Ticagrelor alleviates pyroptosis in myocardial ischemia reperfusion-induced acute lung injury

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Research Article

Keywords: myocardial ischemia-reperfusion injury, acute lung injury, pyroptosis, NLRP3, ticagrelor

Posted Date: April 27th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1550421/v1

Abstract

Background: Pulmonary infection is highly prevalent in patients with acute myocardial infarction undergoing percutaneous coronary intervention. However, the potential mechanism is unknown. Myocardial ischemia-reperfusion injury (MIRI) was shown to induce acute lung injury (ALI) related to pulmonary infection and inflammation. Whether MIRI induces pyroptosis in the lungs remains unclear.

Method: Sprague-Dawley rats were randomly divided into four groups: control, MIRI, low ticagrelor (30 mg/kg), and high ticagrelor (100 mg/kg). Rats were treated with ticagrelor or saline via intragastric gavage before undergoing surgery. Serum parameters of CK-MB and LDH were measured using automatic biochemistry analyzers. HE staining to obtain HE scores was performed following the calculation of the wet-to-dry ratio. NLRP3, ASC, and cleaved caspase-1 in lung tissue were detected by western blot, and IL-1β was assessed by ELISA. Immunohistochemistry was used to determine the MPO^+, $NLRP3^+$, and cleaved $caspase\text{-}1^+$ area.

Results: The HE score, wet-to-dry ratio, and MPO^+ area were increased in the MIRI group, and attenuated after ticagrelor treatment. Pyroptosis-associated proteins including NLRP3, ASC, and cleaved caspase-1 were elevated in MIRI, and eliminated by ticagrelor. Similar results were observed using immunohistochemistry assays.

Conclusions: Pyroptosis was augmented in lung tissue after MIRI, and pre-treatment with ticagrelor attenuated these effects.

Background

Pulmonary infection is highly prevalent in patients with acute myocardial infarction (AMI) undergoing percutaneous coronary intervention, which significantly increases mortality despite the use of antibiotics[1–3]. The potential mechanism is critical, yet remains unexplored.

Previous studies have shown that myocardial ischemia-reperfusion injury (MIRI) was capable of inducing acute lung injury (ALI) and activating the apoptosis pathway[4, 5]. ALI typically presents as the characteristic features of neutrophilic infiltration and diffuse alveolar damage, and it has been associated with pneumonia development [6]. In the experimental models, ALI was widely used to describe the lung damage[7–9]. Pyroptosis is a type of discovered programmed cell death, closely related to the inflammatory response, and a novel potential treatment target for inflammatory diseases, including AMI[10]. NLRP3 plays a central role in pyroptosis-its regulation is associated with various infectious pulmonary diseases[11]. Together, pyroptosis and NLRP3 may be active in lung injuries after MIRI, and this phenomenon has not been reported before.

Ticagrelor is a potent anti-platelet agent in patients with myocardial infarction[12], and it might reduce infection occurrences in the clinic[13, 14]. A growing number of studies have also shown that ticagrelor can alleviate the ALI and/or inflammatory reaction, even against bacteria in vivo[15–17]. Some studies

demonstrated that ticagrelor leads to the inhibition of pyroptosis-associated proteins, such as NLRP3, adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1 in cells as well as several experimental models[18–21]. However, none of these studies focused on the therapeutic effects of ticagrelor in ALI. Therefore, we aimed to investigate the MIRI-induced pyroptosis in a rat model by activating the NLRP3 inflammasome and assessing the effect of ticagrelor under these conditions.

Methods

Animals

Pathogen-free male Sprague-Dawley (SD) rats weighing 250–300 g (8–12 weeks old) were

purchased from the Guangdong experimental animal center in China. The rats were maintained under sterile conditions and provided food and water ad libitum. The study protocol was in compliance with the legislation on protecting of animals and was approved by the research ethics committee of Guangdong Provincial People's Hospital (No. GDREC2018063H(R1)).

Experimental protocol

The rats were randomly separated into the following four groups: control (n = 6), MIRI (n = 6), low ticagrelor (30 mg/kg; n = 6), and high ticagrelor (100 mg/kg; n = 6). Ticagrelor was dissolved and given by intragastric gavage 2 hours before MIRI surgery[17]. Rats in the control and MIRI groups were administrated with equal volumes of saline.

