

Ticagrelor Attenuates Apoptosis of Lung and Myocardial Cells Induced by Abdominal Aorta Ischemia/Reperfusion

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Abstract. Aim: This study aimed to analyze the effect of ticagrelor pretreatment on the prevention of lung and heart injury induced by abdominal aorta ischemia and reperfusion (I/R) and also to determine the effective dose. Materials and Methods: Thirty-five male Sprague-Dawley rats weighing 350-400 g were randomized into five groups. The animals received ticagrelor at doses of 7.5 mg/kg, 15 mg/kg and 25 mg/kg or normal saline 0.1 ml/kg orally via gastric gavage before the ischemic period. In the control and study groups, I/R injury was induced by clamping the aorta infrarenally for 2 hs, followed by 4 h of reperfusion. After sacrifice, hearts and lungs of the animals were extracted for both histopathological and biochemical analysis. Results: There was a significant difference between the animals that received 7.5 mg/kg and 25 mg/kg and 15 mg/kg and 25 mg/kg dose of ticagrelor regarding tissue malonaldehyde (MDA), and glutathione reductase levels in both lung and heart. Ticagrelor treatment at 25 mg/kg led to significant cardiac remodeling activity and normal lung architecture against I/R induced injury. The number of TdT-mediated dUTP nick-end labeling (TUNEL)-positive cells in alveolar epithelium and myocytes were increased in the sections from saline (I/R) group rats, and decreased following 25 mg/kg ticagrelor treatment. Conclusion: Ticagrelor dose-dependently inhibits platelet aggregation, increases cyclooxygenase-2 and also inhibits cellular uptake of adenosine all resulting in

attenuation of I/R injury. Ticagrelor at 25 mg/kg was determined as the dose effective against I/R-induced injury in lung and heart in Sprague-Dawley rats in the present study.

Abdominal aortic surgery is one of the main procedures performed in clinical practice by cardiovascular surgeons. The main step in this procedure is the cross-clamping of the aorta that is 'ischemia' and declamping after completion of anastomosis that is 'reperfusion'. Reperfusion initiates both local and systemic damage, particularly through inflammatory mediators and rapid release of oxygen-free radicals, mainly from polymorphonuclear leukocytes (1, 2). These products may lead to dreadful complications during reperfusion and even death due to systemic inflammatory response and multi-organ failure (3). Remote organ damage after ischemia and reperfusion (I/R) mainly occurs in lung, kidney and heart.

Ticagrelor is a direct-acting antagonist of P2Y₁₂, a purinergic receptor of adenosine diphosphate (ADP) expressed by thrombocytes. P2Y₁₂ plays important roles in hemostasis and thrombosis (4, 5). It is essential for ADP-induced platelet aggregation and its defects result in bleeding (6, 7). Ticagrelor is therefore a widely used drug for the prevention of cardiovascular events and stroke (8). Ticagrelor is reported to inhibit cellular uptake of adenosine, a purine nucleoside produced by metabolism of ADP (9, 10). Adenosine levels in plasma increase after inflammation, injury or I/R (11). As ticagrelor inhibits cellular uptake of adenosine, the level of endogenous adenosine concentration increases, resulting in reduction of inflammatory markers (12).

This study was planned to analyze the effect of ticagrelor pretreatment on the prevention of lung and heart injury induced by abdominal aorta I/R and also to determine the effective dose for achieving this.

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Materials and Methods

This study was approved by the Institution of Animal Care and Use Committee at Kocaeli University (date and number: 2014/41) and complied with the Guide for the Care and Use of Laboratory Animals.

