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Research Article

Clopidogrel Attenuates Sepsis-Induced Acute Lung Injury and Is Associated With Increased Pulmonary FOXP3 Expression in Rats

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Abstract

Objective: Acute lung injury (ALI) resulting from sepsis and acute respiratory distress syndrome (ARDS) are leading causes of mortality and disability in critically ill patients. Clopidogrel, a P2Y12 receptor antagonist, has demonstrated potential anti-inflammatory and antioxidant effects alongside its antiplatelet activity. This study aimed to investigate the protective effects of clopidogrel against cecal ligation and puncture (CLP)-induced ALI and to evaluate the association between clopidogrel treatment and pulmonary FOXP3 expression, systemic inflammation, and oxidative stress. **Methods:** A total of 36 female Wistar albino rats were divided into three groups: control (n = 12), CLP+Saline (n = 12), and CLP+Clopidogrel (n = 12; 10 mg/kg/day). The CLP model was used to induce sepsis. Biochemical analyses included measurements of plasma malondialdehyde (MDA) and tumor necrosis factor-alpha (TNF-α) levels, as well as FOXP3 expression in lung tissue. Histopathological evaluations assessed lung injury parameters, including alveolar congestion (AC) and septal thickening. Arterial blood gas parameters (partial pressure of arterial oxygen (PaO_2) and partial pressure of arterial carbon dioxide ($PaCO_2$)) were also analyzed. **Results:** Rats in the CLP+Saline group showed significantly increased plasma MDA and TNF-α levels ($p < 0.001$) and decreased pulmonary FOXP3 levels ($p < 0.05$) compared with the Normal Control group. Clopidogrel treatment significantly reduced plasma MDA and TNF-α levels ($p < 0.001$) and partially restored pulmonary FOXP3 levels ($p < 0.05$) compared with the CLP+Saline group. Histopathological examination revealed reduced leukocyte infiltration, AC, edema, hemorrhage, and alveolar septal thickening in animals treated with clopidogrel. Arterial PaO_2 was significantly higher in the CLP+Clopidogrel group than in the CLP+Saline group ($p < 0.01$), whereas the difference in $PaCO_2$ between the groups was not statistically significant. **Conclusion:** Clopidogrel attenuated CLP-induced ALI, as evidenced by reduced plasma TNF-α and MDA levels, improved lung histopathology and arterial oxygenation, and partial restoration of pulmonary FOXP3 expression. Further mechanistic studies are required to clarify the pathways underlying these effects.

Keywords: sepsis; acute lung injury; clopidogrel; forkhead transcription factors; oxidative stress; tumor necrosis factor-alpha

1. Introduction

Severe organ failure and high death rates are hallmarks of sepsis, a potentially fatal disease caused by an uncontrolled immune response to infection. Mortality rates in intensive care units (ICUs) may range from 30% to 50% due to this syndrome, making it one of the leading causes of death in these settings [1,2]. The enormous global burden of sepsis is shown by the fact that it affects over 19 million people every year [3,4].

Both acute lung injury (ALI) and its severe variation, acute respiratory distress syndrome (ARDS), are critically harmful sepsis complications. Multiple organ dysfunction syndrome (MODS) often begins in septic individuals with pulmonary impairment. ALI and ARDS are major causes of death and disability in ICUs [5,6]. The activation of neutrophils and macrophages, leading to damage to the alveolar epithelium and pulmonary capillary endothelium, is the pathophysiological characteristic of ALI. These immune cells worsen pulmonary inflammation and prolong tissue destruction by releasing pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukins (ILs), and reactive oxygen species (ROS) [7]. Protein leakage, alveolar-capillary barrier breakdown, oxidative stress, and leukocyte infiltration are the underlying processes [8,9,10].

As of right now, ALI therapy is mostly supportive, and there are no targeted medicines for the condition. The foundation of supportive care, mechanical ventilation, unknowingly adds to ventilator-induced lung injury (VILI) by subjecting the lungs to excessive mechanical stress, which in turn increases inflammation and damage [11]. Reducing the impact of sepsis-related ALI requires the creation of pharmaceuticals with strong anti-inflammatory and antioxidant capabilities.

To reduce myocardial infarction (MI) and stroke in patients with acute coronary syndromes (ACS) and pe-

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ripheral arterial disease, the antiplatelet effects of the oral P2Y12 receptor antagonist clopidogrel have been well established [12]. Clopidogrel is most often used to treat cardiovascular disease, but new research shows it may have pleiotropic benefits that include lowering inflammation and modifying immunological responses as well. Neutrophil activation and ROS generation represent important mechanisms involved in the pathogenesis of ALI, and modulation of platelet signaling may influence these inflammatory processes [13]. FOXP3-expressing regulatory T cells (Tregs) are important regulators of immune homeostasis and inflammatory responses, providing a biological rationale for examining pulmonary FOXP3 expression in sepsis-associated inflammation [14].

Studying the anti-inflammatory effects of clopidogrel in sepsis-induced lung damage is an exciting new direction for ALI research, especially since there are currently no specific treatments for this condition. The current research tests the theory that clopidogrel reduces ALI in a rat model of sepsis caused by cecal ligation and puncture (CLP) via regulating the FOXP3 pathway, decreasing oxidative stress, and suppressing pro-inflammatory cytokines. The results hope to open the door to new treatments for this terrible syndrome by filling in knowledge gaps in sepsis-related ALI.

2. Materials and Methods

2.1 Animals

The 36 female Wistar albino rats used in this investigation ranged in weight from 200 to 250 g. The study was carried out in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) 2.0 guidelines and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The Demiroğlu Bilim University Animal Ethics Committee gave its approval to the research (Ethical Approval Number: 1524105819) (Clinical trial number: not applicable). The rats were obtained from the Demiroğlu Bilim University Experimental Animal Laboratory. In their steel cages, which were kept at a regulated temperature of 22 ± 2 °C and followed a 12-h light/dark cycle, the animals were given unlimited access to food and kept in pairs.

2.2 Experimental Procedures

A total of 36 rats were randomly allocated to three experimental groups (n = 12 per group). Twenty-four rats assigned to the two sepsis groups underwent CLP, whereas animals in the Normal Control group did not undergo surgery or sham laparotomy. The experimental groups were as follows:

Group 1 (Normal Control group, n = 12): Animals received no surgical intervention.

Group 2 (CLP+Saline group, n = 12): Animals underwent the CLP procedure and received sterile 0.9% NaCl intraperitoneally at an equivalent injection volume and according to the same administration schedule used for the clopidogrel-treated group. Thus, this group served as the vehicle-treated septic control group.