The rats were administered with pentobarbital sodium (50 mg/kg) by intraperitoneal injection, and then the hearts were exposed by surgery. Myocardial ischemia was induced by left anterior descending coronary artery ligation using a 7/0 nylon suture, at about 2 mm below the left auricle. The ligations lasted for 30 minutes. Rats were euthanized after another 120 minutes of reperfusion. Serum parameters of CK-MB and LDH were measured with automatic biochemistry analyzers and kits (Changchun huili, C060-e and C018-e), which aimed to confirm the success of the MIRI surgery. The blood samples and tissues were pre-treated and then stored at -80°C.

Wet to dry weight ratio

The left lung was used to calculate the wet-to-dry weight (W/D) ratio. The wet weight was measured immediately after excision. The lung tissue was then preserved in an oven at 65°C for 72 hours to obtain the corresponding tissue dry weight.

HE staining and damage evaluation

The right lung tissue was immersed in 10% paraformaldehyde for 48 hours, and then embedded in paraffin blocks and cut into 4-μm sections. The paraffin sections were stained with H&E.

The pathological sections were observed with an optical microscope. Lung damage was evaluated by two independent blinded pathologists, with three horizons randomly obtained from each section based on the hemorrhage, edema, congestion, and inflammation. The extent of lung damage was assessed with one of the following scores: 0, no obvious damage; 1, slight damage; 2, moderate damage; 3, severe damage; and 4, maximal damage.

Western blotting

The protein samples of lung tissue were extracted, and the concentration measured using the BCA protein determination method. Samples containing the same amount of protein (20–30 μg) were fractionated by SDS-PAGE, then transferred to a nitrocellulose membrane (Gelman Laboratory, Ann Arbor, MI, USA). Membranes were blocked with 5% BSA for 1 hour and then incubated at 4°C overnight with following primary antibodies from Abcam: NLRP3 (1:500; Cat# ab214185), cleaved caspase-1 (1:2000, Cat# ab179515), and ASC (1:1000, Cat# ab18193); β-actin (1:2000, Cat# ab8227) was used as an internal control. Membranes were then incubated with goat anti-rabbit IgG antibody (1:5000, Cat# ab6721) at room temperature for 1 hour. After the washing steps, electrochemiluminescence reagents (Merck Millipore, Hayward, CA, USA) were used and the immunoreactive bands were visualized with a fluorescence system. Band densities were calculated using Image-J software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

Paraffin sections of lung tissue were used following standard immunohistochemistry steps. The primary antibodies of myeloperoxidase (MPO) (Servicebio, 1:500; Cat# GB11224), NLRP3 (Abcam, 1:400; Cat# ab214185) and cleaved caspase-1 (Affinity, 1:200, Cat# AF4022) were added to the sections and incubated at 4°C overnight, followed by the incubation of FITC-labeled secondary antibodies for 1 hour at room temperature in the dark. All of the images were obtained using the optical microscope. The percentage of positive areas were measured with Image-J software.

Statistical analysis

Results are presented as the mean ± SD. One-way ANOVA, followed by the LSD test, was used for the comparison between the groups when the data followed the Gaussian distribution and homogeneity of variance; otherwise, the Kruskal-Wallis test was used, followed by Dunnett's T3 test. The P values were reported according to the LSD or Dunnett's T3 tests. GraphPad Prism 8.0 software (La Jolla, CA) was used for the statistics analysis; two tails and $P < 0.05$ was considered to be statistically significant.

Results

Ticagrelor attenuates MIRI-induced ALI

The serum myocardial markers were significantly increased when including LDH by 1.7-fold ($P < 0.01$) and CKMB ($P < 0.01$) by 2.5-fold following the occurrence of MIRI compared to the control group, which

generally implies surgery success. These alterations were attenuated by pre-treatment with high or low ticagrelor ($P < 0.01$ with MIRI group) (Fig. 1a-b).

Elevated W/D ratio was observed in MIRI rats when compared to the control group, indicating that MIRI accelerated the pulmonary edema ($P < 0.01$). W/D ratios were significantly reduced in both ticagrelor groups ($P < 0.01$ compared with MIRI group) (Fig. 2a). Additionally, the deteriorated histology of lung tissue, including hemorrhage, edema, congestion, and inflammation, was detected using HE staining and scores ($P < 0.01$) (Fig. 2b and 2d).

