The effects of inhaled NO on plasma vasoactive factor and CTnI level in rabbits with acute massive pulmonary embolism[1]

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Abstract

Purpose: To investigate changes in the plasma concentrations of cardiac troponin I (CTnI), thromboxane A2 (TXA2), prostaglandin I2 (PGI2) and endothelin-1 (ET-1) in rabbits with massive pulmonary embolism (AMPE) and the impact of nitric oxide inhalation (NOI) on these indices.

Methods: A total of 30 Japanese rabbits were used to construct an MPE model and were divided into 3 groups equally (n=10), including an EXP group (undergoing modeling alone), an NOI group (receiving NOI 2 h post-modeling) and a CON group (receiving intravenous physiological saline).

Results: In the model group, plasma concentration of CTnI peaked at 16 h following modeling (0.46±0.10 µg/ml) and significantly decreased following NOI. Plasma levels of TXB2, PGI2 and ET-1 peaked at 12, 16 and 8 h following modeling, respectively, and significantly decreased at different time points (0, 2, 4, 8, 12, 16, 20 and 24 h) following NOI. A significant correlation was observed between the peak plasma CTnI concentration and peak TXB2, 6-keto prostaglandin F1α and ET-1 concentrations in the model and NOI groups.

Conclusion: Increases in plasma TXA2, PGI2 and ET-1 levels causes myocardial damage in a rabbit model of AMPE; however, NOI effectively down regulates the plasma concentration of these molecules to produce a myocardial-protective effect.

Key words: Pulmonary Embolism. Nitric Oxide. Thromboxane A2. Epoprostenol. Endothelin-1. Rabbits.

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■ Introduction

Acute massive pulmonary embolism (AMPE) is associated with high early mortality[1,2] of ~30%, particularly in patients who present with concomitant shock and heart failure[3,4]. Therefore, early assessment of mortality risk as well as early and effective treatment is recommended for the successful management of AMPE[5-7]. The European Society of Cardiology (ESC) has divided the prognostic risk into three groups: High-risk, medium-risk and low-risk[5]. Patients presenting with right ventricular (RV) dysfunction and hemodynamic instability are in the high-risk group[5]. The mechanisms responsible for myocardial damage in patients with PE in the high-risk group are unclear[5,6]; however, when patients present with concomitant heart failure, they typically have elevated cardiac troponin I (CTnI) levels[7,8]. Elevated CTnI expression is negatively correlated with patient prognosis[9]. Elucidating the mechanisms and factors that precipitate myocardial damage in patients with PE may be useful for predicting and improving patient prognoses.

AMPE damages the vascular endothelium, induces platelet activation and increases the plasma concentration of thromboxane A2 (TXA2)[10], which leads to prostaglandin I2 (PGI2) metabolic disorders and elevated plasma concentrations of endothelin-1 (ET-1)[11,12]. Elevated plasma TXA2, PGI2 and ET-1 concentrations may cause pulmonary thrombosis and hypertension, which may further aggravate pulmonary embolism and exacerbate the myocardial damage associated with pulmonary embolism[10-12].

Inhaled nitric oxide (NO) has been reported as an effective treatment for AMPE due to its vasodilatory effects[13,14], as well as its ability to reduce neutrophil chelation, promote lung endothelium integrity, improve lung ventilation/perfusion, increase the density of blood vessels and repair vascular endothelium to restore injured lung tissue[15,16].

NO is an important signaling molecule in the cardiovascular system and is considered to be a cardioprotective mediator[17]. However, its cardioprotective role in AMPE requires further investigation.

In the present study, a rabbit model with acute MPE-induced myocardial damage (AMPE-MD) was prepared to study the mechanism of myocardial damage in MPE and correlations between the plasma concentrations of CTnI, TXA2, PGI2 and ET-1, were examined. The curative effect of NO inhalation (NOI) AMPE-MD was assessed and the results revealed that NOI may be an effective treatment for AMPE.

■ Methods

The present study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MA, USA). The animal use protocol has been reviewed and approved by the Medical Ethics Committee of Hebei University (approval number 21041109).

