International Journal of Pharmaceutical and Medical Research
Volume – 5 Issue – 3 June 2017
Website: www.woarjournals.org/IJPMR *ISSN: 2348-0262*

Platelets Prevent Systemic and Pulmonary Inflammatory Responses in a Mouse Model of Extracorporeal Circulation

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Abstract: ***Extracorporeal circulation (ECC) is necessary for conventional cardiac surgery and life support, but it often triggers systemic inflammation, which can lead to systemic inflammatory response syndrome (SIRS) and acute lung injury (ALI). Clinical evidence shows that platelet-transfusion after ECC can reduce the risk of lung injury, but the underlying mechanisms are still unknown. We recently established a mouse-ECC model that can effectively mimic many features of ECC-induced SIRS and ALI. Using this model, we tested the hypothesis that platelets play a considerable role in alleviating inflammatory responses in both the blood and lungs, thereby reducing the occurrence of ECC-related ALI. The right carotid artery and external jugular vein of anesthetized adult male C57BL/6 mice were cannulated to direct blood flow through a 1/32-inch external tube. All animals subjected to ECC (the ECC group and the platelet-transfusion-ECC (PLTs-ECC) group; each n = 10) survived 30 min of ECC and the sequent 60 min of observation. The mice in the PLTs-ECC group were given 0.1 mL of platelet-rich-plasma (PRP) at 25 min after the start of ECC. Blood analysis after ECC showed significant decreases in the levels of tumor necrosis factor-alpha, interleukin-6 and neutrophils elastase in plasma and lung tissue. Additionally, PRP treatment protected lung function and led to a notable decrease in circulating lactate. Histopathology showed that ECC induced lung inflammation, as expected, including alveolar congestion, haemorrhage, neutrophil infiltration, and alveolar wall thickening. The abovementioned changes were less pronounced in the PLTs-ECC group. Double immunofluorescence staining of lung sections showed a "mixed" yellow fluorescence signal in platelets and vascular endothelial cells (VECs) that was speculated to be from the tight binding of activated platelets with VECs and sub-endothelial matrices. Our results showed that PRP treatment led to significant respiratory function protection and mitigated the inflammatory responses in both the blood and lungs. Based on those observations, we propose two potential platelet-pulmonary protection mechanisms: (1) platelets reduce the release of inflammatory cytokines in the lung to prevent an inflammatory response and (2) platelets tightly bind to the endothelium and sub-endothelial matrices via the association of glycoprotein IB/IX/V with von Willebrand factor to repair damaged VECs and secrete sphingosine-1-phosphate (S1P), which helps maintain the integrity and promote the growth of vessel walls to reduce exudation and the retention of neutrophils in the lungs.***

Keywords: platelet transfusion; inflammation; vascular endothelial cells repair; pulmonary function preservation; mice.

1. Introduction

Extracorporeal circulation (ECC) is essential for conventional cardiac surgery and life support. Despite the profound benefits that ECC has brought to patients around the world, it often induces a vigorous systemic inflammatory response that can lead to poor clinical outcomes [1]. ECC can activate the complement system (CS), which is thought to be triggered by contact between the blood components and non-physiological surfaces of the ECC apparatus, as well as haemodynamic changes and low temperatures, thereby further activating leukocytes and other inflammatory cells [1] -[2]. Systemically inflamed endothelium is substantially different from normal vascular endothelium (VE). During an inflammatory response, leukocytes, especially neutrophils, are activated and adhere to vascular endothelial cells (VECs), subsequently migrating into the interstitium where they may release proteases, free oxygen radicals, and pro-inflammatory factors that can damage tissues [1]- [3]. The increased release of inflammatory cytokines will eventually lead to increased permeability of the VE, VECs injury, and a systemic inflammatory response (SIR), therefore resulting in pulmonary edema, decreased pulmonary compliance, and lung dysfunction, which may further develop into acute lung injury (ALI) or even acute respiratory distress syndrome (ARDS) [1]- [4]. Improving clinical outcomes after ECC therefore depends on preventing a SIR.

Numerous studies using animal models have sought to clarify the mechanism of ECC-induced SIR in an effort to identify methods to prevent its occurrence. Our laboratory has been working to develop a much simpler, minimally invasive small-animal model that mimics the clinical effects of ECC. Recently, we described a murine model of arterial-venous partial bypass that may allow detailed molecular studies of ECC-induced SIR. The model showed that the bypass

procedure mimics the strong SIR and ALI observed in mice and requires only minimally invasive microsurgical techniques and a low priming volume; additionally, the technique has a high survival rate [5].