Group 3 (CLP+Clopidogrel group, n = 12): Animals underwent the CLP procedure and received clopidogrel bisulfate (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. PHR1431) at a dose of 10 mg/kg intraperitoneally once daily. Clopidogrel was freshly suspended in sterile 0.9% NaCl immediately before administration at a concentration of 10 mg/mL and was administered at an injection volume of 1 mL/kg. The first dose was administered immediately after completion of the CLP procedure, representing an early post-CLP intervention rather than pretreatment, and treatment was subsequently continued once daily throughout the five-day experimental period. The intraperitoneal route was selected to provide consistent systemic drug administration and to minimize variability related to oral intake and gastrointestinal absorption during sepsis. The dose of 10 mg/kg/day was selected based on previous experimental evidence using P2Y12 inhibition in rat models of systemic inflammation, including a CLP-induced polymicrobial sepsis model in which clopidogrel at this dose attenuated platelet activation and inflammasome-associated inflammatory responses [15].

Postoperative fluid resuscitation was administered separately from the experimental drug/vehicle injections. Both CLP groups received 0.9% NaCl for fluid replacement, consisting of an initial dose of 30 mL/kg within the first hour after surgery, followed by 10 mL/kg/day divided into two equal doses administered every 12 h. The unoperated Normal Control group did not receive postoperative fluid resuscitation.

All surgical procedures were performed under general anesthesia induced by intraperitoneal ketamine (75 mg/kg) and xylazine (10 mg/kg). The experimental period lasted five days. During this period, five animals in the CLP+Saline group and three animals in the CLP+Clopidogrel group died before the scheduled day-5 endpoint, whereas no deaths occurred in the Normal Control group. Animals that died before the scheduled endpoint were not included in the terminal biochemical, histopathological, or arterial blood gas analyses. Consequently, the final numbers of animals available for terminal analyses were n = 12 in the Normal Control group, n = 7 in the CLP+Saline group, and n = 9 in the CLP+Clopidogrel group.

2.3 Perioperative Care, Monitoring, and Humane Endpoints

Postoperative analgesia was provided with buprenorphine (0.05 mg/kg, s.c.) every 12 h during the first 48 h after CLP. No antibiotics were administered during the experimental period. During the first 24 h following CLP, animals were monitored at least every 6 h and subsequently at least twice daily until the scheduled day-5 endpoint. Monitoring included general activity, posture, grooming, respi-

ratory pattern, food and water intake, wound condition, responsiveness to external stimuli, and clinical signs of pain or distress. Prespecified humane endpoints included persistent inability to ambulate or access food and water, severe respiratory distress, marked loss of responsiveness to external stimuli, moribund appearance, or other signs of severe and irreversible distress. Animals reaching a humane endpoint were euthanized under deep ketamine/xylazine anesthesia according to the approved experimental protocol.

On day 5, surviving animals were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg), administered intraperitoneally (stock concentrations: ketamine, 100 mg/mL; xylazine, 20 mg/mL). Adequate anesthetic depth was assessed by absence of the pedal withdrawal reflex. Arterial blood was collected from the carotid artery for blood-gas analysis while the animals were breathing spontaneously under room-air conditions. Subsequently, while the animals remained deeply anesthetized, terminal blood collection and euthanasia were performed by exsanguination through cardiac puncture using a 21-gauge needle attached to a syringe, with slow withdrawal of blood. Death was confirmed by cessation of spontaneous respiration and absence of heartbeat. Collected blood and subsequently harvested lung tissues were used for biochemical and histopathological analyses.

2.4 CLP Procedure

To induce sepsis, a sterile 3 cm midline laparotomy was performed, providing access to the cecum and adjacent intestinal structures. The cecum was securely ligated near its base, just below the ileocecal valve, using 3.0 silk sutures, and a single perforation was made with a 22-gauge needle. Prior to repositioning the cecum into the peritoneal cavity, a tiny quantity of fecal matter was delicately expelled from the puncture site. Closure of the incision was achieved under sterile circumstances using 4-0 polyglactin sutures. The animals were given time to recuperate after surgery before being put back in their cages. The rats were deemed septic five hours after the CLP operation in this experimental scenario [16].

2.5 Sample Size, Exclusion Criteria, and Animal Disposition

No formal a priori sample-size calculation was performed. The predefined group size of 12 animals was selected based on group sizes used in comparable experimental CLP studies and the laboratory's previous experience with the model. No prespecified data-exclusion criteria were applied on the basis of observed experimental results. Animals that died before the scheduled day-5 endpoint were necessarily unavailable for terminal biochemical, histopathological, and arterial blood gas measurements and therefore were not included in those endpoint analyses.

Thirty-six rats entered the experiment (n = 12 per group). No deaths occurred in the Normal Control group. During the five-day observation period, five animals in the CLP+Saline group and three animals in the CLP+Clopidogrel group died before the scheduled day-5 endpoint. Animals that died before the scheduled endpoint were not included in the terminal biochemical, histopathological, or arterial blood gas analyses, and measurements obtained at death were not pooled with the scheduled day-5 measurements. Therefore, the final biological sample sizes for the terminal analyses were n = 12 for the Normal Control group, n = 7 for the CLP+Saline group, and n = 9 for the CLP+Clopidogrel group.

2.6 Histopathological Examination of Lung

Lung tissues were fixed in 10% formaldehyde prepared in 0.1 M phosphate-buffered saline (PBS), embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E). Representative lung parenchymal sections were examined using an Olympus BX51 light microscope equipped with a C-5050 digital camera. Histopathological assessment was performed according to the previously described protocol [17]. Five randomly selected sections were evaluated from each animal, comprising a total of 25 randomly selected microscopic fields per animal.

Lung injury was evaluated according to five histopathological parameters: alveolar congestion (AC), hemorrhage (H), leukocyte infiltration or aggregation within the air spaces or vessel walls (AL), perivascular/interstitial edema (PE), and alveolar wall thickening and/or hyaline membrane formation (TA). Each parameter was scored separately in each microscopic field using a 0–4 semiquantitative scale: 0, no detectable histopathological injury; 1, >0–25% involvement of the examined field; 2, >25–50% involvement; 3, >50–75% involvement; and 4, >75–100% involvement. The following issues exist: 25% fall into both 1 and 2 categories; 0% fall into both 2 and 3 categories; and 75% fall into both 3 and 4 categories.

Histopathological evaluation was performed on coded slides by an investigator blinded to experimental group allocation. Individual microscopic fields were not considered independent experimental units. For each histopathological parameter, the scores obtained from the multiple fields examined within an individual animal were averaged to generate a single animal-level mean score. Thus, the animal, rather than the individual microscopic field, was used as the experimental unit for subsequent statistical comparisons. Because the reported values represent animal-level averages derived from multiple field-level ordinal scores, fractional mean values, including values below 1, can occur despite the use of integer scores from 0 to 4 at the individual-field level.