A total of 30 white Japanese rabbits aged 6-9 months and weighing 2-2.5 kg were provided by the Laboratory Animal Center, Medical Department of Hebei University (Baoding, China). Animals were housed at a temperature of 16-28°C with a humidity of 40-70% and a 14/10 h light/dark cycle. All rabbits received two meals at 09:00-09:30 and 16:00-16:30 consisting of, 50-75 g pre-meal and *ad libitum* access to water. The ratio of male: female rabbits was 1:1. Rabbits were randomly divided into the experimental (EXP, n=10), NOI (n=10) and control (CON, n=10) groups. The EXP group underwent modeling alone without any anti-coagulant therapy, rabbits in the NOI group received NOI 2 h post-modeling and the CON group received intravenous physiological

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saline instead of emboli. Rabbits in the EXP and NOI groups were treated with first-line therapy (dopamine; Changzhou Yabang Pharmaceutical Co., Ltd, Changzhou, China; 10-20 µg/kg/min, intravenous as required following modeling) to stabilize vital signs. The EXP and NOI groups did not receive the subcutaneous injection of low molecular heparin calcium as anti-coagulant therapy. Mechanical ventilation was the same in all groups.

Preparation of rabbit AMPE-MD model

Following disinfection and injection of 20% urethane (Shanghai Debang Chemical Co., Ltd., Shanghai, China) via the ear vein (1.0 g/kg), then urethane 0.5 g/kg was maintained in the whole experiment. The rabbits underwent tracheal cannulation to facilitate spontaneous breathing and ensure protection of the airway. A 2 cm conventional transverse incision was made on the anterior chest. The right jugular vein and left external carotid artery were separated and the left femoral vein was exposed and clearly separated. A pulmonary artery catheter (made in house) was inserted simultaneously and a microvascular catheter (5Fr TI; Tyler & Company, Atlanta, GA, USA) was inserted through the right jugular vein and left carotid artery. Mechanical ventilation was applied. Pulmonary artery catheterization was monitored throughout the process using an oscilloscope to determine the positions of catheter tips based on pressure waveforms detected by the oscilloscope and the inserted length of the pulmonary catheters. Throughout the experiment, the pulmonary artery catheters were fed with physiological saline (0.3 ml/min) continuously using one micro-peristaltic pump (Cole-Parmer Instrument Company, LLC, St. Neots, UK). The two catheters were connected to one multi-channel physiological parameter analyzer (MP150; BIOPAC, Goleta, CA, USA) for synchronous measurement of the mean arterial pressure and mean pulmonary arterial pressure

(mPAP). The left femoral vein was punctured and implanted with one microcatheter for in-time rehydration and blood sampling. Rabbits were repeatedly injected (once every 3 min) with 0.5 ml of prepared emboli into the right jugular vein via the pulmonary artery catheter using a 5 ml syringe, followed by 2 ml of physiological saline, until the mPAP was reduced to 40% of the baseline measurement and hypotension was maintained at 55-60 mmHg, the heart beats were between 120-160per min, the respiratory rate was more than 40 per min for ~40 min, at which point the AMPE-MD model was considered to be successful. Rabbits in the CON group received saline only. During this process a ventilator for short-term mechanical ventilation was used if the rabbit developed serious breathing difficulties. Rabbits in the NOI group received NOI following modeling (Inhaled concentration was 10-20 ppm immediately following surgery; administered for 2 h). Rabbits in all groups received mechanical ventilation at the same time following tracheotomy using a SERVO-i infant ventilator (Rontgenvagen 2, SE-171 54; Maquet Critical Care AB, Solna, Sweden) with 50% oxygen.

Perfusion and observation of results

Following modeling, rabbits were perfused with acrylonitrile butadiene styrene (ABS) perfusion agent (made of byacrylonitrile butadiene and styrene) and subjected to BIOPAC pressure monitoring (18-25 mmHg; BIOPAC).