When endothelial cells (ECs) are injured, circulating endothelial progenitor cells (EPCs) and nearby mature ECs participate in endothelial repair. However, endothelial repair is difficult and thus requires a long time to complete [6]- [8].

Platelets are the main blood component involved in endothelial repair. Glycoprotein (GP) Ib/IX/V is expressed on the surface of platelets and binds with von Willebrand factor (vWF) within the endothelium and sub-endothelial matrices, causing circulating platelets to recruit and adhere to the vessel wall [9]- [10]. Activated platelets express beta 1 and beta 3 integrin (alphaIIbbeta3 and GP IIb-IIIa), which mediate the tight binding of platelets with VECs and sub-endothelial matrices to repair the damaged vascular wall [11]- [12]. Therefore, we propose that platelets are the primary component involved in the early restoration of endothelium injured by ECC.

However, platelet counts are significantly reduced during ECC due to the activation and adhesion of platelets and the use of heparin, especially at the beginning of the early period [13]. Therefore, we asked whether the addition of fresh platelets during ECC could mitigate ECC-induced pulmonary edema. A previous clinical study indicated that platelet transfusion after ECC could reduce lung injury, but the observation was not confirmed by additional research [14]. Therefore, the present study sought to explore whether platelet transfusion could alleviate inflammatory responses and reduce the occurrence of ECC-induced postoperative complications.

2. Material and Methods

The study protocol was performed in accordance with the legislation on the protection of animals and approved by the Regional Ethical Committee for Animal Experimentation of Sichuan University.

2.1. Animals

Inbred male C57BL/6 mice aged 10-12 weeks and weighing 25-30 g were purchased from Sichuan University (Chengdu, China). All animals received standard care according to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Surgical Procedures and ECC

Animals were anesthetized by the intraperitoneal administration of 0.2 mg/Kg pentobarbital; they were then orotracheally intubated with a 20-gauge angiocatheter (Becton Dickinson Medical Devices, USA), which was connected to a small rodent ventilator (Taimeng Keji, Chengdu, China). The respiratory rate was set to 130 breaths/min, and the tidal volume was set to 0.5 mL of room air. All animals were kept on a heating pad to maintain a body temperature of 37℃ during the entire experiment.

Surgical procedures were performed with the aid of an operating microscope, which offered 10-40x magnification (Precision Stereo Zoom Trinocular Microscope III, World Precision Instruments, USA). After the administration of heparin (500 U/Kg), the right carotid artery, which is located between the right strap muscle and sternocleidomastoid muscle. The omohyoid muscle was cut to improve visibility during the surgical procedures. One 24-gauge intravenous catheter (Becton Dickinson Medical Devices) was inserted into the right carotid artery, and another was inserted into the external jugular vein; then, they were connected to a tube (inner diameter, 1/32 inch) primed with 0.4 mL of normal saline and a roller pump (Stock II, Munich, Germany) for ECC (Fig. 1(a)-1(b)).

During ECC, the flow rate was maintained at 5 mL/min, which corresponds to approximately 25% of cardiac output. ECC was performed for 30 min, after which the mice were observed for another 60 min.

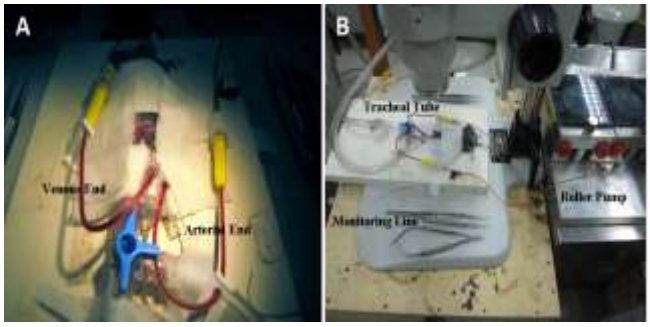

Fig. 1 The mouse extracorporeal circulation circuit. Mice were anesthetized and mechanically ventilated. Both the right carotid artery (a.) and the external jugular vein (v.) were cannulated and connected to a simplified ECC circuit comprised of a sterile tube with a 1/32-inch inner diameter and a roller pump. Blood traveled from the right carotid artery (a. catheter) to the right external jugular vein (v. catheter).

2.3. Experimental Protocol

Mice were randomly divided into four groups: the naïve, sham-control, ECC and platelet-transfusion-ECC (PLTs-ECC) groups (n = 10 in each). In the ECC and PLTs-ECC groups, mice were heparinized, cannulated, perfused for 30 min, and observed for 60 min, as described in Section 2.2. In the PLTs-ECC group, mice were treated as in the ECC group, but they were also given 0.1 mL of platelet-rich-plasma (PRP, containing $5.58 \pm 3.92 \times 10^8$ platelets/mL) from the centrifugal enrichments of 50% whole blood after 25 min of ECC. Mice in the ECC group were similarly given 0.1 mL of platelet-poor-plasma (PPP) 25 min after the start of ECC. In the sham-control group, mice were orotracheally intubated for mechanical ventilation during the 90-minute observation until the termination of the experiments. In the naïve group, mice were anesthetized for 90 min. All samples were immediately harvested.