2.7 Lung Biochemical Analysis

Immediately following euthanasia by exsanguination under deep ketamine/xylazine anesthesia, lung tissues were

rapidly excised and stored at –20 °C until biochemical analysis. Lung tissues were homogenized on ice in five volumes of ice-cold PBS (pH 7.4), and homogenates were centrifuged at 5000 rpm for 15 min at 4 °C. Supernatants were aliquoted and kept frozen until enzyme-linked immunosorbent assay (ELISA) analysis, avoiding repeated freeze–thaw cycles. Total protein concentration was determined using the Bradford method with bovine serum albumin as the standard. Pulmonary FOXP3 concentrations were measured using a commercially available rat FOXP3 sandwich ELISA kit (Cloud-Clone Corp., Wuhan, Hubei, China; Cat. No. SEB877Ra) according to the manufacturer's instructions. The assay has a reported detection range of 0.312–20 ng/mL, a sensitivity of <0.115 ng/mL, an intra-assay coefficient of variation (CV) of <10%, and an inter-assay CV of <12%. Samples and standards were assayed in duplicate, and absorbance was measured at 450 nm. The mean of the duplicate measurements was used for analysis. FOXP3 concentrations were normalized to the total protein content of the corresponding lung homogenate and expressed as pg/mg protein.

2.8 Determination of TNF-α Levels in Plasma

Blood samples intended for plasma analysis were collected into EDTA-containing tubes and centrifuged promptly after collection. Plasma was separated by centrifugation at 3000 ×g for 10 min at 4 °C, aliquoted, and stored at –80 °C until analysis. Samples were thawed only once before ELISA measurements. Plasma TNF-α concentrations were determined using a commercially available rat-specific sandwich ELISA kit (Abcam Ltd., Cambridge, UK; Cat. No. ab100784) according to the manufacturer's instructions. The assay has a reported detection range of 82.3–20,000 pg/mL and a sensitivity of <25 pg/mL, with intra-assay and inter-assay coefficients of variation of <10% and <12%, respectively. The reported mean recovery for rat plasma is 93.79% (range, 82–102%). Samples and standards were assayed in duplicate using 100 µL per well, and absorbance was measured at 450 nm. The mean of duplicate measurements was used to determine the final TNF-α concentration from the corresponding standard curve.

2.9 Evaluation of Lipid Peroxidation

The levels of malondialdehyde (MDA), which are evaluated as thiobarbituric acid reactive substances (TBARS), were used to evaluate lipid peroxidation in plasma samples. To summarize, after completely mixing the plasma samples with trichloroacetic acid and TBARS reagents, they were incubated at 100 °C for 60 min. The samples were centrifuged at 3000 rpm for 20 min after cooling on ice after incubation. A spectrophotometer was used to measure the absorbance of the supernatant at 535 nm. Quantities of MDA were measured in nM, with tetraethoxypropane serving as the reference standard for calibration.

2.10 Arterial Blood Gas Analysis

On day 5, arterial blood gas measurements were obtained while the animals were breathing spontaneously under room-air conditions (fraction of inspired oxygen (FiO_2) approximately 0.21). Animals were anesthetized with ketamine (75 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) before arterial sampling. A 0.2-mL arterial blood sample was collected directly from the carotid artery into a heparinized syringe, with care taken to avoid exposure to air, and was analyzed immediately after collection using an ABL 700 Series blood gas analyzer (Radiometer, Copenhagen, Denmark). Partial pressure of arterial oxygen (PaO_2) and partial pressure of arterial carbon dioxide ($PaCO_2$) were recorded. Animals were breathing spontaneously and were not mechanically ventilated during blood-gas sampling.

2.11 Randomization and Blinding

Animals were allocated to the three experimental groups by simple randomization using a draw-based procedure. No separate formal allocation-concealment procedure was used. To minimize observer bias, tissue and plasma samples were coded before outcome assessment. Investigators performing histopathological scoring and biochemical measurements were blinded to group allocation. Investigators conducting the CLP procedure and administering experimental treatments were necessarily aware of group allocation because of the nature of the interventions. Group identities were revealed only after completion of outcome assessment and data recording.

2.12 Statistical Analysis

Quantitative data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple-comparison test for pairwise comparisons among the Normal Control, CLP+Saline, and CLP+Clopidogrel groups. Data are presented as mean ± standard deviation (SD). Tukey's procedure was used to account for multiple pairwise comparisons. A two-sided p-value < 0.05 was considered statistically significant. For histopathological outcomes, individual field scores were first averaged within each animal, and these animal-level mean scores were used for group comparisons; individual microscopic fields were not treated as independent observations.

3. Results

3.1 Mortality and Final Sample Sizes

During the five-day experimental period, no animals died in the Normal Control group, whereas five animals died in the CLP+Saline group and three animals died in the CLP+Clopidogrel group. Accordingly, the numbers of animals available for terminal biochemical, histopathological, and arterial blood gas analyses were 12, 7, and 9 in the Normal Control, CLP+Saline, and CLP+Clopidogrel groups, respectively. Mortality was numerically lower in

Table 1. Effects of clopidogrel on plasma MDA, TNF-α levels, and lung FOXP3 expression in CLP-induced sepsis.

	Normal control group	CLP+Saline group	CLP+Clopidogrel group
Plasma MDA (nM)	52.3 ± 4.5	163.7 ± 8.5***	98.7 ± 10.3###
Plasma TNF-α (pg/mL)	10.6 ± 2.9	159.7 ± 7.1***	67.8 ± 7.2###
Lung FOXP3 (pg/mg protein)	9.05 ± 0.6	4.4 ± 0.2*	5.92 ± 0.1#

Data are presented as mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc multiple-comparison test. *$p < 0.05$ and ***$p < 0.001$ versus the Normal Control group; #$p < 0.05$ and ###$p < 0.001$ versus the CLP+Saline group. MDA, malondialdehyde; TNF-α, tumor necrosis factor-alpha; FOXP3, forkhead box P3; CLP, cecal ligation and puncture; SD, standard deviation; ANOVA, analysis of variance.

clopidogrel-treated animals; however, the study was not designed or powered to evaluate survival as an outcome.

3.2 Plasma Biochemical Markers

Table 1 summarizes plasma MDA and TNF-α levels, as well as FOXP3 expression in lung tissue (Fig. 1). Plasma MDA and TNF-α levels were significantly increased in the CLP+Saline group compared with the Normal Control group (***$p < 0.001$). Clopidogrel treatment (10 mg/kg) significantly reduced both MDA and TNF-α levels compared with the CLP+Saline group (###$p < 0.001$). Lung FOXP3 expression was significantly decreased in the CLP+Saline group compared with the Normal Control group (*$p < 0.05$), whereas clopidogrel treatment was associated with a modest but significant increase in FOXP3 expression compared with the CLP+Saline group (#$p < 0.05$).

3.3 Histopathological Lung Analysis

Histopathological examination revealed marked differences among the experimental groups (Table 2, Figs. 2,3). The CLP+Saline group showed significantly increased leukocyte infiltration/aggregation (AL), AC, PE, alveolar wall thickness (TA), and H compared with the Normal Control group (***$p < 0.001$). Clopidogrel treatment significantly reduced all histopathological injury scores compared with the CLP+Saline group, with significant reductions in H, AL, and PE (#$p < 0.05$) and more pronounced reductions in AC and TA (###$p < 0.001$).

3.4 Arterial Blood Gas Analysis

Arterial blood gas parameters (PaO_2 and $PaCO_2$) showed significant alterations in the CLP+Saline group compared with the Normal Control group, indicating impaired oxygenation and an altered ventilatory response (Table 3, Fig. 4). PaO_2 was significantly reduced in the CLP+Saline group (**$p < 0.01$), whereas clopidogrel treatment significantly increased PaO_2 compared with the CLP+Saline group (##$p < 0.01$). $PaCO_2$ was also significantly reduced in the CLP+Saline group compared with the Normal Control group (**$p < 0.01$). Although $PaCO_2$ was numerically higher in the CLP+Clopidogrel group, the difference compared with the CLP+Saline group did not reach statistical significance.

Table 2. Histopathological lung damage scores in CLP-induced sepsis and the effects of clopidogrel.

	Normal control group	CLP+Saline group	CLP+Clopidogrel group
AC	0.1 ± 0.1	2.8 ± 0.1***	1.5 ± 0.2###
H	0.1 ± 0.1	1.9 ± 0.1***	1.3 ± 0.2#
AL	0.2 ± 0.1	2.3 ± 0.3***	1.6 ± 0.1#
PE	0.2 ± 0.2	2.4 ± 0.2***	1.1 ± 0.2#
TA	0.2 ± 0.1	2.2 ± 0.1***	0.9 ± 0.1###

Data are presented as mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc multiple-comparison test. ***$p < 0.001$ versus the Normal Control group; #$p < 0.05$ and ###$p < 0.001$ versus the CLP+Saline group. AC, alveolar congestion; H, hemorrhage; AL, leukocyte infiltration/aggregation; PE, perivascular/interstitial edema; TA, alveolar wall thickening/hyaline membrane formation.

4. Discussion

ARDS and sepsis-related lung damage (ALI) are major causes of death and disability in severely sick patients, and they often signal the beginning of MODS. Therefore, important treatment goals are ALI prevention and good management.

Despite advancements in understanding the complex pathophysiology of sepsis and the development of novel therapeutic strategies, sepsis remains the foremost cause of mortality in ICUs when accompanied by ALI/ARDS. The molecular mechanisms underlying sepsis-induced immune dysfunction and systemic inflammation remain poorly understood, significantly impeding the development of effective therapeutic interventions for this devastating condition.

The present study evaluated the potential of clopidogrel to mitigate CLP-induced ALI in rats in association with changes in pulmonary FOXP3 expression. This research highlights clopidogrel's potential as a therapeutic agent for sepsis-associated ALI, with a focus on reducing inflammatory responses and oxidative stress. ALI prevention, as demonstrated in this study, remains a pivotal goal in managing critically ill septic patients.

The CLP model is widely regarded as the gold standard for experimental sepsis studies in rodents [18]. By perforating the cecum, this model induces bacterial peri-

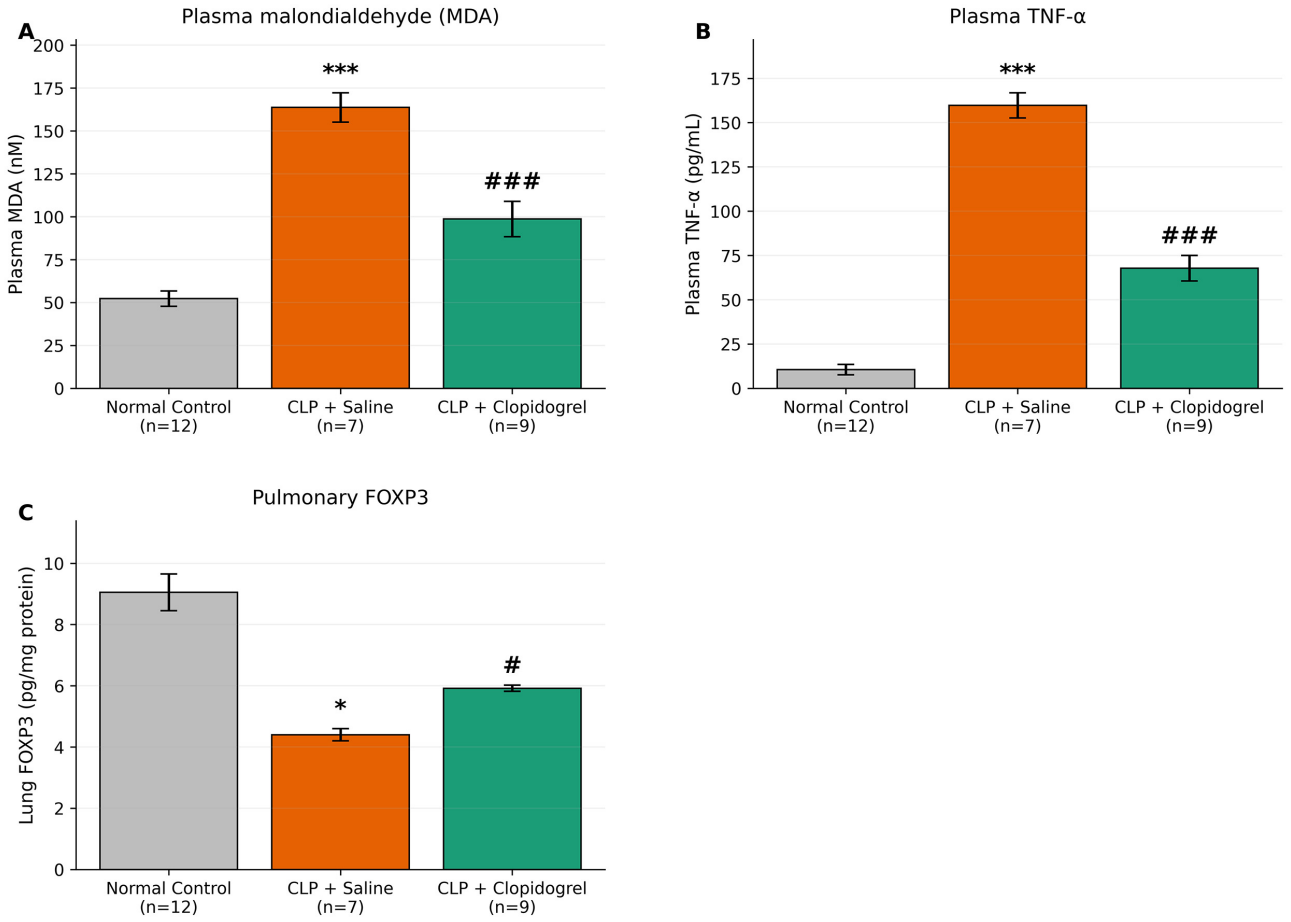


Fig. 1. Effects of clopidogrel on biochemical markers in CLP-induced sepsis. (A) Plasma malondialdehyde (MDA) levels. (B) plasma TNF-α levels. (C) lung FOXP3 levels in the Normal Control, CLP+Saline, and CLP+Clopidogrel groups. Data are presented as mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc multiple-comparison test. *$p <$ 0.05 and ***$p < 0.001$ versus the Normal Control group; #$p < 0.05$ and ###$p < 0.001$ versus the CLP+Saline group.

Table 3. Effects of clopidogrel on arterial blood gas parameters (PaO_2 and $PaCO_2$) in CLP-induced sepsis.

	Normal Control group	CLP+Saline group	CLP+Clopidogrel group
PaO_2 (mmHg)	99.2 ± 7.9	78.3 ± 4.5**	92.5 ± 6.1##
$PaCO_2$ (mmHg)	40.7 ± 3.5	31.7 ± 2.8**	36.3 ± 3.7

Data are presented as mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc multiple-comparison test. **$p < 0.01$ versus the Normal Control group; ##$p < 0.01$ versus the CLP+Saline group. PaO_2, partial pressure of arterial oxygen; $PaCO_2$, partial pressure of arterial carbon dioxide.

tonitis and systemic bacteremia, triggering an inflammatory cascade that can progress to septic shock, MODS, and ultimately death [19]. This model effectively replicates the clinical features of sepsis and allows for the evaluation of therapeutic agents.

Previous studies, such as the work by Xiao et al. [20], demonstrated that curcumin could reduce CLP-induced ALI by decreasing pulmonary edema, protein content, and inflammatory cell infiltration into lung tissue. These effects were attributed to curcumin's ability to suppress polymorphonuclear leukocyte (PMN) infiltration, reduce ROS production, enhance antioxidant status, and regulate pro-inflammatory cytokine levels. In the current study, the CLP+Saline group exhibited severe histopathological changes, including significant alveolar inflammation and septal thickening, which were notably reduced in the CLP+Clopidogrel group. These findings align with the results of Xiao et al. [20], underscoring clopidogrel's potential protective effects.

Neutrophils play a central role in limiting bacterial dissemination during sepsis and are a key component of the body's first line of defense. PMN migration to the infec-

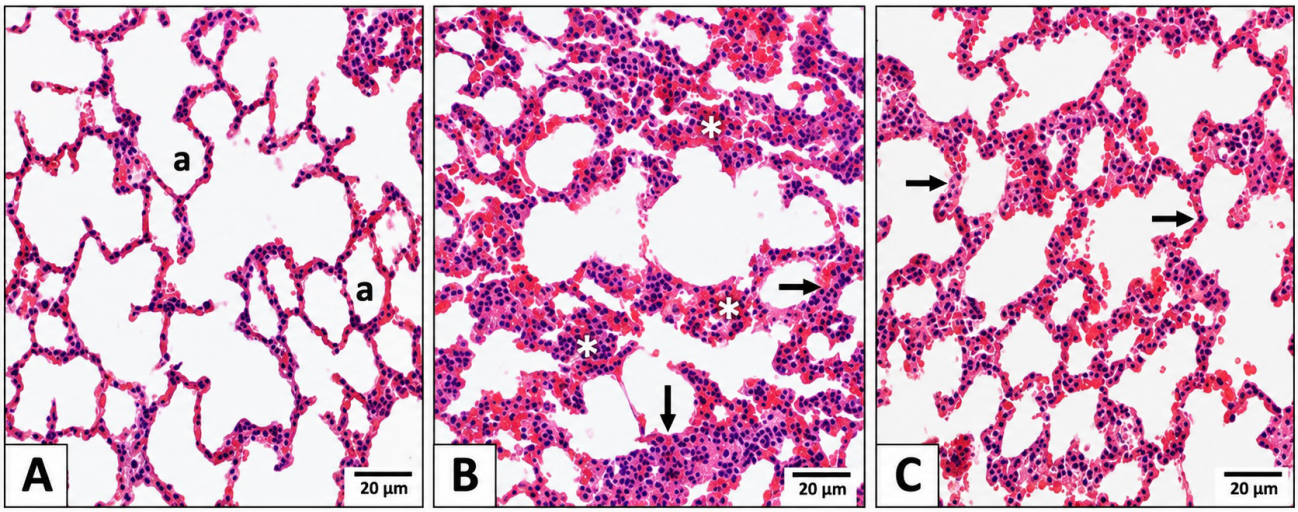


Fig. 2. Representative histopathological changes in lung tissue following CLP-induced sepsis and clopidogrel treatment. Representative H&E-stained lung sections are shown. (A) Normal Control group showing preserved alveolar architecture and open alveolar spaces (a). (B) CLP+Saline group showing marked inflammatory cell infiltration (*), alveolar congestion, edema, and increased alveolar septal thickness (arrows). (C) CLP+Clopidogrel group showing attenuation of inflammatory changes and reduced alveolar septal thickening (arrows) compared with the CLP+Saline group. a, alveolar air space; *, inflammatory cell infiltration; arrows, alveolar septal thickening. Original magnification: ×20. Scale bar = 20 μm. H&E, hematoxylin and eosin.

tion site is critical for controlling sepsis. Changes in PMN counts in peripheral blood are also an important marker of sepsis severity [21]. A study by Tavares et al. [22] demonstrated that immunoneutralization of endogenous aminoprocalcitonin reduced sepsis-induced ALI and mortality in rats. Anti-NPCT treatment increased PMN counts and facilitated their recruitment to the peritoneum, emphasizing the role of localized inflammatory responses in host defense during severe sepsis [22]. Although Tavares et al. [22] demonstrated a pathogenic role for aminoprocalcitonin in CLP-induced sepsis-associated ALI, experimental findings regarding the calcitonin/procalcitonin pathway are not entirely uniform. Tuvim et al. [23] reported that deletion of the calcitonin/CGRP-α gene did not significantly alter the outcome of severe infection in mice, suggesting that the contribution of this pathway may depend on the experimental model, species, and pattern of procalcitonin expression. In the present study, clopidogrel treatment was associated with reduced pulmonary inflammatory changes, suggesting that modulation of inflammatory cell recruitment may represent one component of its protective effect in sepsis-induced ALI.

The pathophysiology of sepsis-induced ALI is mostly influenced by pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 [24,25]. Experimental models of inflammatory lung injury have further demonstrated that pro-inflammatory cytokine signaling is closely associated with NF-κB activation, pulmonary neutrophil recruitment, edema, and subsequent tissue injury [26]. In this study, clopidogrel significantly reduced TNF-α levels in the plasma of CLP-induced septic rats, consistent with findings from previous studies by Tavares and Miñano [27] and Xiao et al. [20], where interventions targeting TNF-α demonstrated protective effects in sepsis models.

Experimental evidence supports a role for TNF-α in acute lung injury. In a porcine model involving cardiopulmonary bypass followed by LPS administration, treatment with a soluble TNF receptor-binding protein prevented lung injury without reducing pulmonary neutrophil sequestration [28]. Yan et al. [29] further demonstrated that cyanidin-3-O-glucoside (C3G) reduced IL-1β, TNF-α, and IL-6 levels in septic rats, providing evidence of its protective effects against systemic inflammation and lung injury. These findings highlight the importance of targeting inflammatory mediators to mitigate ALI progression.

The inflammatory response in sepsis-associated ALI is multifactorial and extends beyond TNF-α. In particular, IL-1β, IL-6, IL-18, and inflammasome-associated pathways contribute substantially to sepsis-induced organ injury. Previous experimental work using a CLP model demonstrated that clopidogrel at 10 mg/kg/day attenuated platelet NLRP3 inflammasome activation and modulated IL-1β and IL-18 responses, supporting a broader immunomodulatory role of platelet P2Y12 inhibition in sepsis [15]. However, because the present study quantified TNF-α as the principal inflammatory cytokine, our findings cannot establish the effect of clopidogrel on the wider cytokine network involved in sepsis-associated ALI.

Oxidative stress contributes to the pathophysiology of sepsis-induced ALI. In the present study, oxidative injury was assessed using plasma MDA, measured as TBARS, as an index of lipid peroxidation. Plasma MDA levels were

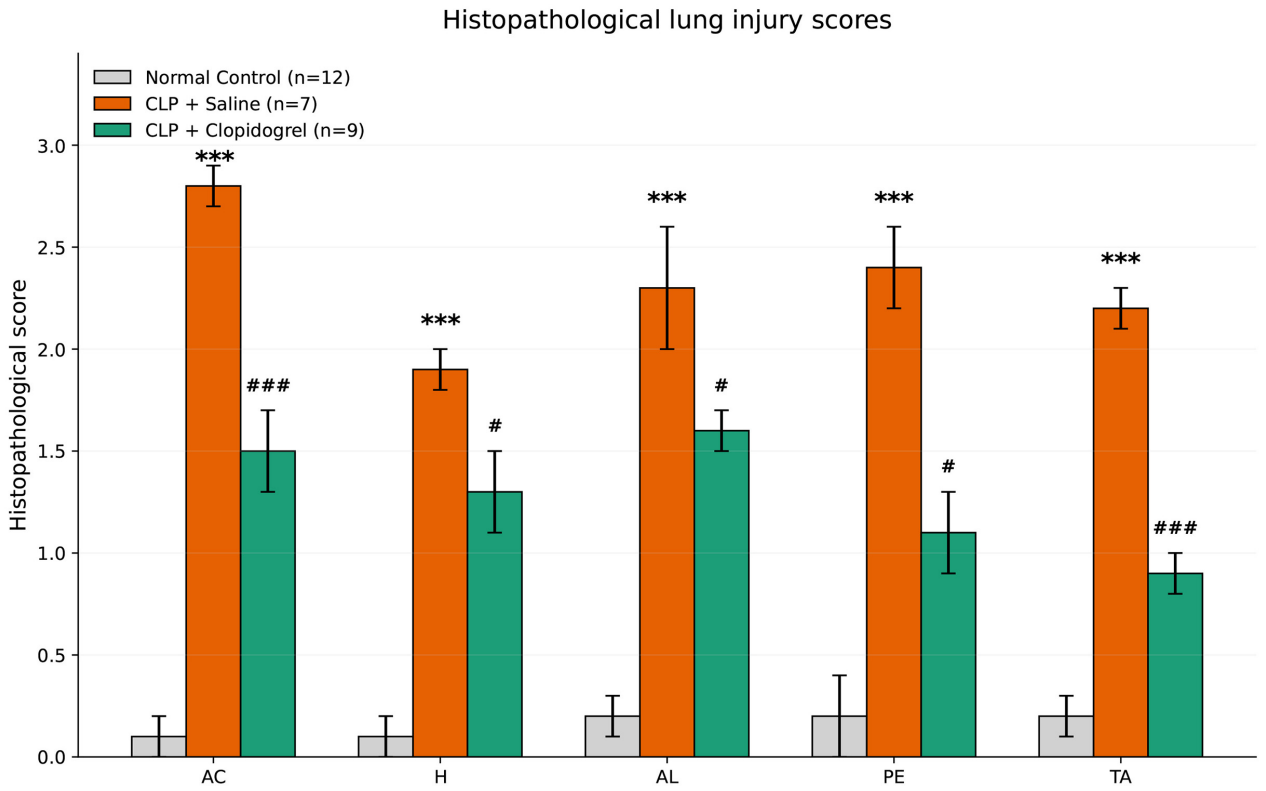


Fig. 3. Effects of clopidogrel on histopathological lung injury scores in CLP-induced sepsis. Histopathological lung injury scores, including alveolar congestion (AC), hemorrhage (H), leukocyte infiltration/aggregation (AL), perivascular/interstitial edema (PE), and alveolar wall thickness (TA), in the Normal Control, CLP+Saline, and CLP+Clopidogrel groups. Data are presented as mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc multiple-comparison test. ***$p < 0.001$ versus the Normal Control group; #$p < 0.05$ and ###$p < 0.001$ versus the CLP+Saline group.

markedly increased in the CLP+Saline group and were significantly reduced by clopidogrel treatment. These findings are consistent with attenuation of lipid peroxidation; however, because ROS production and endogenous antioxidant enzyme activities were not directly measured, the present data do not establish a direct effect of clopidogrel on ROS generation or antioxidant defense systems.

Clopidogrel is a widely used P2Y12 receptor antagonist that has also been reported to exert anti-inflammatory effects in experimental settings. Its established pharmacological action is the inhibition of platelet activation and aggregation through blockade of platelet P2Y12 receptors. Beyond their role in thrombosis, activated platelets can participate in inflammatory signaling through interactions with leukocytes and the release of inflammatory mediators, thereby potentially contributing to systemic and pulmonary inflammation [12,13]. Accordingly, inhibition of platelet P2Y12 signaling may represent one mechanism through which clopidogrel could modulate inflammatory responses during sepsis. However, platelet activation, platelet–leukocyte interactions, and direct P2Y12 target engagement were not assessed in the present study; therefore, these mechanisms should be regarded as biologically plausible explanations supported by previous literature rather than mechanisms directly demonstrated by our findings.

Pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, contribute to the inflammatory response in sepsis-associated acute lung injury (ALI), involving endothelial activation, leukocyte recruitment, and tissue injury [24,30]. Platelet signaling may also contribute to inflammatory responses beyond its established role in thrombosis [31]. In the present study, however, only plasma TNF-α was measured. Clopidogrel significantly reduced plasma TNF-α levels in CLP-induced septic rats compared with saline-treated septic animals, supporting an attenuation of the measured systemic inflammatory response. IL-1β and IL-6 were not assessed in the present study; therefore, no conclusions regarding the effects of clopidogrel on these cytokines can be drawn from our data.

Experimental sepsis has been associated with increased lipid peroxidation [16], while studies of sepsis-associated and endotoxin-induced acute lung injury support the involvement of oxidative stress in pulmonary injury [17,32]. In the present study, oxidative injury was assessed specifically by measuring plasma MDA as TBARS. Clopidogrel treatment significantly reduced plasma MDA

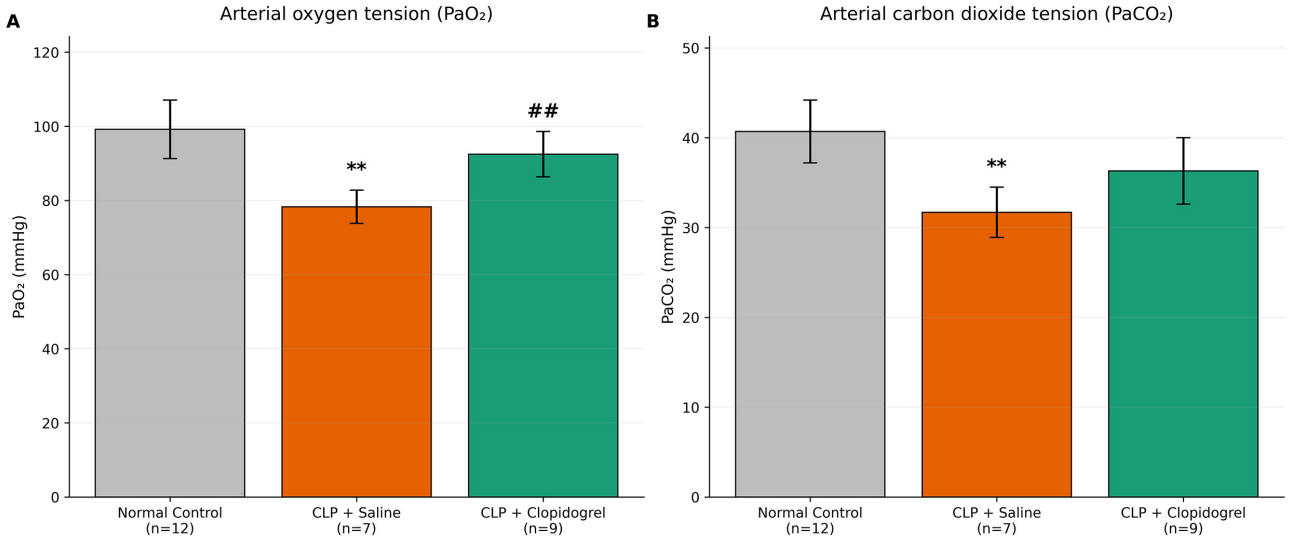


Fig. 4. Effects of clopidogrel on arterial blood gas parameters in CLP-induced sepsis. (A) Arterial oxygen tension (PaO_2) and (B) arterial carbon dioxide tension ($PaCO_2$) in the Normal Control, CLP+Saline, and CLP+Clopidogrel groups. Data are presented as mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc multiple-comparison test. $^{**}p < 0.01$ versus the Normal Control group; $^{\#\#}p < 0.01$ versus the CLP+Saline group.

levels compared with the CLP+Saline group, indicating lower lipid peroxidation under the conditions of this experiment. However, ROS production, superoxide dismutase activity, and other enzymatic or non-enzymatic antioxidant defense systems were not directly measured. Therefore, the present findings should be interpreted as evidence of reduced TBARS-derived lipid peroxidation rather than as direct demonstration of ROS scavenging or a comprehensive antioxidant effect of clopidogrel.

Another proposed mechanism is clopidogrel's effect on neutrophil recruitment and activation. Platelet-leukocyte aggregates formed during sepsis exacerbate inflammatory responses by promoting neutrophil infiltration into the lungs. Experimental studies of acute lung injury and neutrophil biology provide a basis for understanding inflammation-associated tissue damage [7,21]. In our study, clopidogrel-treated rats exhibited reduced inflammatory cell infiltration and better-preserved alveolar architecture compared with saline-treated controls. These findings suggest attenuation of pulmonary inflammation, although the underlying cellular mechanisms were not directly investigated.

These observations are consistent with recent studies demonstrating that sepsis-associated inflammation and organ injury are regulated by multiple interconnected signaling pathways. Increased IL-27 activity has been associated with enhanced production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and with more severe sepsis-related inflammatory responses [33]. In addition, ADAM10 has emerged as an important regulator of endothelial–immune interactions, vascular barrier dysfunction, cytokine shedding, and microcirculatory injury during sepsis [34]. More specifically, modulation of ADAM17 by miR-145 has been shown to reduce TNF-α, IL-1β, IL-6, endothelial activation, and acute lung and kidney injury in CLP-induced sepsis [35], whereas inhibition of the HMGB1/RAGE/NF-κB pathway similarly attenuated systemic inflammation and organ injury in experimental sepsis [36]. Taken together, these findings support the concept that therapeutic strategies capable of modulating multiple inflammatory and endothelial pathways may be particularly relevant in sepsis-associated ALI.