After induction of adequate anesthesia by 20% urethane (Shanghai Debang Chemical Co., Ltd., Shanghai, China) via the ear vein (1000 mg/kg). Full experiment anaesthesia was maintained by injecting additional doses of the drug (500 mg/kg i.v,). Rabbits were fixed on the operating table. Fur was removed from the chest and abdomen and a 6 cm incision was made along the linea alba to expose the

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abdominal organs. The abdominal aorta was exposed, freed and threaded for further use. Prepared heparin (GSK, Middlesex, UK) saline was connected to the infusion device and, using a cutting needle, abdominal aortic cannulation, knotting, and fixation were performed, cutting the accompanying inferior vena cava and using the infusion device to rinse the blood vessels. Heparin rinsing was discontinued when the washed lung lobe appeared pale. A 50 ml syringe was used to inject 50 ml of acetone until the lung tissue exhibited a color change (the embolism became white, whilst the remainder became red) and the inferior vena cava emitted a strong odor. The thoracic cavity and manubrium were opened to ligate the superior vena cava. A catheter was inserted into the inferior vena cava above the diaphragm and connected to a BIOPAC multi-function pressure detector. The prepared ABS perfusion fluid was injected at a pressure of 18-25 mmHg until the pressure continued to rise and could not be reduced, at which point the injection was discontinued. The lung was removed for Micro computed tomography scanning (PerkinElmer, Inc., Waltham, MA, USA.). Lung and heart tissue specimens were fixed in 10% formaldehyde at room temperature and for 48 h, dehydrated and embedded in paraffin. Tissues were sectioned into 4-6 μm and Hematoxylin-Eosin stained (Hematoxylin stained for 5 min and Eosin for 1-2 min) at room temperature for microscopic observation using an optical microscope (magnification, x200) and pathological assessment.

Method of NOI

Following successful modeling, mechanical ventilation was initiated when mPAP reached 40% and the rabbit exhibited breathlessness or breathing difficulties for 4 h. NO was composed of 800×10^{-6} g/l decompressed NO, nitrogen (N2), oxygen (O2) and air. When the concentration of NOI reached $10\text{-}20 \times 10^{-6}$ g/L, a ventilator pipe was connected to the NOI pipe for invasive mechanical ventilation (pressure, 20 cm water column; respiratory rate, 28 bpm; oxygen concentration with mechanical ventilation, 50%). The concentrations of NO and nitrogen dioxide (NO_2) were continuously monitored using a nitrogen oxide analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the level of methemoglobin was also monitored throughout and did not exceed 0.3 g/L. Blood samples were obtained from Jugular vein at different time points (0, 2, 4, 8, 12, 16, 20 and 24 h) and the mean arterial pressure and mPAP were monitored.

Plasma concentrations of CTnI, TXB2, PGI2 and ET-1

The plasma concentration of CTnI was determined using a microparticle chemiluminescence method with an automatic immunoassay analyzer (Thermo Fisher Scientific, Inc.). The immunoassay kit was provided by Beckman Coulter, Inc. (cat. no. ab47003; Brea, CA, USA). TXA2 and PGI2 are metabolic products of arachidonic acid, which are unstable and are further metabolized into TXB2 and 6-keto prostaglandin F1α (6-K-PGF1α)[18] The plasma concentrations of TXB2 (cat. no. 519031-96) and 6-K-PGF1α (cat. no. C507164) were determined using kits (Beijing North Institute of Biological Technology, Beijing, China) while ET-1 was detected using a radioimmunoassay kit obtained from the Institute of Radioimmunology, Science and Technology Development Center, Beijing PLA General Hospital. All procedures were performed according to the manufacturer's protocol.

Statistical analysis

Data are expressed as the mean ± standard deviation and were analyzed using

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SPSS statistical software 19.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparisons test and paired Student's two-tailed t tests were used. Correlation analyses were performed using Pearson correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