Thirty-five male Sprague-Dawley rats weighing 350-400 g (Kocaeli University, Kocaeli, Turkey) were randomized into five groups. The animals were initially anesthetized with intraperitoneal ketamine hydrochloride (Ketalar; Pfizer, Ortakoy, Istanbul, Turkey) 100 mg/kg bodyweight and received ticagrelor (Brilinta-Astra-Zeneca, Södertälje, Sweden at doses of 7.5 mg/kg, 15 mg/kg or 25 mg/kg, or 0.1 ml/kg normal saline orally *via* gastric gavage before the ischemic period. The abdomen was then explored through a midline incision after shaving and disinfection. In the sham group, only laparotomy was performed. In the control group and the study groups, I/R injury was induced by clamping the aorta with atraumatic vascular clamp infrarenally for 2 hours, followed by 4 h of reperfusion. Cessation of arterial flow was confirmed by means of the absence of an audible continuous-wave Doppler signal. At the end of these procedures, the animals were sacrificed with lethal injection of sodium thiopental (Pentothal Sodium, Abbot, Italy). Immediately after sacrifice, through midline sternotomy, the hearts and lungs of the animals were extracted and washed with 0.9% saline solution for both histopathological [hematoxylin-eosin staining and TdT-mediated dUTP nick-end labeling (TUNEL) method for detection of apoptosis] and biochemical analysis [malondialdehyde assay (MDA), glutathione reductase (GR) and glutathione peroxidase (GPx) assays].

Biochemical analysis. Heart and lung tissues were frozen immediately in liquid nitrogen and stored at -80°C until measurements were started. Twenty-micron-thick sections were prepared and dried under vacuum overnight (at 20°C). Freeze-dried sections were stored at -20°C until biochemical assays were undertaken.

Determination of MDA, GPx and GR levels were performed by enzyme-linked immunosorbent (ELISA) assay. The levels of these oxidant and antioxidant enzymes in heart and lung tissues were measured based on the Biotin double antibody sandwich technology (Bioassay Technology Laboratory, Shanghai, China). MDA concentration was expressed as nmol/ml [level of detection (LOD): 0.024 nmol/ml, level of quantification (LOQ): 10 nmol/ml] and the concentrations of GPx and GR were expressed as ng/ml (LOD: 0.23 ng/ml, LOQ: 200 ng/ml for GPx-1 and LOD: 0.24ng/ml, LOQ: 150 ng/ml for GR).

Histopathological examination. Tissue samples were fixed in 10% formalin and embedded in paraffin with routine follow-up procedure; 4-5 μm sections were cut from paraffin blocks and stained with hematoxylin and eosin (H-E) for light microscopic examination ($\times 200$).

For assessment of apoptosis in heart and lung tissues, ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit, S7101; EMD Millipore Corp., Temeculla, CA, USA) was used. In brief, formalin-fixed, paraffin-embedded tissue slides were rehydrated using xylene to alcohol washings, followed by a hydrogen peroxide-methanol quench. The samples were treated with 25 $\mu\text{g}/\text{ml}$ of proteinase K at 37°C for 15 min. After washing and incubation with equilibration buffer for 5 min, TdT was diluted 1:3.9 (14 μl of TdT enzyme in 40 μl of reaction buffer) and incubated on the slides for 1 hour at 37°C .

After applying stop solution for 15 min and washing, the samples were incubated with anti-digoxigenin peroxidase conjugate at 37°C for 30 min. Slides were developed with a 1:20 dilution of 3,3'-diaminobenzidine substrate, counterstained with methyl green, dehydrated, and coverslipped for detection of DNA fragmentation.

Statistical analysis. Statistical analyses were performed using SPSS 20 (IBM Corp, New York, USA). Values are expressed as mean \pm SD. Kruskal-Wallis nonparametric analysis of variance and Dunn's multiple-comparison tests were used to compare the groups receiving ticagrelor at various doses and the group receiving normal saline. *p*-Values of less than 0.05 were considered significant.

Results

Biochemical assay results. MDA was studied here as a marker of free radical-mediated lipid peroxidation. Animals that received ticagrelor showed a general trend of less lipid peroxidation product both in lung and cardiac tissues than the control group and this decrease was statistically significant (Table I).

Our results revealed that both GPx-1 and GR were elevated in ticagrelor-treated groups at all doses regarding both lung and heart tissues (GPx-1, $p=0.034$ and $p<0.0001$; and GR, $p=0.004$ and $p=0.014$, respectively, in all ticagrelor-treated groups).

Although there was no significant difference between the animals that received 7.5 mg/kg and those that received 15 mg/kg dose of ticagrelor regarding MDA, GPx-1 and GR levels in both lung and heart tissues, in animals that received 25 mg/kg compared with those 15 mg/kg and 7.5 mg/kg ticagrelor, MDA was significantly reduced, while GPx-1 and GR levels were significantly increased, in both lung and heart tissues (Table I).

Findings of myocardium stained with H-E. Histological changes in myocardium and lung alveolar sections are illustrated in Figure 1. The sham group exhibited normal cardiac architecture and arrangement (Figure 1a). There was disruption in cardiomyocytes, with fragmented and feathery appearance, heterogeneity in sarcoplasm, irregular arrangement, and increasing of interfibrillar distance of cardiomyocytes in the saline control (I/R) group (Figure 1b). Numerous coronary capillaries were seen in the saline control (I/R) group (Figure 1b) and the groups treated with 7.5 mg/kg or 15 mg/kg ticagrelor (Figure 1c and d) compared with the sham control group (Figure 1a). Increasing dose of ticagrelor gradually reduced feathery appearance, heterogeneity in sarcoplasm, irregular arrangement disruption of cardiomyocytes in treatment groups compared with saline control (I/R) group. In rats treated with 25 mg/kg ticagrelor, the histological appearance observed similar to that of the sham-operated group (Figure 1a-e). Treatment with 25 mg/kg of ticagrelor led to significant cardiac remodeling activity against I/R-induced

Table I. Lung and heart tissue malonaldehyde (MDA), glutathione peroxidase (GPx-1) and glutathione reductase (GR) levels compared between animals receiving different doses of ticagrelor before induction of ischemia and reperfusion. Kruskal–Wallis non-parametric analysis of variance and Dunn's multiple-comparison tests were used to compare the groups.

Tissue	Analyte	Sham	Control	Ticagrelor dose			p-Value		
				7.5 mg/kg	15 mg/kg	25 mg/kg	7.5 vs. 15 mg/kg	7.5 vs. 25 mg/kg	15 vs. 25 mg/kg
Lung	MDA nmol/ml	1.05±0.23	3.20±0.86	2.22±0.11	2.8±0.37	1.30±0.25	0.830	<0.0001	<0.0001
	GPx-1 ng/ml	31.66±9.86	30.20±5.39	34.48±13.03	33.29±14.18	47.56±9.33	0.875	0.048	0.032
	GR ng/ml	25.53±6.27	17.83±6.78	24.52±2.42	24.42±7.56	33.83±8.09	0.976	0.019	0.031
Heart	MDA nmol/ml	2.42±0.65	3.42±1.22	2.40±0.29	2.76±0.41	1.76±0.18	0.900	<0.0001	<0.0001
	GPx-1 ng/ml	41.29±3.75	32.31±8.51	40.90±4.51	42.78±7.02	50.34±3.44	0.331	0.001	0.016
	GR ng/ml	35.59±6.10	33.97±12.25	32.19±5.95	35.10±4.80	45.55±7.87	0.331	0.005	0.006

injury in rat heart sections resulting in approximately normal cardiac architecture, arrangement of myofibrils, and absence of necrotic areas (Figure 1a-e).

