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Effects of different antiplatelet therapy drugs on platelet activation and platelet-leukocyte aggregate formation in early septic ARDS

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Abstract

Background In patients with sepsis, platelets are activated and adhere to neutrophils, forming platelet-leukocyte aggregates (PLAs) that lead to the development of MODS. ARDS is one of the main manifestations of septic MODS. We designed this study to explore the effects of different anti-plate therapy drugs on platelet activation and platelet-leukocyte aggregate (PLA) formation in the early stage of septic ARDS.

Methods Sixty adult male SD rats were randomly divided into: Control group; ARDS group, ARDS + aspirin group, ARDS + clopidogrel group and ARDS + tirofiban group. ARDS was performed via instill lipopolysaccharide (LPS) intratracheally at a dose of 5 mg/kg. Aspirin or clopidogrel were given by gavage immediately after modeling. Tirofiban were given by intraperitoneal injection immediately after modeling. Rats in every group were euthanized by rapid decapitation 6 h after modeling. Platelet activation and PLA were assessed using flow cytometry and immunofluorescence staining. Histology of lung was performed by hematoxylin and eosin staining.

Results Aspirin, clopidogrel and tirofiban decreased CRP, IL-1 and TNF-α significantly in septic ARDS ($P < 0.05$). Aspirin, clopidogrel and tirofiban decreased platelet function and ratio of wet/dry significantly in septic ARDS ($P < 0.05$). Aspirin, clopidogrel and tirofiban increased PaO_2 significantly in septic ARDS ($P < 0.05$). Platelet activation and PLA in the ARDS + aspirin group, ARDS + clopidogrel group and ARDS + tirofiban group decreased significantly compared to the ARDS group ($P < 0.05$). At 6 h after ARDS operation, obvious histological damage was observed in the lungs. All of these histological changes were quantitatively evaluated using injury scores. Aspirin, clopidogrel and tirofiban reduced the histological damages in ARDS group ($P < 0.05$).

Conclusions Aspirin, clopidogrel and tirofiban alleviated the inflammatory response and pulmonary edema, reduced platelet function, and alleviated hypoxemia in early septic ARDS. Aspirin, clopidogrel and tirofiban reduced platelet activation and PLA formation in early septic ARDS. Aspirin, clopidogrel and tirofiban ultimately alleviated lung injury in early septic ARDS.

Keywords Platelet-leukocyte aggregate, ARDS, Sepsis, Aspirin, Clopidogrel, Tirofiban

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Introduction

Sepsis is a dysregulated immune response to an infection that leads to life-threatening organ dysfunction [1]. Sepsis is a medical emergency with a high incidence, mortality and disability [2]. In 2017, the World Health Organization declared that improving the prevention, recognition and treatment of sepsis is a global health priority. In patients with sepsis, platelets are activated and adhere to neutrophils, forming platelet-leukocyte aggregates (PLAs) that induce pathogenic neutrophil extracellular traps (NETs) formation, microcirculation arrest, amplify inflammation, and play a vital role in organ injury mediated by dysregulated inflammation [3–5]. Acute respiratory distress syndrome (ARDS) is one of the main manifestations of septic multiple organ dysfunction syndrome (MODS). Activated platelets play an important role in sepsis-related lung injury [6, 7]. Recent studies have found that antiplatelet drugs can reduce acute lung injury [8–11]. Antiplatelet therapies target distinct pathways of platelet activation: thromboxane A2 synthesis, adenosine diphosphate-mediated signaling, integrin αIIbβ3 (glycoprotein [GP] IIb/IIIa) [12]. Effects of different antiplatelet drugs, especially drugs target integrin αIIbβ3 (GP IIb/IIIa), on platelet activation and platelet-leukocyte aggregate (PLA) formation in early septic ARDS are still unclear, so we designed this study in order to provide a new site for the treatment of ARDS.

Materials and methods

Ethics statement

The study was approved by the Institutional Animal Care and Use Committee (IACUC) at Peking Union Medical College, Beijing, China (XHDW-2023-029).

Animal surgical procedures were carried out in strict accordance with the guidelines for the care and use of laboratory animals established by the Animal Use and Care Committee of the Beijing Committee on Animal Care.