■ Results

Pathological results

The embolized lungs were harvested and visualized using computed tomography (Figure 1A and B). Following perfusion, the embolized lung lobes appeared pale (Figure 1C) and emboli were observed in the pulmonary arteries (Figure 1D). The right ventricular wall was thinner in rabbits from the EXP group compared with those in the NOI group (Figure 1E and F). A greater degree of myocardial necrosis was observed in tissues from the EXP group compared with the NOI group (Figure 1G and H). Cells exhibited disappearing striated cytoplasm, forming tone bands, with nucleation or disintegration. Inflammatory cell infiltration was also observed, the cell structure is blurred, nuclear debris was found and inflammatory cells are invaded.
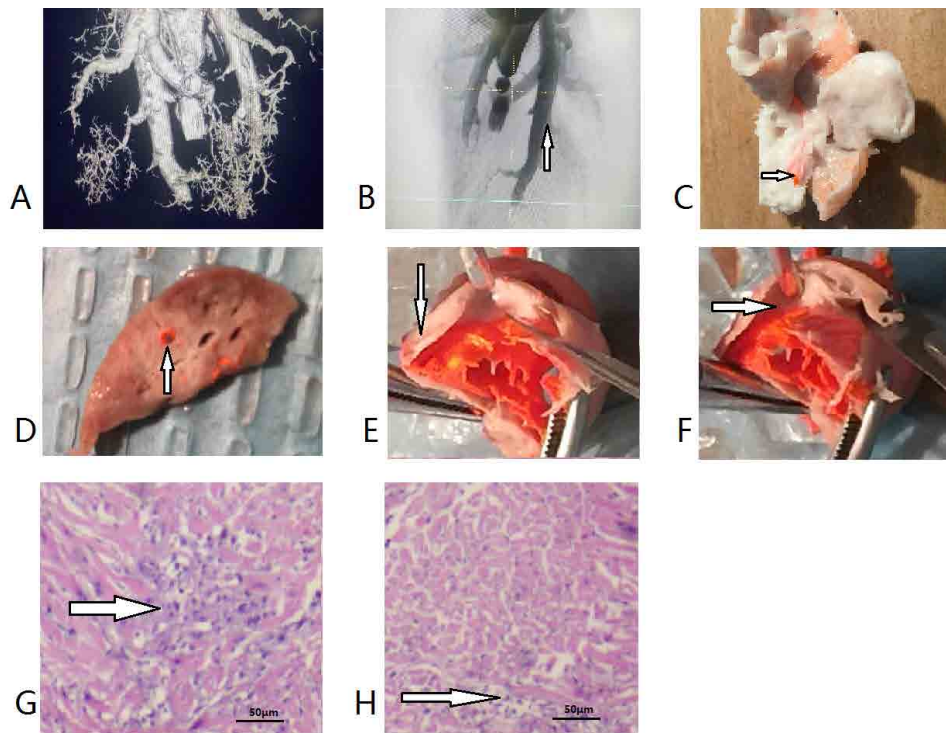
Figure 1 - Images and pathology of pulmonary embolism. (**A**) Computed tomography (**B**) X-ray angiography of pulmonary embolisms. (**C**) Arrow indicating embolized arteries in whole lungs following modeling. (**D**) Arrow indicating an embolized pulmonary lobe of a cross-sectioned lung following modeling. Arrow indicates the embolus. The right ventricle of a model rabbit (**E**) following and (**F**) prior to NOI. Pathological changes (**G**) prior to and (**H**) following NOI. A greater amount of myocardial necrosis is evident prior to NOI. Magnification, x40. NOI, nitric oxide inhalation.

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Changes in plasma CTnI concentration

The plasma CTnI concentration in the EXP group began to increase at 2 h following modeling and significantly increased at 4 h, reaching a peak at 16 h. CTnI concentrations were significantly higher in the EXP group compared with the CON group at 4, 8, 12, 16, 20 and 24 h (Table 1, Figure 2). Furthermore, CTnI concentrations were significantly higher in the EXP group compared with the NOI group at 4, 8, 12 and 16 h (Figure 2A).

Changes in plasma TXB2 concentration

The plasma TXB2 concentration in the EXP group began to increase 2 h following modeling and peaked at 12 h. The TBX2 concentration in the EXP group was significantly higher compared with the CON and NOI groups at 4, 8, 12 and 16 h (Table 1, Figure 2B).

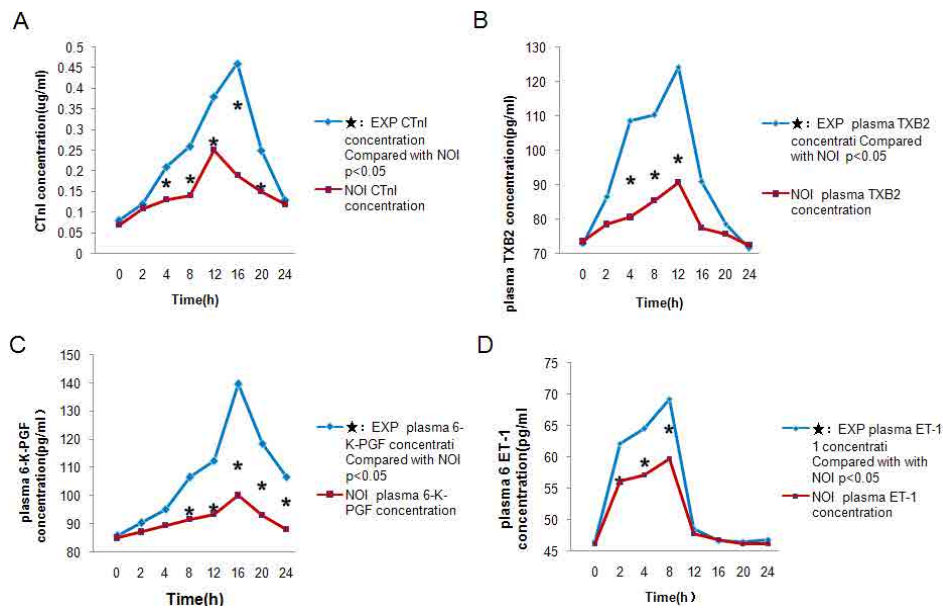### Changes in plasma 6-K-PGF1α concentration

The plasma 6-K-PGF1α concentration in the EXP group began to increase 2 h following modeling and peaked at 16 h (Figure 2C). The plasma 6-K-PGF1α was significantly lower in the CON group at 8, 12, 16, 20 and 24 h and in the NOI group at 8, 12 and 16 h (Figure 2C) compared with the EXP group.

Changes in plasma ET-1 concentration

The plasma ET-1 concentration in EXP group began to increase 2 h following modeling and reached its peak concentration at 8 h. ET-1 levels were significantly lower in the CON group at 2, 4, 8 and 12 h and in the NOI group at 2, 4 and 8 h (Table 1, Figure 2D) following modeling compared with the EXP group. The increase in plasma ET-1 concentration occurred earlier compared with the increase in plasma TXA2 concentration, whereas the increase in plasma 6-K-PGF concentration immediately followed that of TXA2. Changes in plasma ET-1, TXA2 and 6-K-PGF concentrations prior to and following the administration of NOI were similar.

**Table 1** - CON Group plasma ET-1, 6-K-PGF, TXB2, CTnI concentrations in test animals ($\bar{x}$ ±s, n=10).

Items	0h	2h	4h	8h	12h	16h	20h	24h
ET-1 (pg/ml)	46.5±4.9	46.7±5.1	45.9±5.3	46.2±5.9	45.9±5.1	46.2±5.4	45.9±5.4	46.2±5.6
6-K-PGF (pg/ml)	82.5±6.9	84.7±8.4	85.6±9.3	81.6±6.9	83.4±7.8	84.6±6.2	84.2±7.9	84.7±6.6
TXB2 (ng/ml)	74.8±15.4	72.5±18.1	71.3±16.8	70.5±16.1	71.8±16.2	73.3±15.9	72.9±15.3	72.7±16.8
CTnI (ug/ml)	0.06±0.01	0.07±0.01	0.07±0.01	0.08±0.01	0.06±0.01	0.06±0.01	0.07±0.01	0.08±0.01

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Figure 2 - Serum levels of CTnI, TXA2, 6-K-PGF1α and ET-1 in the EXP and NOI groups. *P<0.05 *vs.* EXP. CTnI, cardiac troponin I; TXA2, thromboxane A2; 6-K-PGF1α, 6-keto prostaglandin F1α; ET-1, endothelin-1; EXP, experimental; NOI, nitric oxide inhalation.

Correlation between CTnI and peak plasma concentrations of TXB2, 6-K-PGF1α, and ET-1

A marked positive association was observed in EXP group between CTnI and TXB2 (Figure 3A), CTnI and PGI2 (Figure 3B), and CTnI and ET-1 (Figure 3C).
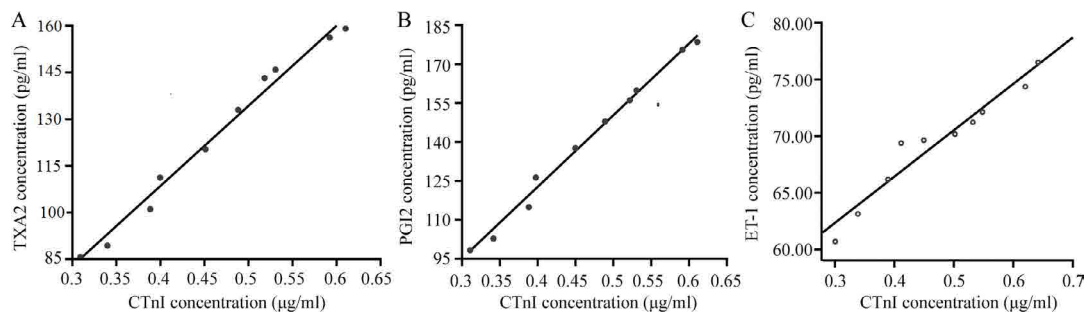
Figure 3 - Correlation between CTnI peak concentration and (**A**) TXA2, (**B**) PGI2; and (**C**) ET-1. CTnI, cardiac troponin I; TXA2, thromboxane A2; PG12, prostaglandin 12; ET-1, endothelin-1

■   **Discussion**

The results of the present study demonstrate that plasma TXA2 concentration is increased in rabbits with AMPE and its peak concentration in the plasma is significantly positive correlated with CTnI. CTnI is a marker for myocardial damage[9], indicating that TXA2 serves an important role in AMPE-MD. A possible mechanism for this is that movement of emboli through the pulmonary vessels causes vascular endothelial damage, leading to activation of platelets and subsequent adsorption on the surface of the emboli. These activated platelets undergo degranulation to release TXA2[10,19,20]. AMPE leads to ischemia, hypoxia and acidosis in rabbits; H+ promotes platelet degranulation to release TXA2, which further increases the formation and enlargement of emboli, thereby forming a vicious cycle[21-23] and

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aggravating right heart failure. Furthermore, AMPE-induced ischemia and hypoxia damages the coronary endothelium and these local and systemic factors act synergistically to enhance platelet activation and cause coronary artery thrombosis, myocardial ischemia and hypoxia, all of which result in myocardial damage and necrosis[22,23].

The present study demonstrated that plasma PGI2 concentration increases in rabbits with AMPE and peak PGI2 concentration is significantly positively correlated with CTnI. PGI2 is primarily associated with the inhibition and prevention of platelet aggregation[24], relaxation of vascular smooth muscles and vasodilatation, particularly in capillaries and resistance vessels[25]. Plasma PGI2 concentrations were significantly increased in rabbits with AMPE, which may be due to the fact that blood vessels are mechanically blocked by emboli following modeling, which increases vascular pressure. The emboli and high blood flow subsequently provide a shear force that damages the vascular endothelium to precipitate the release of PGI2, which is significantly delayed compared to TXA2. The increase in plasma PGI2 concentration may therefore act as a protective response to counteract/neutralize the increase of TXA2, which is most likely a reactionary increase. When TXA2 increases, PGI2 levels increase as a compensatory mechanism to maintain a balance between TXA2 and PGI2 levels.

The results of the present study demonstrate that plasma ET-1 concentration is upregulated in a AMPE model. This may be because emboli cause mechanical injury to the pulmonary vascular endothelium, leading to pulmonary ischemia, hypoxia or acidosis, followed by sustained structural damage to and dysfunction of the pulmonary vascular endothelium, as well as an increased release of ET-1[11]. The damaged pulmonary vasculature is subsequently unable to eliminate ET-1 in the blood[12], which leads to a significantly elevated plasma ET-1 concentration.

Elevated plasma ET-1 concentration causes extensive contraction of pulmonary vessels, increased resistance in the pulmonary circulation and secondary pulmonary hypertension[26]. Furthermore, mechanical blockage of the pulmonary vasculature secondary to emboli may also significantly increase pulmonary artery pressure[27]. The initial effect of AMPE on pulmonary circulation is an increase in pulmonary resistance[28]. In the early stages of AMPE there is an increase in right ventricular post load, causing an increase in the energy consumed by the right ventricle per beat, increased right ventricular end-stage systolic pressure and enlargement of the right ventricle[29].

This in turn significantly reduces right ventricular output. Additionally, ischemia and hypoxia damage the coronary endothelium, inducing increased production/release of ET-1 in the coronary arteries[22]. These factors act synergistically to cause severe coronary artery vasoconstriction[22]. Due to these effects, the coronary blood supply dramatically decreases, causing myocardial ischemia and injury and/or myocardial necrosis. The results of the present study demonstrated that, in rabbits with AMPE, the increase in plasma ET-1 occurred earlier compared with the other substances (2 h following modeling) and peaked at 8 h. Furthermore, this increase was positively correlated with plasma CTnI concentrations, indicating that the increased plasma ET-1 concentration serves a key role in inducing myocardial damage in rabbits with AMPE. This is consistent with the fact that the initial impact of AMPE on the pulmonary circulation is an increase in pulmonary resistance, affecting the stability of the circulatory system and causing early myocardial damage[22]. It was also observed that the increase in plasma ET-1 concentration occurs earlier than the increase in TXA2 and that the consequent increase of ET-1 and TXA2 is associated with

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the AMPE-regulated synthesis of TXA2. The increase in plasma PGI2 is correlated with increased TXA2 concentration and serves as a compensatory mechanism to maintain the balance between TXA2 and PGI2 levels. Increased concentrations of ET-1 and TXA2, as well as the balance maintained between TXA2 and PGI2, may serve a key role in the increased resistance in pulmonary circulation, pulmonary hypertension, and myocardial damage.

This present study demonstrated that NOI is able to significantly reduce plasma concentrations of TXA2, PGI2, ET-1 and CTnI in rabbits with AMPE. NOI may produce this beneficial effect inhibiting inhibiting the activity of protease C[30], which inhibits the deregulation of activated platelets as well as reducing platelet aggregation and TXA2 production. NOI treatment may therefore reduce plasma TXA2 concentration, inhibit platelet aggregation and block multiple pathogenic steps in the development of AMPE. NOI also reduced plasma PGI2 concentrations, which may be responsible for maintaining the TXA2/PGI2 balance. This suggests that the TXA2/PGI2 ratio balance is more important that independent effect of PGI2 in pulmonary thromboembolism. NOI is able to reduce the plasma concentrations of ET-1 by reducing pulmonary arterial pressure and regulating the balance between TXA and TXA2/PGI2. The reduction in plasma ET-1 concentration in turn affects the levels of these other factors. NOI appears to effectively protect against myocardial damage and alleviate myocardial damage in PE via reducing plasma TXA2, PGI2 and ET-1.

AMPE treatment with NO remains a controversial topic and has not been widely used in the clinic. Kline *et al.*[31] reported that the NOI combined with oxygen alleviated right ventricular dysfunction in patients with pulmonary thromboembolism and decreased the level of high-sensitivity troponin, which is not consistent with the results of the present study. Diasjunior *et al.*[32] also demonstrated that intravenous administration of NO was not able to reduce pulmonary hypertension caused by AMPE. The use of high dose intravenous NO does not exhibit a selective effect on the pulmonary circulation, which can lead to severe systemic hypotension. When NO and vasodilatation are combined, the use of intravenous NO may not result in additional pulmonary vasodilation[31]. It has been reported that NOI as an auxiliary or palliative treatment for acute pulmonary thrombosis was able to reduce respiratory frequency and respiratory difficulties and improve the function of the right ventricle, as well as the average shock index[13].

■ Conclusions

Plasma concentrations of TXA2, PGI2 and ET-1 are significantly increased in MPE and are positively correlated with myocardial damage. NOI is able to reduce plasma concentrations of TXA2, PGI2 and ET-1, maintaining the balance between TXA2 and PGI2 to protect against myocardial damage. These results provide a theoretical basis for the treatment of pulmonary thromboembolism with clinical NOI.

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