The MPO staining showed that MPO^+ cell infiltration was significantly increased in the MIRI group and a decline was present in both the low and high ticagrelor groups (both $P < 0.01$) (Fig. 2c and 2e). Together, these results verified the occurrence of ALI after MIRI, while pre-treatment with ticagrelor attenuated the pathological changes of ALI.

Ticagrelor attenuates MIRI-induced pyroptosis, as indicated by western blot and ELISA

The western blot analysis showed that a rise in pyroptosis-associated proteins (NLRP3 2.1-fold, $P < 0.05$; ASC 3.0-fold, $P < 0.01$, and cleaved caspase-1 9.0-fold, $P < 0.01$) was obtained in the MIRI group when compared to the control (Fig. 1d-g). Consistent with these observations, the release of IL-1β in lung tissue was elevated by 1.5-fold ($P < 0.01$) (Fig. 1c).

Given the therapeutical effect of ticagrelor in ALI, we found that pre-treatment with high ticagrelor markedly reduced the expressions of NLRP3 (0.46-fold reduction, $P < 0.05$), ASC (0.64-fold reduction, $P < 0.01$), cleaved caspase-1 (0.80-fold reduction, $P < 0.01$), and IL-1β (0.09-fold reduction, $P < 0.01$), when compared to the MIRI group (Fig. 1c-g). The low concentration of ticagrelor also exhibited a trend of reducing NLRP3 (0.22-fold reduction), ASC (0.28-fold reduction), cleaved caspase-1 (0.42-fold reduction), and IL-1β (0.04-fold reduction).

Immunohistochemical analysis demonstrates ticagrelor attenuates MIRI-induced pyroptosis

Additionally, immunohistochemical assay confirmed that the $NLRP3^+$ and cleaved $caspase\text{-}1^+$ area was significantly increased in the MIRI group compared to the control group ($NLRP3^+$: $P < 0.05$, cleaved $caspase\text{-}1^+$: $P < 0.01$). The positive area of NLRP3 was reduced in both high and low ticagrelor groups ($P < 0.05$), while the positive area of cleaved caspase-1 was reduced in the high ticagrelor group ($P < 0.01$) and showed a trend of reduction in the low ticagrelor group (Fig. 3a-d).

Discussion

The present study discovered that the pyroptosis and pyroptosis-associated proteins NLRP3, ASC, and caspase-1 were increased in lung tissue after MIRI. Pre-treatment with ticagrelor significantly attenuated

these alterations.

A number of studies have shown that MIRI was able to induce ALI in rat models, which is consistent with our results[4, 5, 22, 23]. The lung appeared to be the frailest organ influenced by MIRI. Triggered by the clinical phenomenon of high pulmonary infection incidence in patients with AMI, we further investigated the MIRI-induced pyroptosis in the lungs. Organs' ischemia-reperfusion injury is known to increase the local expression of pyroptosis[24, 25]. However, studies that evaluate one organ ischemia-reperfusion-induced pyroptosis in another organ are rare. Zhao et al. revealed that renal ischemia-reperfusion induced ALI and pyroptosis in lungs[26], while similar results were found in limb ischemia-reperfusion [27]. Hailin and colleagues also showed that renal graft ischemia–reperfusion injury could lead to pyroptosis in the remote liver[28]. In the present study, we found that pyroptosis occurred in lungs after MIRI, and the following two features might explain this observation. First, MIRI produced circulating cellular debris, which was recognized as a damage-associated molecular pattern, which activated NLRP3 and its downstream signaling pathway[29]. Second, MIRI generated pro-inflammatory cytokines, including TNF, that might also induce NLRP3-associated pyroptosis[30]. Thus, targeting these features may reduce the lung damage after MIRI. Further evaluation of the molecular mechanism is required.