The animal protocol was designed to minimize pain or discomfort to the rats. The rats were housed in Plexiglas cages under controlled temperature (22±2 °C), humidity (54±2%), and 12 h light/dark cycle for one week prior to experimentation. Food and water were freely available during the study period. All rats were intraperitoneally anesthetized with 3% sodium pentobarbital (50 mg/kg) prior to surgery and decapitation.

Animal model

Sixty adult male Sprague-Dawley rats (specific pathogen free [SPF], 8wk, 250–300 g) were purchased from the Si Bei Fu Animal Centre of Beijing (Beijing, China; License: SCXK, Beijing, 2019-0010). The rats were randomly divided into 5 groups of twelve rats per group applying a table of random numbers: Control group; ARDS group, ARDS+aspirin group, ARDS+clopidogrel group and ARDS+tirofiban group. Rats in ARDS group, ARDS+aspirin group, ARDS+clopidogrel group and ARDS+tirofiban group were intraperitoneally anesthetized with sodium pentobarbital. Make a small incision in the ventral region of the rat neck, carefully exposing the trachea, and then secure it on a 45-degree inclined plate. Intubate with a 26-gauge needle and instilled lipopolysaccharide (LPS) (dissolved in 0.9% normal saline, 1 mg/0.05mL) intratracheally at a dose of 5 mg/kg. To ensure that LPS is evenly distributed throughout the rat lung, place the rat in the prone position after 30 s of vertical rotation. When normal spontaneous breathing is evident, the neck incision is sutured with silk sutures. The time of the tail SPO2<90% lasted for more than 5 min was the indicator of success of septic ARDS modeling. Then, rats were free-breathing without ventilation and anesthesia. Rats in the ARDS+aspirin group were given aspirin (10 mg/kg) by gavage immediately after modeling [13, 14]. Rats in the ARDS+clopidogrel group were given clopidogrel (10 mg/kg) by gavage immediately after modeling [15]. Rats in the ARDS+tirofiban group were given tirofiban (60 ug/kg) by intraperitoneal injection immediately after modeling [16]. Rats in every group were euthanized by rapid decapitation 6 h after modeling.

Enzyme-linked immuno sorbent assay (ELISA)

Specific ELISA kits and the instructions provided by the manufacturer were used to measure C reactive protein (CRP), interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) in plasma. The measured absorbance of the samples in a microplate reader was compared with an established standard curve in the same measurement, and the cytokines concentrations were calculated.

Immunofluorescence

After dewaxing and rehydrated, the sections were incubated in 3% hydrogen peroxide to quench any endogenous peroxidase activity. Sections were placed in ethylene diamine tetraacetic acid (EDTA) antigen retrieval solution (pH 9.0) to repair antigens. A 10% nonimmune goat serum was applied to eliminate nonspecific staining. Sections were incubated overnight at 4 °C with an optimally diluted rabbit polyclonal anti-rat CD45 antibody (1:100). The sections were washed with phosphate buffered saline (PBS) and incubated with a horseradish peroxidase (HRP) multipolymeric anti-rabbit/mouse secondary antibody for 30 min, rewashed. Sections were incubated with D-594 marked tyramide for 10 min, rewashed. Sections were incubated overnight at 4 °C with an optimally diluted rabbit polyclonal anti-rat CD41 antibody (1:100). The sections were washed with PBS and incubated with a HRP multipolymeric anti-rabbit/mouse secondary antibody for 30 min, rewashed. Sections were incubated with

D-488 marked tyramide for 10 min, rewashed. Finally, an antifluorescent quencher was added. Examine the slides with a fluorescence microscope Eclipse TE300 (Nikon).

Flow cytometry

Blood was stained with PE-anti-CD45, FITC-anti-CD41, and APC-anti-CD62P, and then treated with BD Phosflow™ Lyse/Fix Buffer. Data were recorded in the BD LSR II Flow Cytometer and analyzed with FlowJo V10.8 software. Leukocytes were identified as CD45 positive events and platelets as CD41 positive events. The activation status of platelets was identified as CD62P positive events. Platelet activation and PLA were determined based on forward (FSC) and side (SSC) scatter properties and double positive expression of CD62P/CD41 and CD41/CD45 cells, respectively.

Hematoxylin and eosin (HE) staining

Lung histopathology was evaluated by HE. After dewaxing, the sections were stained with hematoxylin and eosin for microscopic examination. The severity of lung injury was scored as follows: 0, no evidence of injury; 1, mild injury; 2, moderate injury; and 3, severe injury with pulmonary edema, interstitial inflammatory cell infiltration, and hemorrhage. All of the evaluations were performed on six fields per section. Finally, the total scores of six field were the histopathological scores of the lung.