In both the ECC and PLTs-ECC groups, mean arterial blood pressure (MAP) and the heart rate were continually monitored during circulation through a piezometric tube, which connected the arterial end of the circuit to the experimental system (multifunctional monitor, BL-420E+, China), and recorded before ECC (baseline), 15 min after the start of ECC, at the end of ECC, and 10 and 60 min after the end of ECC (Table 1). Levels of circulating leukocytes and neutrophils were measured using an automated hematology analyzer (BC 3000 plus Mindray, Shenzhen, China) at the end of ECC and at the end of the experiment.

2.4. Platelets Collection for PRP and PPP

Platelets were freshly prepared on the same day as treatment for the PLTs-ECC group. The platelet-donor mouse was anesthetized by the intraperitoneal administration of 0.2 mg/Kg pentobarbital and orotracheally intubated as described in

Section 2.2. The left ribs of the mouse were transected for a thoracotomy to expose the heart. Then, the right ventricle was punctured with a 1.0 mL aseptic syringe to extract blood, which was gently injected into heparinized 1.5 mL Eppendorf tubes. After blood collection, the mouse was euthanized by an excessive dose of anesthetic. The whole blood was immediately prepared for concentrated platelets, and the protocol was as follows: (1) the first gradient centrifugation (low-temperature high-speed centrifuge, Legend Mach 1.6R, Sorvall, Germany) was performed at 461 xg and 2,650 xg for 4 min each at 4℃; (2) the upper plasma, the white-film layer, and a small amount of red blood cells was separated into another heparinized 1.5 mL Eppendorf tube and kept at room temperature for 60 min; (3) the second gradient centrifugation was performed at 285 xg for 2 min and 461 xg for 4 min; (4) the upper plasma was transferred into another 1.5 mL Eppendorf tube, and the third centrifugation was performed at 4,560 xg for 6 min; (5) 0.1 mL of the upper fraction of plasma (PPP) was transferred into another 1.5 mL Eppendorf tube for transfusion into the mice in the ECC group; and (6) the remainding 0.1 mL of plasma containing the concentrated platelets (PRP) was transfused into the mice in the PLTs-ECC group.

2.5. Levels of Pro-Inflammatory Factors

To measure plasma markers of ECC-induced systemic inflammation in our study, blood samples were obtained at the end of the experiment, and plasma was separated by centrifuging the total sample for 15 min at 3,500 xg and 4℃. The plasma supernatant was removed and stored at -80℃ until analysis. Plasma levels of tumor necrosis factor-alpha (TNF-a), interleukin-6 (IL-6), neutrophil elastase (NE), and soluble E-selectin (sE-selectin) were assayed using the following commercial ELISA kits according to the manufacturers' instructions: TNF-a, IL-6, and sE-selectin from Thermo Scientific (Rockford, IL, USA); and NE from Cloud-Clone (Houston, TX, USA).

To measure ECC-induced lung damage in our study, the right lung was harvested, homogenized, and centrifuged as previously described. Briefly, tissues were harvested on ice immediately after euthanasia and weighed. An aliquot of tissue (50 mg) was cut into 1 mm^3 pieces, added to 600 μL of normal saline, and homogenized (OMNIBead Ruptor 24 multisample grinding beads homogenizer, China). Samples were then left standing on ice for 5 min and centrifuged at 3,500 xg for 20 min at 40℃. The supernatants were then transferred to Eppendorf tubes and stored at -80℃ until analysis. Samples were assayed for TNF-a, IL-6, and NE using the methods described for plasma samples.

2.6. Assessments of Lung Function, Histopathological Injury Score, and Double Immunofluorescence Staining

Arterial blood samples were taken from the carotid artery, and O_2 tension (P_aO_2) and circulating lactic acid levels (RADUOMETER ABL 800, USA) were measured both immediately at the end of ECC and 60 min after the end of ECC. The oxygen index (OI) was calculated with the equation $OI = F_iO_2/P_aO_2$, where F_iO_2 was assumed to be 0.21. Lung damage was histopathologically assessed by harvesting the upper lobe of the left lung and fixing it in 4% poly-paraformaldehyde overnight at 4℃. Paraffin-embedded sections (3 μm thick) were stained with hematoxylin and eosin and examined under a light microscope (Olympus CAST, