.29)	167.85 (18.45)	260.83 (77.07)[b]	221.21 (61.59)[b]
IL-6 [pg/mL]	163.25 (40.56)	143.17 (49.62)	158.86 (55.20)	189.67 (102.85)	179.61 (41.37)
IMA [ABSU]	0.61 (0.34)	0.84 (0.42)	0.69 (0.48)	0.98 (0.74)[c]	0.90 (0.09)

MDA – malondialdehyde; MPO – myeloperoxidase; TNF-α – tumor necrosis factor alpha; IL-6 – interleukin 6; IMA – ischemia-modified albumin; Sh – sham; CLT – clotrimazole; PEG – polyethylene glycol; SIR – shock+ischemia/reperfusion. Values are given as median (interquartile range (IQR)). The Kruskal–Wallis test was used for the analysis. Post hoc analysis within subgroups was tested with the Dunn's test and Bonferroni adjustment; [a] $p < 0.05$ compared the results between between Sh+CLT and Sh+PEG; [b] $p < 0.05$ compared the results between Sh – SIR and SIR – SIR+CLT; [c] $p < 0.05$ compared the results between Sh and SIR.

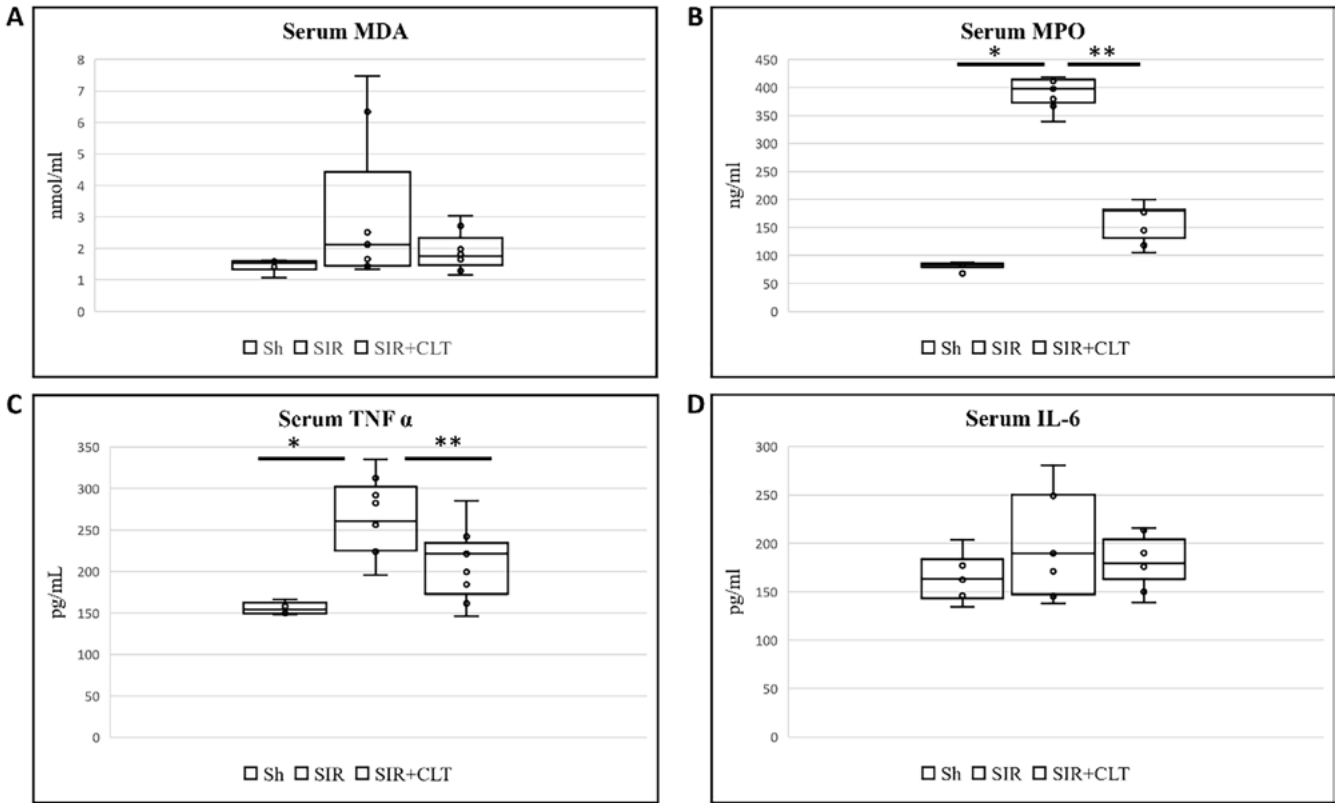

Fig. 1. A. MDA – serum malondialdehyde; B. MPO – serum myeloperoxidase; C. TNF-α –serum tumor necrosis factor alpha; D. IL-6 – serum interleukin 6; Sh – sham; SIR –shock+ischemia/reperfusion; SIR+CLT – SIR+clotrimazole. Boxes show median and interquartile range (IQR) (25–75 percentiles), and the end of whiskers show min–max values; data are presented using the Kruskal–Wallis test. Post hoc analysis within subgroups was assessed with the Dunn's test and Bonferroni adjustment

* $p < 0.001$ for serum MPO and $p = 0.001$ for serum TNF-α when comparing Sh and SIR groups; ** $p = 0.021$ for serum MPO and $p = 0.003$ for serum when TNF-α when comparing SIR and SIR+CLT groups.

values were significantly higher in the SIR group compared to the Sh (Dunn's test statistic = –13.556; adj. $p < 0.001$) and SIR+CLT groups (Dunn's test statistic = 12.400; $p < 0.003$).

There were no statistically significant differences between the levels of serum IL-6 across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 0.246, df = 2; $p = 0.884$), and Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 1.960, df = 2; $p = 0.375$).

Ischemia-modified albumin is a marker that shows tissue hypoxia. It is sensitive to ischemia but not specific to any particular tissue. There were no statistically significant differences between the levels of serum IMA across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 0.667, df = 2; $p = 0.717$), but there was a significant difference between Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 6.529, df = 2; $p < 0.04$). Serum IMA was higher among the SIR group than the Sh group (Dunn's test statistic = –9.333; adj. $p < 0.04$).

Upon examination of arterial blood gases, it was observed that both metabolic and respiratory acidosis developed in the SIR and SIR+CLT groups, in comparison to the sham groups (Table 3).

There were no statistically significant differences between the levels of PH across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test statistics = 2.783, df = 2; $p = 0.249$). Still, there was a significant difference between Sh, SIR and SIR+CLT (Kruskal–Wallis test H-statistic = 14.537, df = 2; $p < 0.001$). The PH in SIR group was significantly lower than in the Sh (Dunn's test statistic = 13.722; adj. $p < 0.001$) and SIR+CLT groups (Dunn's test statistic = –8.333; adj. $p < 0.04$).

There were no statistically significant differences between the levels of PaO_2 across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 2.321, df = 2; $p = 0.994$), and Sh and SIR groups. There were no statistically significant differences in the level of $PaCO_2$ across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 0.012, df = 2; $p = 0.313$), and across Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 1.913, df = 2; $p = 0.384$).

There were no statistically significant differences regarding the level of HCO_3 across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 0.672, df = 2; $p = 0.714$), but there was a significant difference between Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 12.742, df = 2; $p = 0.002$). The HCO_3 in the SIR group was significantly lower than in the Sh group (Dunn's test statistic = 13.278; adj. $p = 0.001$).

Table 3. Arterial blood gas values in all groups

Parameters median (IQR)	Sh (n = 6)	Sh+CLT (n = 6)	Sh+PEG (n = 6)	SIR (n = 9)	SIR+CLT (n = 9)
pH	7.37 (0.05)	7.40 (0.17)	7.43 (0.07)	7.26 (0.18)[a]	7.34 (0.06)
PaO_2	99.60 (25.00)	90.95 (38.70)	103.90 (13.80)	104.00 (20.10)	115.40 (37.40)
$PaCO_2$	41.75 (9.30)	44.05 (21.00)	42.20 (13.70)	37.00 (10.70)	38.00 (15.20)
HCO_3 [mEq/L]	25.75 (2.40)	27.00 (11.50)	25.30 (7.60)	13.60 (8.00)[b]	19.40 (50.30)
BE [mmol/L]	0.90 (2.30)	2.30 (3.30)	1.15 (3.00)	–13.30 (9.80)[b]	–6.00 (6.60)

PaO_2 – arterial partial oxygen pressure; $PaCO_2$ – arterial partial pressure of carbon dioxide; HCO_3 – bicarbonate; BE – base excess; Sh – sham; CLT – clotrimazole; PEG – polyethylene glycol; SIR – shock+ischemia/reperfusion. Values are given as median (interquartile range (IQR)). Data are presented using the Kruskal–Wallis test. Post hoc analysis within subgroups was tested with the Dunn's test and Bonferroni adjustment; [a] $p < 0.05$ compared the results between SIR – Sh and SIR – SIR+CLT, [b] $p < 0.05$ compared the results between Sh and SIR.

Table 4. Lung tissue findings in all groups

Parameters median (IQR)	Sh (n = 6)	Sh+CLT (n = 6)	Sh+PEG (n = 6)	SIR (n = 9)	SIR+CLT (n = 9)
MDA [nmol/mL]	465.59 (70.56)[a]	455.37 (82.04)	479.95 (137.54)	631.81 (95.15)	582.47 (83.10)
MPO [ng/mL]	5361.32 (703.77)	5780.96 (538.36)	5273.26 (264.63)	6626.55 (812.59)[b]	5659.79 (857.31)
Wet/dry weight ratio	2.48 (0.37)	2.26 (0.32)	2.30 (0.64)	5.47 (1.08)[b]	3.76 (0.87)[b]
Lung injury score	0.50 (1.00)	1.00 (1.00)[c]	1.00 (0.00)	3.00 (1.00)[d]	2.00 (2.00)

MDA – malondialdehyde; MPO – myeloperoxidase; Sh – sham; CLT – clotrimazole; PEG – polyethylene glycol; SIR – shock+ischemia/reperfusion. Values are given as median (interquartile range (IQR)). Data are presented using the Kruskal–Wallis test. Post hoc analysis within subgroups was tested with the Dunn's test and Bonferroni adjustment. [a] $p < 0.05$ compared the results between Sh – SIR and Sh – SIR+CLT; [b] $p < 0.05$ compared the results between SIR-Sh and SIR-SIR+CLT; [c] $p < 0.05$ compared the results between Sh and Sh+CLT; [d] $p < 0.05$ compared the results between Sh and SIR.

There were no statistically significant differences concerning base excess (BE) levels across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 1.770, df = 2; p = 0.413). Still, there was a significant difference between Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 15.676, df = 2; p < 0.001). The BE in the SIR group was significantly lower than in the Sh group (Dunn's test statistic = 14.677; adj. p < 0.001).

Lung tissue

Biochemical results, lung injury score and wet/dry ratio in lung tissue are shown in Table 4 and Fig. 2.

There were no statistically significant differences between the levels of lung tissue MDA across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 0.152, df = 2; p = 0.927), but there was a significant difference between Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 13.548, df = 2; p < 0.001). The lung tissue MDA in the Sh group was significantly lower than in the SIR group (Dunn's test statistic = –13.278; adj. p < 0.001) and in the SIR+CLT group (Dunn's test statistic = –10.722; adj. p < 0.02). There were no statistically significant differences between the levels of lung tissue MPO across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 2.889, df = 2; p = 0.236) but there was a significant difference between Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 11.382, df = 2; p = 0.003). The lung tissue MPO in the SIR group was significantly higher than in the Sh group (Dunn's test statistic = –11.333; adj. p = 0.007) and in the SIR+CLT group (Dunn's test statistic = 8.889; adj. p < 0.03).

Histopathological findings of lung injury in the experimental groups are presented in Fig. 3. Diffuse alveolar edema, intra-alveolar and inter-alveolar hemorrhage, and neutrophil infiltration were observed in the SIR group. There were significant differences between the levels of lung injury scores across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 6.611, df = 2; p = 0.037), and Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 13.158, df = 2; p < 0.001). The lung injury scores in the Sh+CLT group (Dunn's test statistic = –6.250; adj. p = 0.045) and in the SIR group (Dunn's test statistic = –13.056; adj. p < 0.001) were significantly higher than in the Sh and SIR+CLT groups (p = 0.011). There were no statistically significant differences between the levels of weight/dry weight ratio across Sh, Sh+PEG and Sh+CLT groups (Kruskal–Wallis test H-statistic = 2.279, df = 2; p = 0.320), but there was a significant difference between Sh, SIR and SIR+CLT groups (Kruskal–Wallis test H-statistic = 19.604, df = 2; p < 0.001). The weight/dry weight ratio in the SIR group was significantly higher than in the Sh group (Dunn's test statistic = –16.222; adj. p < 0.001) and in the SIR+CLT group (Dunn's test statistic = 8.889; adj. p < 0.03).

Discussion

The surgical treatment of RAAA involves 2 essential processes. The 1st is the "hypovolemic shock" phase caused

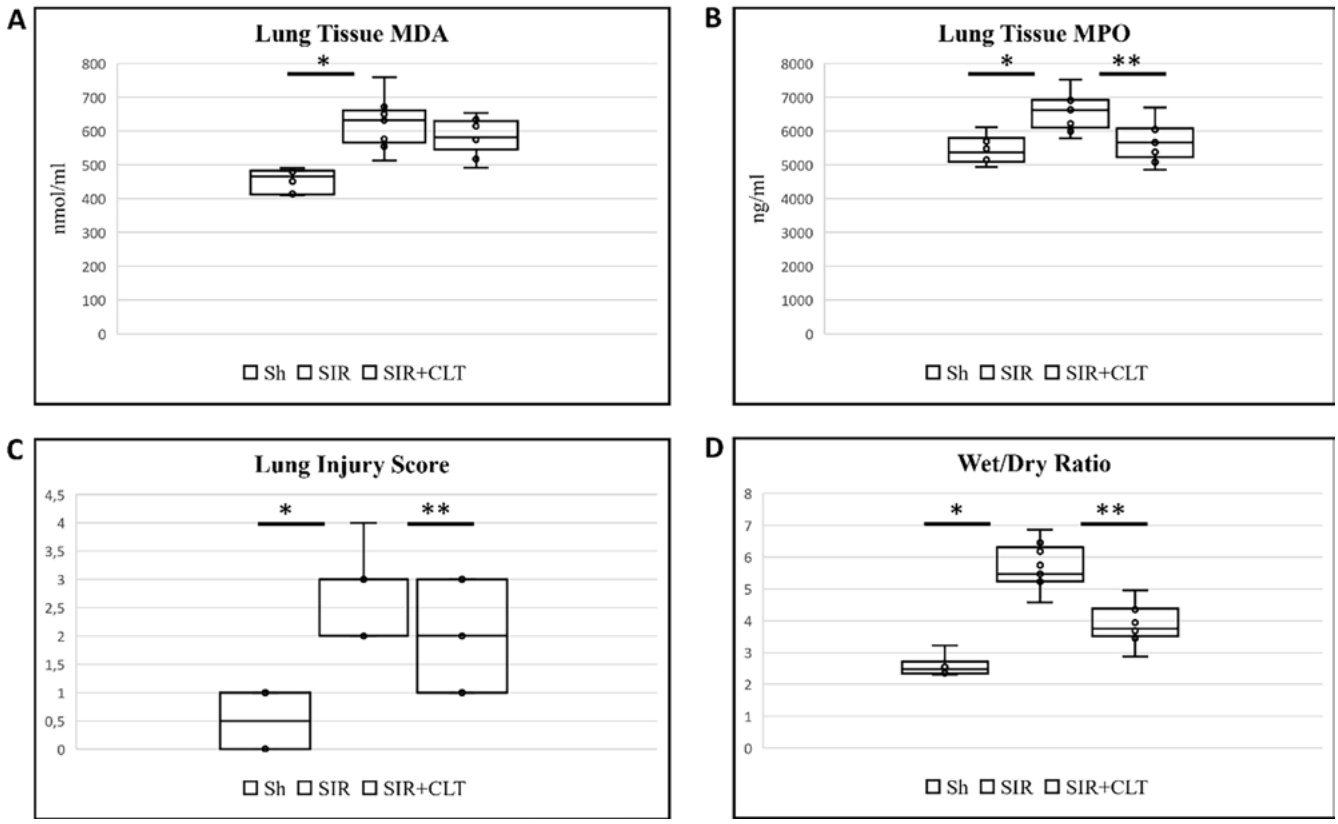

Fig. 2. A. MDA – lung tissue malondialdehyde; B. MPO – lung tissue myeloperoxidase; C. lung injury score; D. Wet/dry ratio in sham (Sh), shock+ischemia/reperfusion (SIR) and SIR+clotrimazole (SIR+CLT) groups. Boxes show median and interquartile range (IQR) (25–75 percentiles), and the end of whiskers show min–max values; data are presented using the Kruskal–Wallis test. Post hoc analysis within subgroups was evaluated with the Dunn's test and Bonferroni adjustment

*p = 0.001 for lung tissue MDA; *p = 0.007 for lung tissue MPO; *p = 0.001 for lung injury score, and *p < 0.001 for wet/dry ratio when comparing Sh and SIR groups; **p = 0.023 for lung tissue MPO; **p = 0.011 for lung injury score; **p = 0.021 for wet/dry ratio when comparing SIR and SIR+CLT groups.

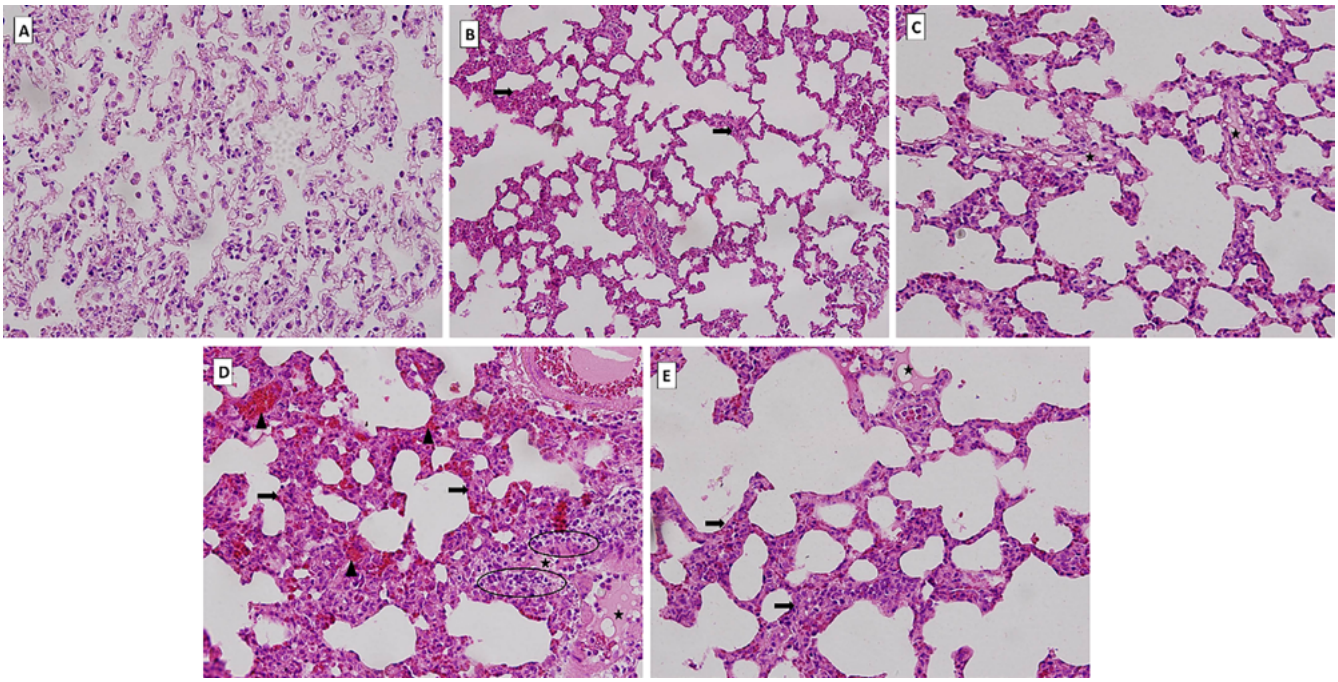

Fig. 3. Histopathological changes of lung tissues in groups. A. Sham (Sh) group; B. Sham+ clotrimazole (Sh+CLT) group; C. Sham+polyethylene glycol (Sh+PEG) group; D. Shock+ischemia/reperfusion (SIR) group; E. SIR+CLT group. Normal histopathological finding in the Sh group. Localized thickening of the alveolar epithelium (arrow) in the Sh+CLT group. There is a small amount of interalveolar edema (star) in the Sh+PEG group. Diffuse alveolar edema (star), intra-alveolar and inter-alveolar hemorrhage (arrowhead), neutrophil infiltration (ellipse), and epithelial thickening (arrow) in the SIR group. Moderate intraalveolar edema (star) and alveolar epithelial thickening (arrow) in the SIR+CLT group (hematoxylin & eosin (H&E) ×200).

by aortic rupture, resulting in total body hypoperfusion. The 2nd is the "clamp" phase, which is the most critical part of the surgical treatment and leads to lower body ischemia.[19] Temporary clamping of the aorta or its major visceral branches at the infradiaphragmatic segment can cause damage to organs that are directly exposed to ischemia, including the bowel and kidney, and to the distant target organs, particularly the lungs.[7] Total body hypoperfusion due to hemorrhagic shock or lower body ischemia due to aortic clamping alone does not cause sufficient organ damage and increased mortality.[6,23–25] Due to these 2 crucial ischemic conditions and subsequent reperfusion in RAAA operations, mortality remains high, and multiple organ failures occur.[5,7] Although many physiopathological factors that cause shock and I/R injury are considered, the main factor is free oxygen radicals that appear in the reperfusion phase. The main source of free radicals that are held responsible for reperfusion injury are neutrophils that are activated both in shock and in ischemia and reperfusion phases.[6,8,26] The systemic inflammatory effects of neutrophils cause varying degrees of damage to many organs and tissues.[7] The lungs have a very capacious endothelial surface due to the pulmonary capillary bed. After reperfusion of ischemic tissues, the pulmonary capillary bed acts as a filter. Platelet microaggregates formed during vascular stasis are retained in the lungs after reperfusion. Following the adhesion of neutrophils to the lung endothelium, proteolytic enzymes and free oxygen radicals are released, and lung damage develops accordingly.[27,28] The severity of lung failure varies depending on the duration, severity of the hemorrhagic shock and the patient's lung reserves.

In our study, we examined lung injury and the effect of CLT on this injury in the RAAA model, where shock and ischemia/reperfusion can be simulated together. We determined serum MDA, MPO, TNF-α, IMA levels, and lung tissue MDA and MPO levels. In addition to biochemical analyses, we evaluated the lung damage by examining the lung tissue histopathologically; we also examined the rats for pulmonary edema using wet/dry weight ratios calculation.

Serum and lung tissue MDA values are an important test showing lipid peroxidation and, therefore, the presence of free oxygen radicals. Although lung damage and lipid peroxidation occurred in the SIR group, the MDA value change was insignificant. Usul et al. found a significant decrease in MDA values in the medulla spinalis tissue after aortic I/R in rats given CLT.[29] We used CLT in similar doses, but both our experimental model and the tissues we examined were different. In our model, more oxygen radicals have been formed, and the drug might have failed to suppress this, or the transition of CLT to the medulla spinalis tissue might have been different. This suggests additional experimental work is needed in this regard.

Clotrimazole suppresses neutrophil activation in the systemic circulation and lung tissue, the most crucial distant organ. Thapa et al. stated that in the experimental colitis model, tissue MPO activity decreased in the CLT group and approached the control group at a dose of 50 mg/kg compared to 10 mg/kg.[16] Cekic et al. showed that the concentration of MPO in the lung and pancreas tissue of rats with necrotizing pancreatitis decreased significantly in the CLT group.[30] Wilson et al. showed that CLT reduces neutrophil cell infiltration in candida-infected vaginal cell cultures, thus inhibiting the production of proinflammatory cytokines, especially IL-8.[31] There is no evidence that CLT suppresses neutrophil activation in both the systemic circulation and the distant organ. Our study is the first in this regard.

Tumor necrosis factor alpha is a proinflammatory cytokine and a potent chemoattractant. Liu et al. showed that transmembrane peripheral blood leukocytes inhibit TNF-α in lung damage caused by lipopolysaccharide and function as a protective agent through its anti-inflammatory effect.[32] In our study, TNF-α values decreased significantly in the SIR+CLT group compared to the SIR group. This showed that CLT significantly suppresses the systemic inflammatory response. Our findings are compatible with other studies. Thapa et al. showed that CLT inhibited the NF-κB-dependent cytokines TNF-α and IL-8 in vivo and in vitro, ameliorating inflammation and abnormal angiogenesis in an experimental colitis model.[16] They also demonstrated that CLT dose-dependently inhibited the expression of TNF-α-induced adhesion molecules and angiogenesis in vivo in the chorioallantoic membrane (CAM) model, inhibiting ROS production and NF-kB translocation, thereby reducing inflammation in CAM tissue.[33] In an in vitro study with cardiac myocytes, Roberge et al. showed that CLT inhibits TNF-α and prevents the death of cardiomyocyte cells by inhibiting the TRPM2 channel.[34] TRPM2 is a Ca^{2+}-permeable cationic channel of the transient receptor potential (TRP) superfamily linked to apoptotic signaling.[35]

Studies have shown that IMA increases due to hypoxia or I/R, sepsis, acute infection, and advanced cancer.[36,37] However, in our research, the IMA value did not decrease significantly in the group receiving CLT. This is the same characteristic as serum MDA values. With these findings, we can conclude that CLT does not show a prominent antioxidant property.

Interleukin 6 is a soluble mediator with pleiotropic effects on inflammation, immune response and hematopoiesis.[38] It initiates and stimulates oxidation in neutrophils and increases ICAM-1 release from endothelial cells and endothelial permeability. Shields and Waldow et al. used IL-6 as a marker in their experimental studies and showed the changes in IL-6 levels with the methods they applied.[39,40] Harkin et al. demonstrated the severity of lung injury with IL-6 levels in a similar experimental study.[23] Swartbol et al. demonstrated that IL-6 reaches peak values between 6 h and 7 days after surgery in different case series during surgical and endovascular treatment of non-RAAAs and RAAAs.[41] Makar et al. found that IL-6 levels were significantly higher in patients with RAAA who

underwent open surgery than those who received endovascular therapy. They showed that they decreased after the 5th day in both groups.[42] The fact that IL-6 levels did not decrease significantly in the SIR+CLT group in our study can be explained by the higher release of IL-6 in the reperfusion phase. The 2-hour reperfusion time in our study may have needed to be increased to show this change.

In the blood gas analysis, significant metabolic and respiratory acidosis developed in the SIR groups. Confirming significant lung damage, this unfortunately did not improve significantly in CLT-treated rats. This situation can also be explained by the spontaneous breathing of the rats and the lack of use of buffering drugs. Also, the reperfusion time of 120 min might have been adequate. Clotrimazole is known to be a CYP2 and TRPM2 channel inhibitor. Shimizu et al. demonstrated the protective effect of CLT as a specific TRMP2 channel inhibitor in experimental focal cerebral ischemia.[43] Studies showing the efficacy of CLT in shock or I/R are needed.

According to our results, when the groups were compared regarding parameters such as histopathological samples, lung injury score and wet/dry weight ratio, the damage that can lead to ARDS in the postoperative period in patients with RAAA, such as alveolar edema, epithelial thickening, alveolar hemorrhage, and leukocyte infiltration seen in the SIR group, was significantly reduced by CLT. Unfortunately, we could not find any other in vivo experimental shock or I/R study that investigated the inhibitory effect of CLT to compare with our findings. The effect of CLT on EETs catalyzed by cytochrome P450 epoxygenases has not been investigated. Examining this pathway may be a good step to see the effects of CLT on blood pressure, pulmonary vascular bed and endothelial functions.[15]

Limitations

In this study, we tried to investigate the protective effect of CLT on lung injury of shock and I/R through inflammation, oxidation and NF-kB-dependent cytokine pathways. It will be more enlightening to show whether CYP2 and TRPM2 channel inhibition are effective in the activity of CLT.

Conclusions

In our study, significant tissue damage occurred in the RAAA model in which hemorrhagic shock, aortic ischemia and reperfusion were simulated together, resulting in substantial pulmonary edema. This damage was significantly reduced when treated with CLT. The administration of CLT provided effective suppression of both systemic and local inflammatory responses, as well as neutrophil infiltration, thereby mitigating lung damage in a rat model of experimental RAAA.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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