Reagents

LPS was purchased from the Beijing Solaibao Technology Co., Ltd (L8880, Beijing, China). The rat TNF-α, CRP and IL-1 ELISA kits were purchased from the purchased from the Beyotime Institute of Biotechnology (PT516, PC188, PI563, Jiangsu, China). The rabbit polyclonal anti-rat CD41 antibody was purchased from the Abcam Company (ab203189, Cambridge, MA, USA). The rabbit polyclonal anti-rat CD45 antibody was purchased from the Abcam Company (ab10558, Cambridge, MA, USA). The fluorescence immunohistochemical mouse/rabbit kit (pH9.0) was purchased from the immunoway Company (RS0036, Plano, TX, USA). PE-anti-CD45 was purchased from the MultiSciences (Lianke) Biotech Company (AR04504, Hangzhou, Zhejiang, China). FITC-anti-CD41 was purchased from the Abcam Company (21851, Cambridge, MA, USA). APC-anti-CD62P was purchased from the Biolegend Company (148303, San Diego, California, USA).

Statistics

Data were analyzed using SPSS 16.0 software. All data are expressed as mean ± SE of mean values and compared using the unpaired Student's t-test. A $P<0.05$ was considered to be statistically significant.

Yun Long was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).

Results

Inflammatory cytokines, platelet function, wet/dry, PaO2

Plasma CRP, IL-1 and TNF-α levels in the ARDS group increased significantly compared to the Control group ($P<0.05$). Plasma CRP, IL-1 and TNF-α levels in the ARDS + aspirin group, ARDS + clopidogrel group and ARDS + tirofiban group decreased significantly compared to the ARDS group ($P<0.05$). Platelet function and the ratio of lung wet/dry in the ARDS group increased significantly compared to the Control group ($P<0.05$). Platelet function and the ratio of lung wet/dry in the ARDS + aspirin group, ARDS + clopidogrel group and ARDS + tirofiban group decreased significantly compared to the ARDS group ($P<0.05$). PaO2 in the ARDS group decreased significantly compared to the Control group ($P<0.05$). PaO2 in the ARDS + aspirin group, ARDS + clopidogrel group and ARDS + tirofiban group increased significantly compared to the ARDS group ($P<0.05$) (Fig. 1).

Platelet activation and PLA in the peripheral blood

In the peripheral blood, platelet activation and PLA in the ARDS group increased significantly compared to the Control group ($P<0.05$). Platelet activation and PLA in the ARDS + aspirin group, ARDS + clopidogrel group and ARDS + tirofiban group decreased significantly compared to the ARDS group ($P<0.05$) (Fig. 2).

PLA in the lung

In the lung, PLA in the ARDS group increased significantly compared to the Control group ($P<0.05$). PLA in the ARDS + aspirin group, ARDS + clopidogrel group and ARDS + tirofiban group decreased significantly compared to the ARDS group ($P<0.05$) (Fig. 3).

Histopathological scores of the lung

At 6 h after ARDS operation, obvious histological damage was observed in the lungs. These damages included intra alveolar oedema, interstitial inflammatory cell infiltration along the septa, epithelial and endothelial cells injury, hyaline membranes formation, and hemorrhaging. A semi-quantitative score of the histological parameters were evaluated using injury scores. Aspirin, clopidogrel and tirofiban alleviated the histological damages in early septic ARDS ($P<0.05$) (Fig. 4).

Discussion

Platelet activation is a prerequisite for platelet-leukocyte interactions and subsequent regulation of immune responses. Platelet activation results in P-selectin onto

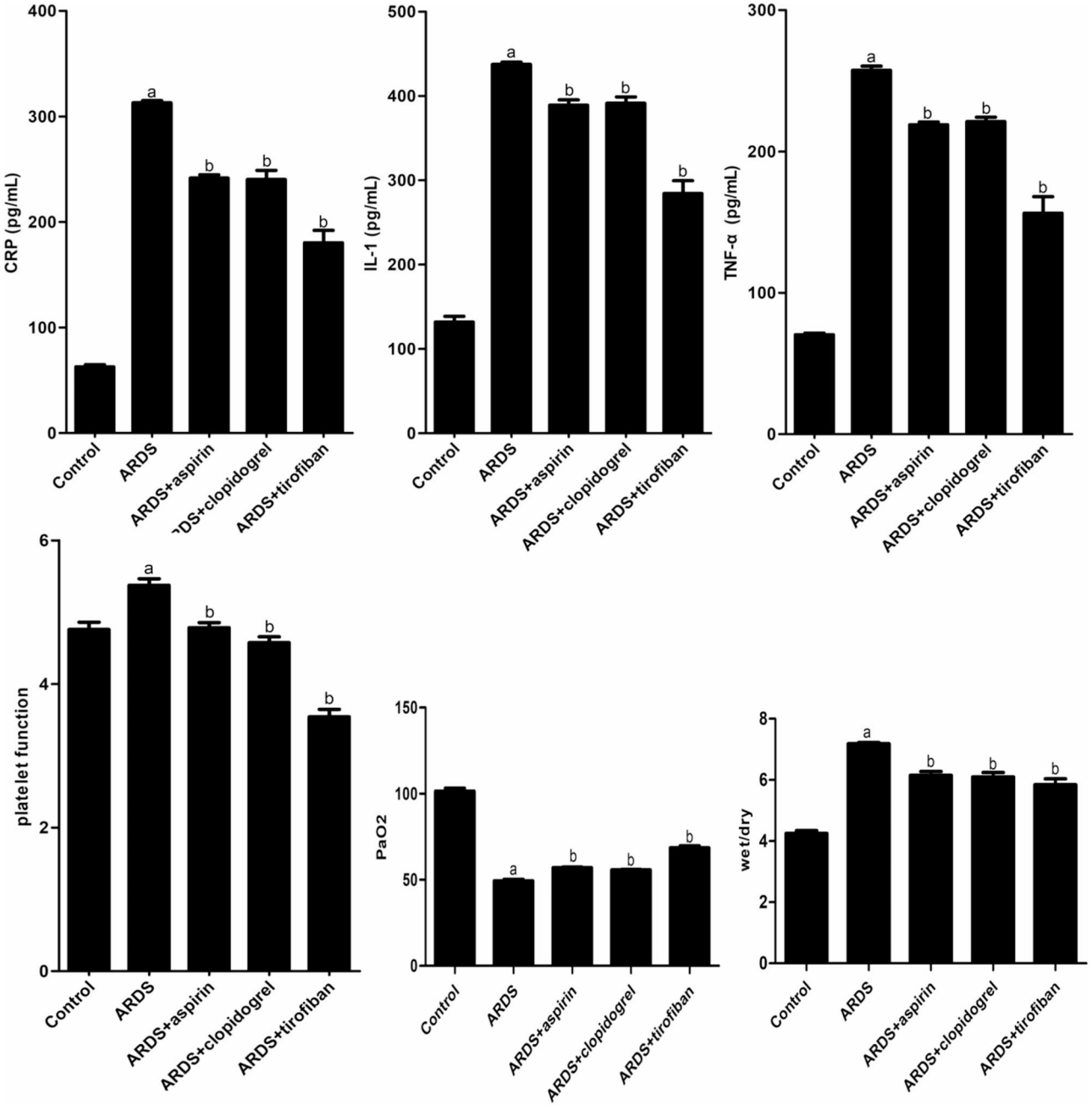

Fig. 1 Inflammatory cytokines, platelet function, wet/dry, and PaO2. Results are presented as mean ± SD ($n = 12$). a = $P < 0.05$ compared to the Control group, b = $P < 0.05$ compared to the ARDS group

platelet membranes, where binding to P-selectin glycoprotein ligand-1 on neutrophils and monocytes initiates, resulting in the formation of PLAs. Subsequently, activated platelets rapidly result in thrombocytopenia [17, 18]. PLA are involved in inflammation and coagulation by promoting the formation of neutrophil extracellular traps and bacterial phagocytosis, leading to organ dysfunction, especially in vascular-rich organs such as the lungs [19]. In this study, the expression of P-selectin in septic ARDS rats was significantly increased and there was obvious inflammatory injury in the lungs. These results are consistent with those reported in the literature.

Due to the important role of platelet activation in lung injury, antiplatelet drugs have important application prospects in the treatment of ARDS [20]. Aspirin has a beneficial role in the prevention and treatment of ARDS [21]. Inhibition of platelet PI3K signaling prevented leukocyte infiltration into the bronchoalveolar compartment during acute lung injury [22]. In septic mice, blocking P-selectin glycoprotein ligand 1 significantly

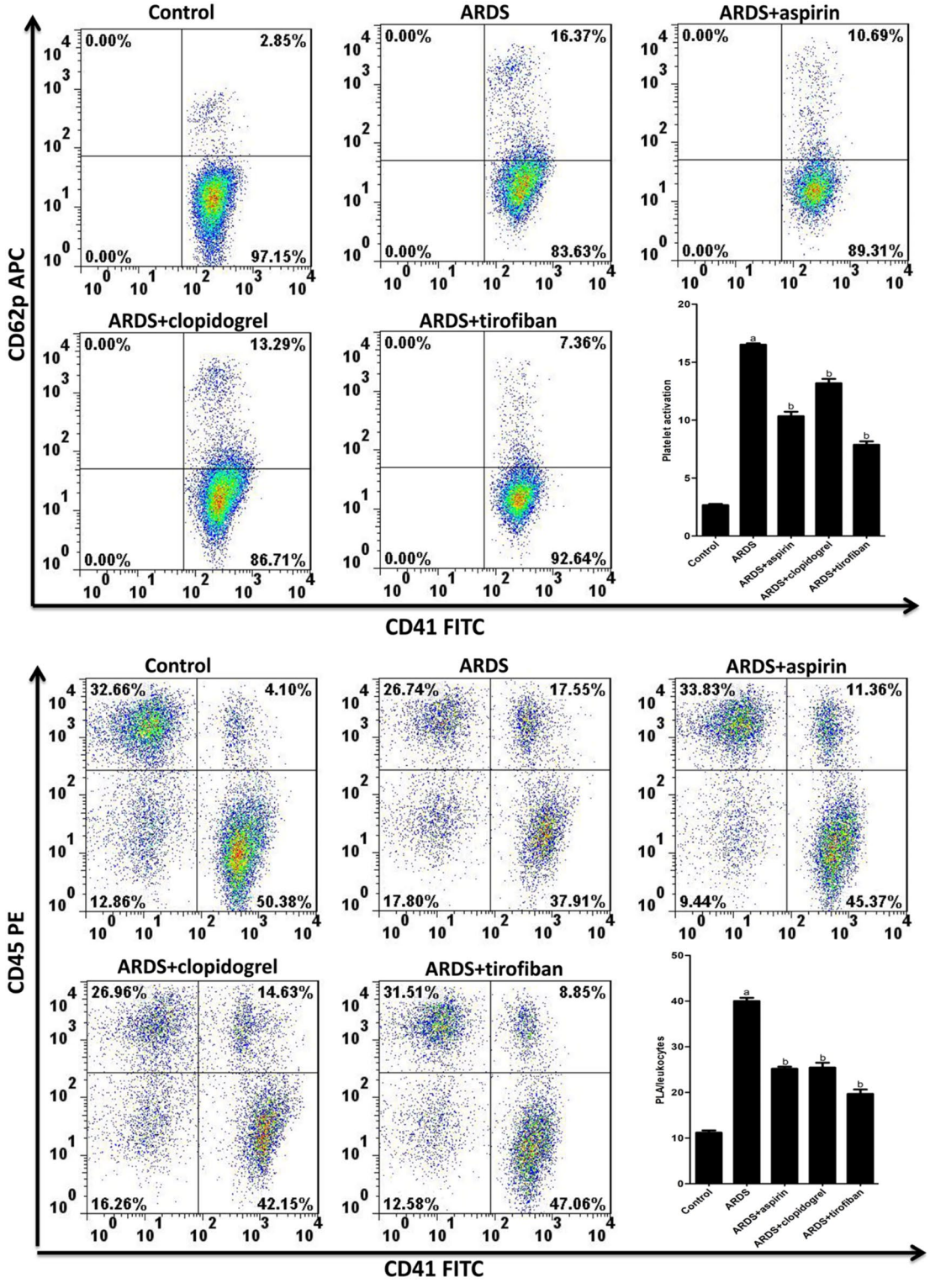

Fig. 2 Platelet activation and platelet-leukocyte aggregate (PLA) in the peripheral blood. Results are presented as mean ± SD ($n = 12$). a = $P < 0.05$ compared to the Control group, b = $P < 0.05$ compared to the ARDS group

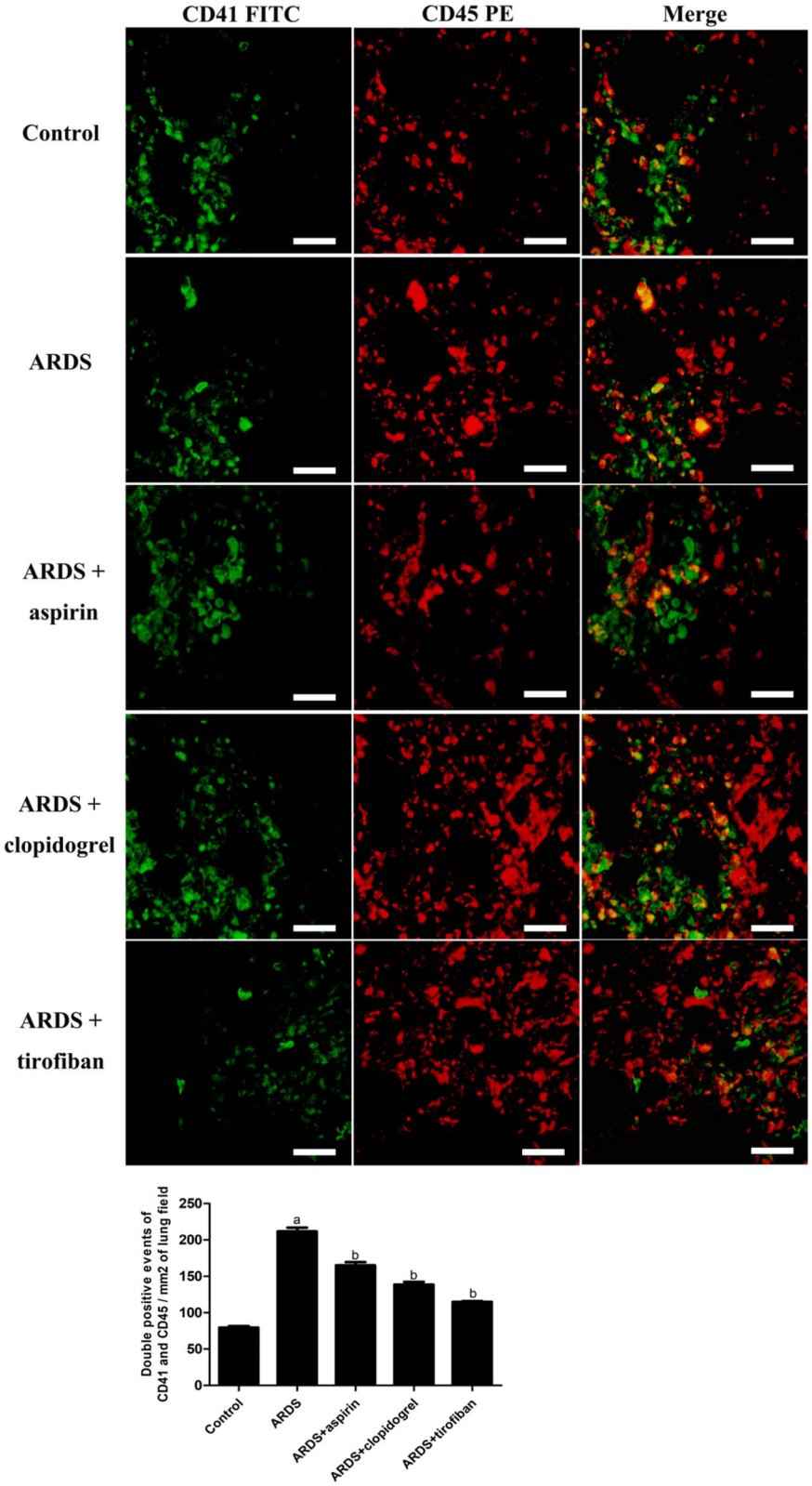

Fig. 3 Platelet-leukocyte aggregate (PLA) in the lung. Scale bars: 20 μm. Results are presented as mean ± SD ($n = 12$). a = $P < 0.05$ compared to the Control group, b = $P < 0.05$ compared to the ARDS group