Findings of lungs stained with H-E. The sham group exhibited normal lung architecture and arrangement. The histological structure of pulmonary interstitial tissue and alveolus was intact and clearly visible in the sham group by light microscopy, without infiltration of inflammatory cells stained with H-E (Figure 1f). Obvious histological changes were observed in the saline (I/R) (Figure 1g) group compared to the sham group: atelectasis, thickening of alveolar interwall, infiltration of inflammatory cells. These changes continued in rats treated with 7.5 mg/kg of ticagrelor (Figure 1h). Treatment with 15 mg/kg of ticagrelor groups continued these changes a little more. Histological changes decreased in the group treated with 25 mg of ticagrelor (Figure 1j) compared with the saline (I/R) and other treatment groups; tissue injury was lower than that in saline (I/R) and 7.5 mg/kg ticagrelor treatment group (Figure 1f-j). Treatment with 25 mg/kg of ticagrelor treatment protected against I/R induced injury, with approximately normal lung architecture in lung sections with normal structure of pulmonary interstitial tissue and alveolus, and less infiltration of inflammatory cells (Figure 1f-j).

TUNEL assay findings. Apoptotic cells in myocardium and alveolar epithelium were counted in five different areas of slides in all groups. Following TUNEL staining, brown-stained cells were TUNEL-positive and considered to be apoptotic (Figure 2).

The number of TUNEL-positive cardiomyocytes were increased in the heart sections from saline (I/R) group rats, and decreased following 25 mg/kg ticagrelor treatment group (Figure 2b-e). Apoptotic cardiomyocytes was significantly lower in the 25 mg/kg ticagrelor treated group (0.0595±0.0058) compared with the saline (I/R)-treated

group (0.1408±0.0178). Compared with the sham group and treatment groups, the apoptotic index in the saline (I/R)-treated group (0.1722±0.0257) was significantly higher than in the sham group (0.0413±0.0240; $p=0.001$) and 25 mg/kg ticagrelor treatment group (0.0595±0.0058; $p=0.003$).

The number of TUNEL-positive cells in alveolar epithelium were increased in the lung sections from saline (I/R)-treated group, and decreased following 25 mg/kg ticagrelor treatment (Figure 2f-j). Ticagrelor (25 mg/kg dose) weakened I/R-induced apoptosis in alveolar epithelium, apoptotic index : 0.0864±0.0149 vs. 0.17188±0.02289, respectively; $p=0.023$. The apoptotic index in the saline (I/R)-treated group was significantly higher than in the sham group (0.17188±0.02289 vs. 0.004143±0.002545, respectively; $p<0.0001$) and 25 mg/kg ticagrelor-treated group (0.08642±0.014983; $p=0.023$).

Discussion

Ticagrelor is a new-generation agent with anti-thrombocytic activity. It is widely and effectively used in patients with acute coronary syndrome. In addition to its P2Y₁₂ receptor antagonist effect, it also inhibits the sodium-independent equilibrative nucleoside transporter-1 transport system and inhibits the cellular uptake of adenosine. Ischemia-reperfusion injury is a widely encountered situation in both cardiac and peripheral arterial surgical procedures. Little is known about the effect of ticagrelor on lower limb ischemia and reperfusion injury nor of its remote organ effects.

The main injury resulting from I/R occurs during the reperfusion period of the ischemic tissue (13). Several inflammatory mediators are released during reperfusion such as interleukins, tumor necrosis factor- α , cialic acid etc. and these mediators cause a systemic inflammatory response resulting in vasodilatation of end-organ arterioles (14-18).

Inflammatory mediators activate circulating leukocytes and vascular endothelial cells and increase the expression of adhesion molecules. Activated endothelial cells in the

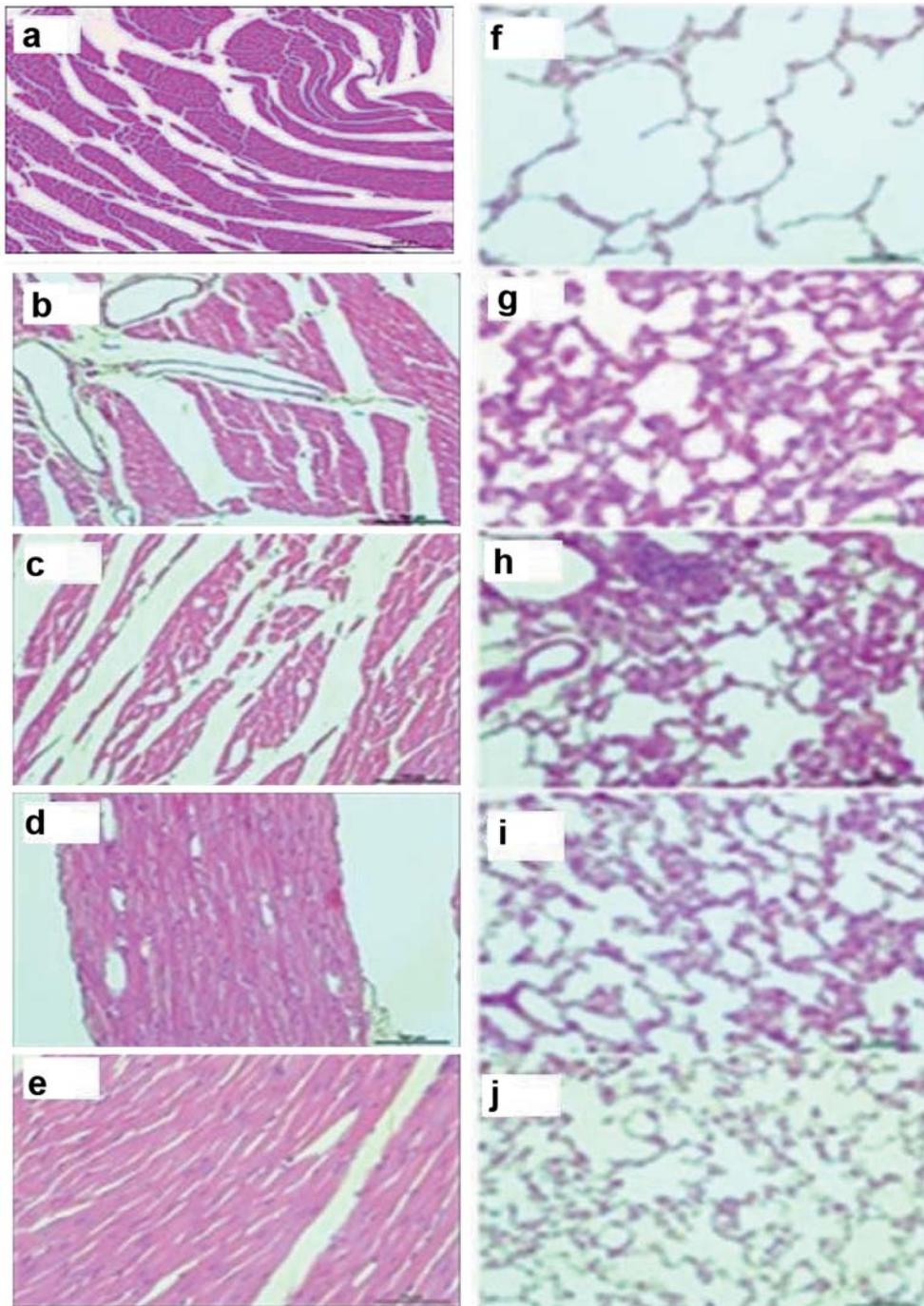


Figure 1. Photomicrographs of sections of cardiac muscle (left column) and lung (right column) stained with H-E. a, f: Sham control group; b, g: saline ischemia and reperfusion group; c, h: 7.5 mg/kg ticagrelor treatment group; d, i: 15 mg/kg ticagrelor treatment group; e, j: 25 mg/kg ticagrelor treatment group. Note heterogeneity of sarcoplasm, fibrocytes and capillaries in b-d. Note intact normal lung histology in the sham control (f) and atelectasis, thickening of alveolar interwall, infiltration of inflammatory cells in g-i. Magnification, $\times 200$; Scale bar, 100 μm .