Similar to our findings, previous studies found that ticagrelor attenuated NLRP3 in other animal models. Yochai and colleagues showed that after treating diabetic ZDF rats with ticagrelor (150 mg/kg daily) for 3 days following MIRI, the level of NLRP3 mRNA was significantly reduced in the ticagrelor group than the control, and ticagrelor reduced the caspase-1 expression in cardiomyocyte as detected by immunoblots[20]. Later, Huan conducted ticagrelor management of daily 100 mg/kg administrations for 12 weeks in mice with type 2 diabetes mellitus, and found that ticagrelor reduced the myocardial NLRP3, caspase-1, and GSDMD-N levels, and attenuated the progress of diabetic cardiomyopathy[21]. Notably, compared with SD rats, diabetes mellitus rats have more interaction with pyroptosis[31]. For this reason, the effect of ticagrelor in diabetes mellitus rats might be attenuated in SD rats, and caution should be exercised when extrapolating to SD rats. Regarding nondiabetic research, Claudia and colleagues conducted a MIRI model and showed that ticagrelor administration for 3 days (150 mg/kg daily) reduced the myocardial expression of NLRP3 and protected the heart. The effects disappeared when isolated myocardial cells were treated. Thus, they concluded that ticagrelor may impart protective effects on the heart through platelets instead of targeting the myocardium directly[19]. This result partly contrasts with the research from another study that showed 50 mg/kg of ticagrelor inhibited the activation of NLRP3 inflammasome by attenuating the oligomerization of ASC in macrophages, and reduced inflammation, including IL-1β, in alum-induced peritonitis and lipopolysaccharide-induced sepsis mice models independent of the $P2Y_{12}$ signaling pathway[18]. The differences might be due to the methodology, which mainly involved the myocardium in the former study versus macrophages, peritoneal cavity lavage fluid, and serum sample in the latter study. This notion indicates ticagrelor has diverse pathways regulating NLRP3 inflammasome in tissues and cells, and thus indicates the need for assessing ticagrelor in various models. Our study reported ticagrelor-reduced NLRP3 depended on pyroptosis in lung tissue. The dosage

of 30 mg/kg and 100 mg/kg ticagrelor was hypothetically generated on the basis of this concentration attenuating ALI[17], and the doses were relatively low when compared to the aforementioned studies.

This study also has some limitations. First, we built the 120-minute ischemia and following 30-minute reperfusion protocol, which was a common protocol to establish MIRI, and a previous study observed apoptosis in lungs using this protocol[5]. However, other timepoints for building a MIRI-induced lung pyroptosis model were not assessed. Second, the interaction between apoptosis and pyroptosis was not investigated in this study. Third, we only preliminarily revealed the association between MIRI and pyroptosis; the molecular mechanism needs to be further explored.

Conclusions

We detected pyroptosis in the lung after MIRI as determined by pathological morphology and elevation of pyroptosis-associated protein in lung tissue. Administration with ticagrelor might attenuate these effects.

Abbreviations

AMI: acute myocardial infarction;

MIRI: myocardial ischemia-reperfusion injury;

ALI: acute lung injury;

ASC: adaptor protein apoptosis-associated speck-like protein containing a CARD;

SD: Sprague-Dawley;

W/D: wet-to-dry weight;

MPO: myeloperoxidase;

Declarations

Ethics approval and consent to participate: The study protocol was in compliance with the legislation on protecting of animals and was approved by the research ethics committee of Guangdong Provincial People's Hospital (No. GDREC2018063H(R1)).

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding: This work was supported by the Shuangqing Talent Program Project of Guangdong Provincial People's Hospital [Grant No. KJ012019095 to Y.H.L]. The funders had no role in the study design, data

collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: None.

Authors' contributions: Study concept and design: Y.H.L. and N.T.. Drafting of the manuscript: Y.N.D. and L.T.W.. Supervisors of the study and guarantee the study data and accuracy: P.C.H. and Y.H.L.. Acquisition, analysis, or interpretation of data: all authors. Critical revision of and final approval the manuscript: all authors.

Standards of reporting: Current study is reported in accordance with ARRIVE guidelines.

Acknowledgements: None.

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Figures

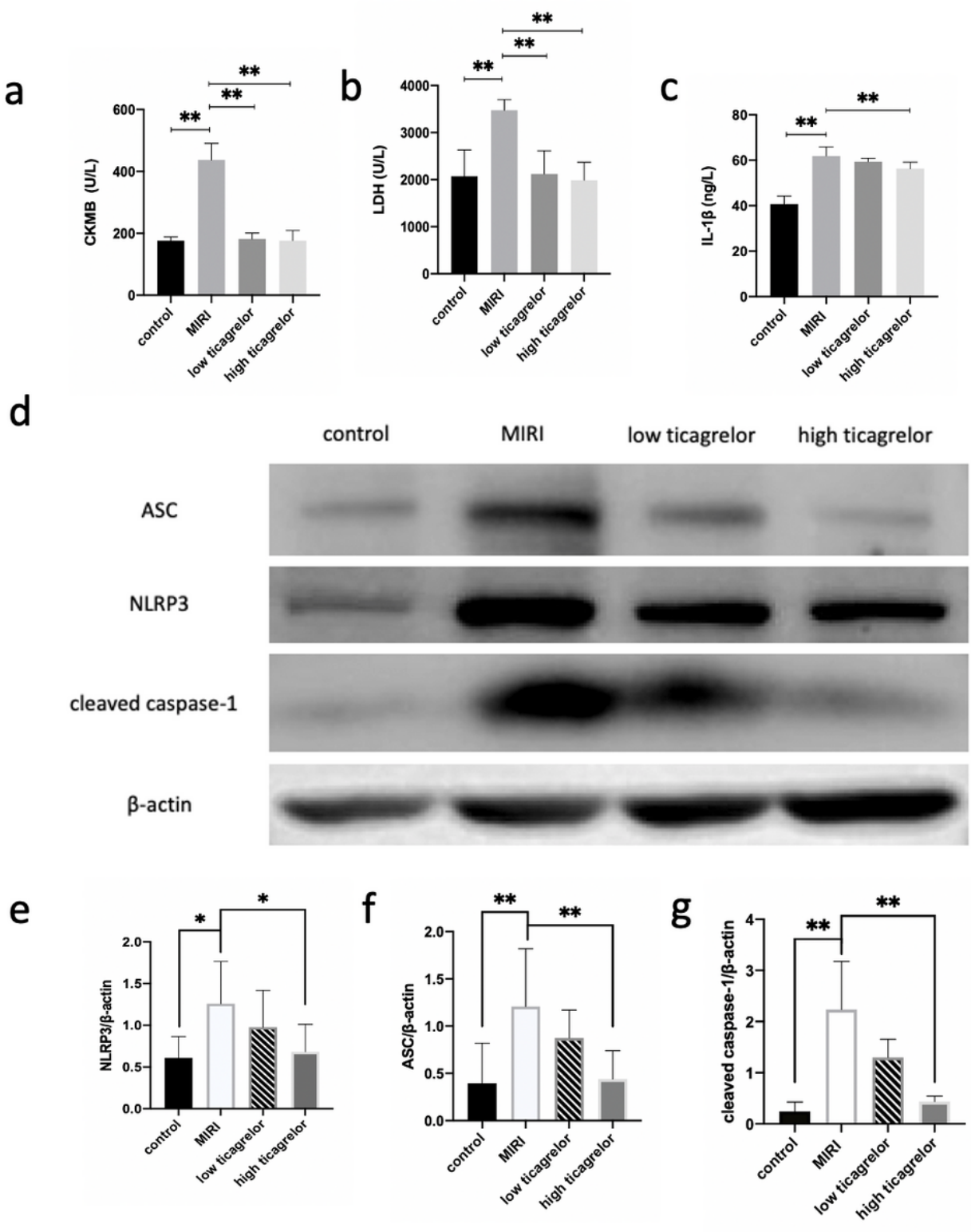

Figure 1

Expression of serum myocardial enzyme and lung pyroptosis-associated protein

a-b, CKMB and LDH secretion in serum. c, IL-1β release in lung tissue. d-g, Immunoblots for NLRP3, ASC and cleaved caspase-1 in lung tissue. Results are presented by mean±SD. Both n=6. Here shows the blot

of NLRP3 and β-actin from the same gel, which were difference with the gel of ASC and cleaved caspase-1; *P < 0.05, **P < 0.01.