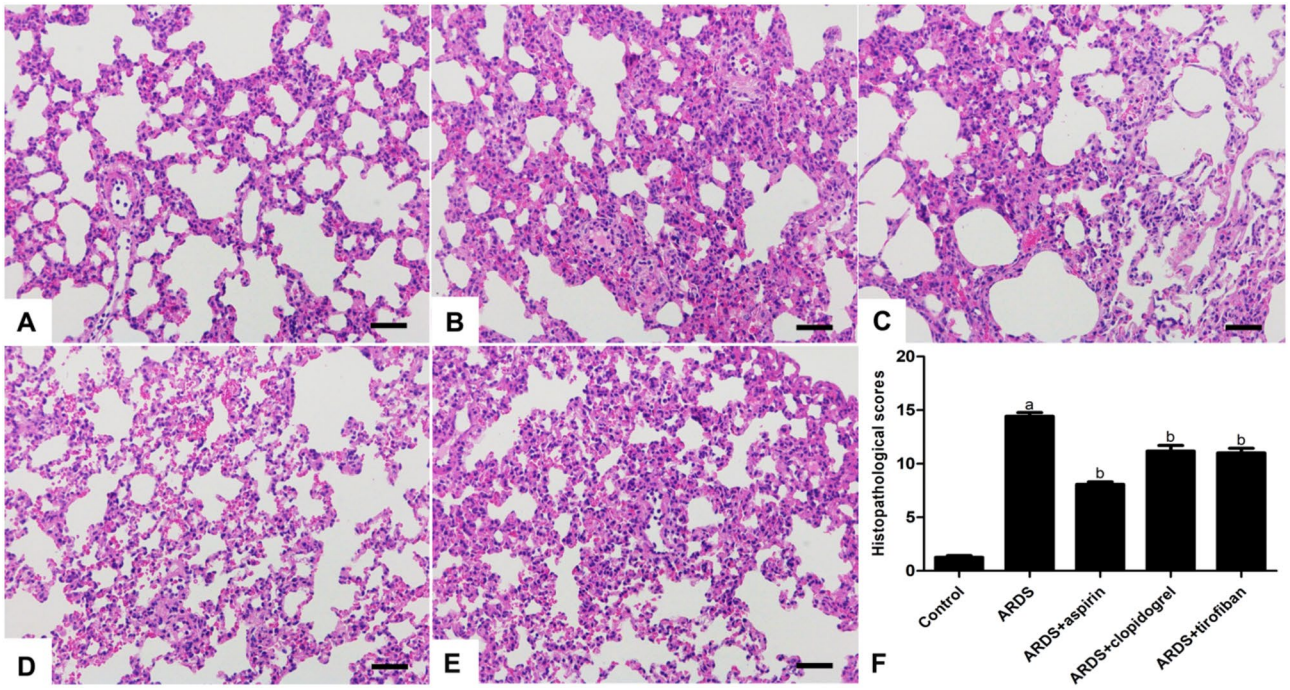

Fig. 4 Histopathological scores of the lung. Scale bars: 100 μm. Results are presented as mean ± SD ($n = 12$). a = $P < 0.05$ compared to the Control group, b = $P < 0.05$ compared to the ARDS group

alleviated lung injury and improved survival [23]. Aspirin can attenuate ventilator-associated lung injury [24] and hyperoxia-induced acute lung injury [25]. Aspirin may reduce the risk of ARDS in high-risk patients [26]. In our study, aspirin, clopidogrel and tirofiban reduced platelet function, platelet activation and PLA formation in early septic ARDS. These results clarified the specific effect of antiplatelet therapy on platelets in septic ARDS.

Inflammation and edema are important manifestations of lung injury in septic ARDS. Aspirin reduces lipopolysaccharide-induced pulmonary inflammation in ARDS [27]. Aspirin attenuates hyperoxia-induced ARDS by suppressing pulmonary inflammation via the NF-κB signaling pathway [28]. Clopidogrel reduces cytokine concentrations (TNF a, IL-1, IL-6) and reduces lung tissue damage in lung tissue of septic mice by regulating pro-inflammatory and oxidative stress cascade signaling pathways [29]. In our study, aspirin, clopidogrel and tirofiban alleviated the inflammatory response and pulmonary edema in early septic ARDS. These results confirm the effect of antiplatelet therapy on the inflammatory response in septic ARDS.

Aspirin treatment before ICU admission is associated with significantly reduced 30- and 90-day mortality rates and decreased length of ICU stay in patients with ARDS [30]. Aspirin therapy in patients with acute respiratory distress syndrome (ARDS) is associated with reduced intensive care unit mortality [31]. Clopidogrel attenuated LPS-induced lung injury in mice [32]. In mice of transfusion-associated acute lung injury, tirofiban can improve coagulation and fibrinolysis abnormalities by inhibiting platelets, reduce lung injury, and improve survival [33]. In our study, aspirin, clopidogrel and tirofiban alleviated hypoxemia and lung injury in early septic ARDS. These results further confirmed the lung-protective effect of antiplatelet therapy in patients with ARDS.

There are some limitations to our study. First, only effects of the intervention were observed, and the mechanism was not involved. There are several possible mechanistic insights involved in the protective effects of these antiplatelet drugs at the molecular level. Aspirin is currently the most widely studied and widely used drug in antiplatelet therapy, mainly by inhibiting arachidonic acid cyclooxygenase to block the synthesis of thromboxane A2 to exert antiplatelet effects. Aspirin inhibits surface GP IIb/IIIa and P-selectin expression on human platelets, inhibiting platelet activation [34, 35]. Irreversibly binding to the P2Y12 adenosine diphosphate receptor on the platelet surface, clopidogrel prevents the exposure of the binding site of the GP IIb/IIIa receptor coupled to the adenosine diphosphate receptor, making the ligand unable to bind and the aggregation of platelets inhibited. Clopidogrel can inhibit the formation of PLA by decreasing the expression of P-selectin on the surface of platelets in patients with atherosclerosis [36–38]. Tirofiban is a specific non-peptide GP IIb/IIIa receptor antagonist that inhibits platelet aggregation by mimicking GP IIb/IIIa receptor recognition of arginine-glycine-aspartate

(RGD) peptides. Tirofiban induces vascular endothelial growth factor (VEGF) production and stimulates migration and proliferation of endothelial cells [39]. Tirofiban counteracts endothelial cell apoptosis through the VEGF/VEGFR2/pAkt axis [40]. Endothelial cells are closely related to platelet activation [41], and it is unclear whether the above effects are related to platelet activation. Further studies are needed to identify the specific mechanism by which antiplatelet therapy mitigates early septic ARDS. Second, the observation time was limited so we cannot ensure the relationship between time and effects of antiplatelet therapy on septic ARDS. Third, the six-hour post-treatment observation period cannot fully capture the longer-term effects and delayed responses of antiplatelet therapies. Extending the observation period are needed in further study.

Conclusions

Aspirin, clopidogrel and tirofiban alleviated the inflammatory response and pulmonary edema, reduced platelet function, and alleviated hypoxemia in early septic ARDS. Aspirin, clopidogrel and tirofiban reduced platelet activation and PLA formation in early septic ARDS. Through these above effects, aspirin, clopidogrel and tirofiban ultimately alleviated lung injury in early septic ARDS.

Abbreviations

PLA	Platelet-leukocyte aggregate
NETs	Neutrophil extracellular traps
ARDS	Acute respiratory distress syndrome
MODS	Multiple organ dysfunction syndrome
GP	Glycoprotein
SPF	Specific pathogen free
IACUC	Institutional Animal Care and Use Committee
LPS	Lipopolysaccharide
ELISA	Enzyme-linked immuno sorbent assay
CRP	C reactive protein
IL-1	Interleukin-1
TNF-α	Tumor necrosis factorα
EDTA	Ethylene diamine tetraacetic acid
PBS	Phosphate buffered saline
HRP	Horseradish peroxidase
VEGF	Vascular endothelial growth factor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40360-024-00806-x.

Supplementary Material 1

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Author contributions

L.W., L.Y.M., X.Y.C., H.W.H. and Y.L. drafted the manuscript. L.W., L.Y.M., X.Y.C., and H.W.H. participated in the surgical procedure. L.W. and H.W.H. performed the statistical analysis. Y.L. conceived of the study, and participated in the design of the study. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval

This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Peking Union Medical College, Beijing, China. (IACUC protocol number: XHDW-2023-029).

Consent for publication

All authors have agreed to publish it.

Competing interests

The authors declare no competing interests.

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