microcirculation release more free oxygen radicals and less nitric oxide (19). These reactive oxygen species oxidize membrane lipids, essential cellular proteins and DNA, and result in cellular damage. MDA is an end-product of lipid

peroxidation (20). The elevated levels of MDA in plasma or tissue reflect an increased level of oxidative stress. On the other hand, GPx-1 and GR are antioxidative enzymes that are used to eliminate reactive oxygen radicals.

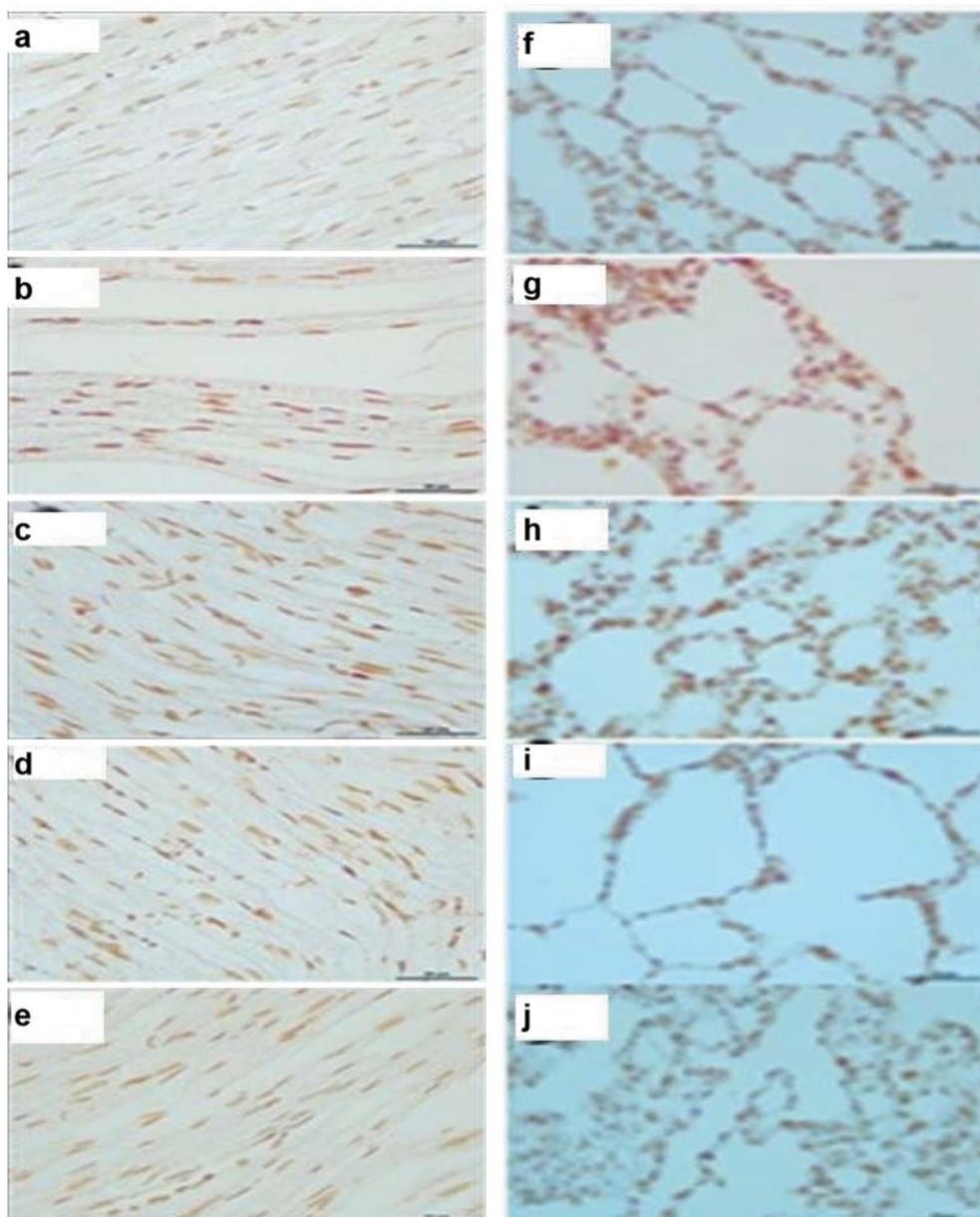


Figure 2. Photomicrographs of sections of cardiac muscle (left column) and lung (right column) stained by TdT-mediated dUTP nick-end labeling (TUNEL). a, f: Sham control group; b, g: saline ischemia and reperfusion (I/R) group; c, h: 7.5 mg/kg ticagrelor treatment group; d, i: 15 mg/kg ticagrelor treatment- group; e, j: 25 mg/kg ticagrelor treatment group. Note apoptotic cells are visible as dark brown spots. The number of TUNEL-positive cells increased in the alveolar epithelium from saline (I/R) group rats, and decreased following treatment with ticagrelor (25 mg/kg). Magnification, $\times 400$; scale bar, 50 μm .

We found that the animals that received 25 mg/kg dose of ticagrelor had lower levels of MDA than the control group, both in lung and heart tissues. Additionally, GPx-1 and GR levels were significantly higher in this treatment group than in the control groups meaning that higher doses of ticagrelor protected both lung and heart tissues against injury due to abdominal aorta I/R.

Histopathological analyses revealed that apoptotic indices of myocardial and alveolar epithelium were significantly lower in the animals that received 25 mg/kg ticagrelor group than the control group. TUNEL-positive cells in both alveolar epithelium and myocardial cells were increased in the control group revealing the protective effect of high-dose ticagrelor on programmed cell death.