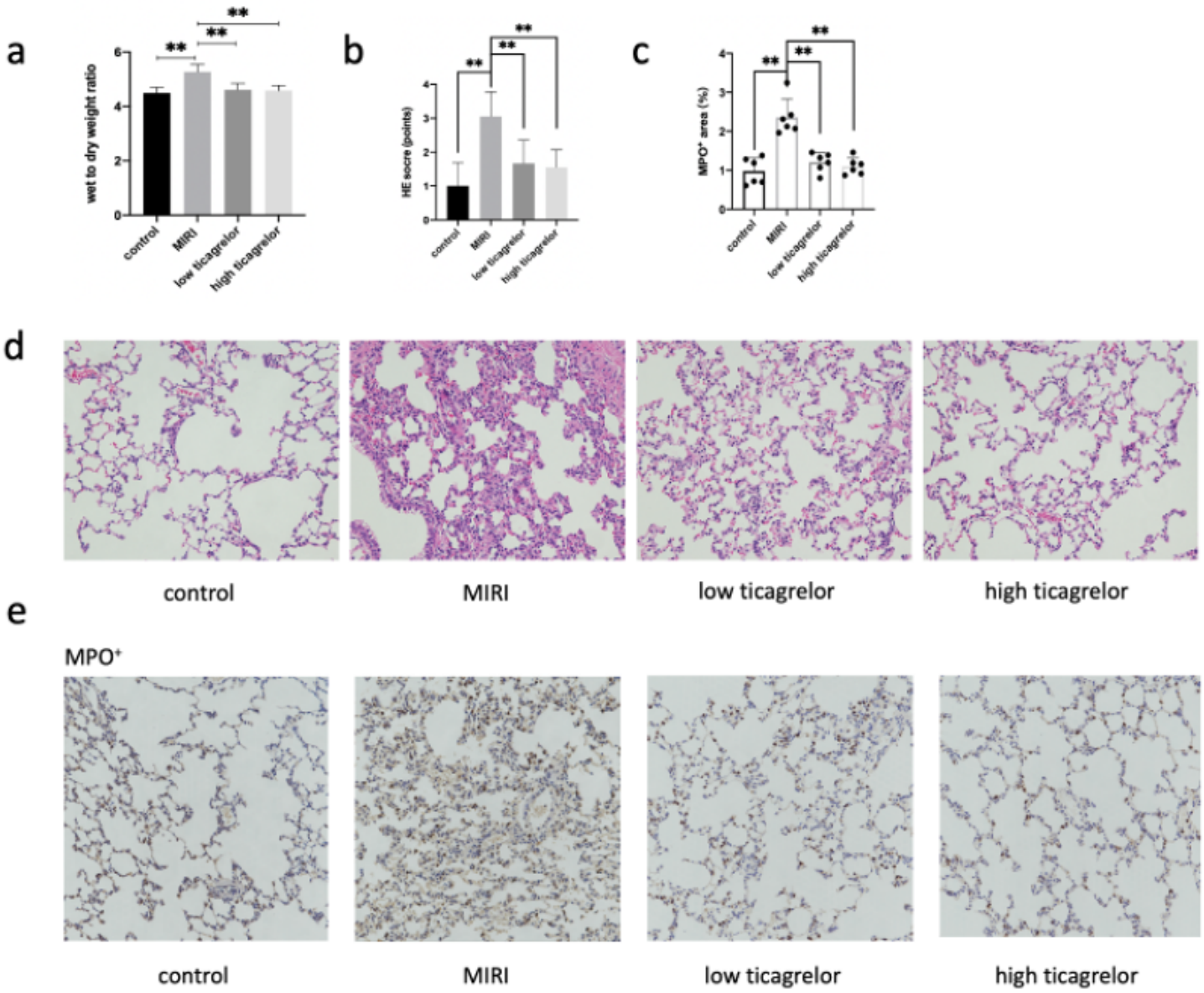

Figure 2

Expression of acute lung injury

a-b, Wet to dry weight (W/D) ratio (n=6), damage score (3 pieces from each sample are analysis) and neutrophil detected by MPO staining of lung tissue (6 pieces from 3 samples are analysis in each group).

c, HE staining showing neutrophil exudation, alveolar walls thickened accompanying with hemorrhage in MIRI, and attenuated by ticagrelor, original magnification is 200x;

d, HE score increased in MIRI and reduced when pre-treated with ticagrelor.

e-f, MPO staining showing MPO+ cells are obviously in MIRI and could be attenuated by ticagrelor; original magnification is 200x; Results are mean±SD. *P < 0.05, **P < 0.01.