The arterial blood-gas findings should be interpreted cautiously. CLP-induced sepsis was associated with a significant reduction in PaO_2, whereas clopidogrel treatment significantly increased PaO_2 compared with the CLP+Saline group, supporting an improvement in arterial oxygenation. $PaCO_2$ was also reduced in septic animals. This finding may reflect an increased ventilatory drive and hyperventilatory response associated with systemic sepsis rather than impaired CO_2 elimination per se. Although $PaCO_2$ was numerically closer to the control value following clopidogrel treatment, the difference between the CLP+Clopidogrel and CLP+Saline groups was not statistically significant. Therefore, the present data do not support a definitive effect of clopidogrel on ventilation or CO_2 clearance. Moreover, because a complete acid–base assessment was not performed, the physiological basis of the $PaCO_2$ changes cannot be determined from the present data alone.

5. Limitation

Despite the promising findings of this study, several limitations should be acknowledged. First, this investi-

gation was conducted in a preclinical rat model of CLP-induced sepsis, which, although widely used, does not fully reproduce the complexity and heterogeneity of human sepsis and sepsis-associated ALI. Moreover, only female Wistar rats were included; therefore, potential sex-related differences in inflammatory responses, platelet biology, pharmacological effects, and sepsis pathophysiology could not be evaluated, and the findings may not be generalizable to male animals. The study also lacked a sham-operated control group. Because the Normal Control animals did not undergo laparotomy, anesthesia, or other perioperative procedures associated with CLP, comparisons with this group cannot completely distinguish the effects of CLP-induced sepsis from those attributable to surgery and perioperative factors. In addition, only a single clopidogrel dose (10 mg/kg/day) was investigated, preventing assessment of dose-response relationships and identification of an optimal therapeutic dose. The experimental period was limited to five days, and the principal biochemical, histopathological, and arterial blood gas outcomes were assessed only at the scheduled terminal time point; consequently, the temporal evolution of lung injury and whether the observed treatment effects were early, transient, or sustained could not be determined. Differential mortality between the septic groups also warrants consideration. Five animals in the CLP+Saline group and three in the CLP+Clopidogrel group died before the day-5 endpoint, leaving n = 7 and n = 9 animals, respectively, for terminal analyses. Because biochemical, histopathological, and blood gas measurements were obtained only from animals surviving to the scheduled endpoint, survivorship bias cannot be excluded. Although mortality was numerically lower in the clopidogrel-treated group, our study was not designed or powered to evaluate survival, and no conclusion regarding a survival benefit can be drawn. Mechanistically, FOXP3 was measured only by ELISA in whole-lung homogenates. Therefore, the present findings do not identify the cellular source of FOXP3, demonstrate changes in regulatory T-cell abundance or function, or establish activation or causal involvement of the FOXP3 pathway. Similarly, direct pharmacodynamic evidence of platelet P2Y12 target engagement was not obtained because platelet aggregation, platelet activation markers, and receptor-specific functional assays were not included; accordingly, attribution of the observed effects specifically to P2Y12 inhibition should be interpreted cautiously. Bleeding outcomes were also not prespecified or systematically quantified, precluding a formal assessment of clopidogrel-related bleeding risk. The biochemical assessment of inflammation and oxidative injury was limited to plasma TNF-α and TBARS-derived MDA, respectively. Other inflammatory cytokines, including IL-1β, IL-6, and IL-18, as well as direct measures of ROS and endogenous antioxidant enzymes such as SOD, were not evaluated. Therefore, the present data support an association between clopidogrel treatment and reduced systemic TNF-α levels and lipid peroxidation, but they do not establish broad anti-inflammatory or antioxidant mechanisms. Finally, pulmonary barrier injury was not directly quantified using complementary measures such as lung wet-to-dry weight ratio or bronchoalveolar lavage fluid protein concentration. Thus, although the histopathological findings and improvement in arterial oxygenation support attenuation of lung injury, the study does not provide a direct quantitative assessment of pulmonary edema or alveolar-capillary barrier permeability. Future studies incorporating both sexes, sham-operated controls, multiple clopidogrel doses and time points, direct platelet/P2Y12 functional assays, cell-resolved assessment of FOXP3-positive regulatory T cells, broader inflammatory and oxidative-stress profiling, and direct measures of pulmonary barrier injury will be required to more clearly define the mechanisms, safety, and translational relevance of clopidogrel in sepsis-associated ALI.

6. Conclusion

Clopidogrel attenuated sepsis-induced ALI in this rat CLP model, as demonstrated by reduced plasma TNF-α and TBARS-derived MDA levels, improved pulmonary histopathological findings, and higher arterial PaO_2 compared with untreated septic animals. Although $PaCO_2$ was reduced in the CLP+Saline group relative to the Normal Control group, clopidogrel did not produce a statistically significant change in $PaCO_2$ compared with CLP+Saline; therefore, no conclusion regarding an improvement in ventilatory function can be drawn from this parameter. Clopidogrel treatment was also associated with partial restoration of pulmonary FOXP3 levels; however, the present study does not establish a causal role for FOXP3-mediated immunoregulation. The reduction in plasma MDA is consistent with decreased lipid peroxidation, but direct ROS production, antioxidant enzyme activity, and other oxidative stress pathways were not assessed, precluding broader conclusions regarding antioxidant mechanisms. Similarly, TNF-α was the only pro-inflammatory cytokine measured, and the effects of clopidogrel on other cytokines were not directly evaluated. Further well-designed preclinical studies incorporating additional inflammatory and oxidative stress markers, direct measures of pulmonary barrier injury, comprehensive acid–base and ventilatory assessment, platelet/P2Y12 target engagement, cell-specific analysis of FOXP3, and different dosing regimens are required to clarify the mechanisms underlying the observed protective effects before clinical translation can be considered.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author, AE, upon reasonable request.

Author Contributions

AE, MAE, and OE: conceived and designed the research, formal analysis, wrote the manuscript, edited the text, conceptualization. AE, MAE, YU, and OE: Data curation, conducted experiments. All authors read and approved the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures were reviewed and approved by the Demiroğlu Bilim University Animal Ethics Committee (Approval No. 1524105819). The study was carried out in accordance with the ARRIVE 2.0 guidelines and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to obtain scientifically valid results. Consent to participate was not applicable because this was an experimental animal study.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/IJP57087.

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