After acute lower extremity I/R, hypoxemia, pulmonary hypertension, reduced lung compliance and nonhydrostatic pulmonary edema are frequently encountered conditions due to lung injury. Clinically, these conditions may result in sub-clinical lung injury and also a very severe form in adult respiratory distress syndrome (21-24).

Ticagrelor inhibits the re-uptake of adenosine in the cells by inhibiting the ENT1 transport system (25, 26). Adenosine results in vasodilatation *via* its effect on A2AR receptors, and it induces progenitor cell migration (27, 28). Adenosine also strongly inhibits the aggregation of platelets. It has an inhibitory effect on inflammatory mediators, production of reactive oxygen radicals and neutrophils in high doses in contrary to lower doses (29). Adenosine reduces I/R injury in both animal models and clinical studies (11, 30, 31). In a meta-analysis, Singh *et al.* reported the significant effect of intracoronary administration of adenosine in early periods but that there was no effect in the late period (32). Amstrong *et al.* reported an association between ticagrelor use and coronary blood flow rates. This effect was suggested to be related to locally increased adenosine levels in coronary circulation (9). Van Giesen *et al.* reported a significant association between different ticagrelor doses and coronary blood flow in a canine model with an adenosine-induced augmentation (10). Birnbaum *et al.* reported that ticagrelor, *via* endogenous adenosine, reduced the myocardial infarct area compared to clopidogrel (33). The Platelet Inhibition and Patient Outcomes (PLATO) study revealed that ticagrelor was more effective than clopidogrel regarding reducing mortality due to cardiovascular causes, myocardial infarction and stroke in patients with acute coronary syndrome (34, 35). Ticagrelor was found to be superior than clopidogrel regarding platelet inhibition and rapid action in the DISPERSE phase II study (35, 36).

Conclusion

Our results showed that ticagrelor attenuates lung and heart injury due to I/R of the abdominal aorta at high doses, confirmed by biochemical and histopathological analysis.

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