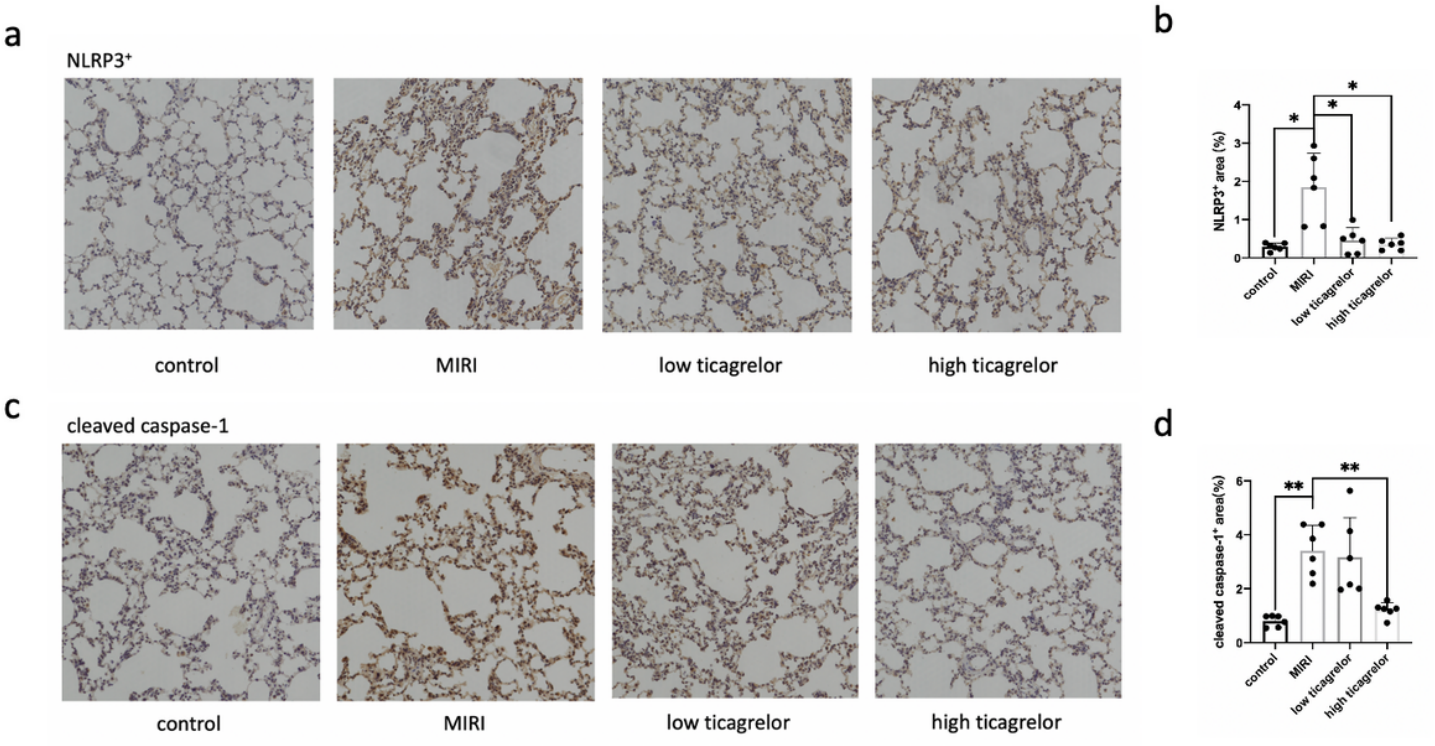

Figure 3

Immunohistochemical of pyroptosis-associated protein

a-b, immunohistochemical of NLRP3, and cleaved caspase-1 in lung tissue (6 pieces from 3 samples are analysis in each group). c-d, NLRP3+ and cleaved caspase-1+ area is increased by MIRI and is decreased when pre-treated with ticagrelor; original magnification is 200x; Results are mean±SD. *P < 0.05, **P < 0.01.

Supplementary Files

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- SupplementaryAppendix.docx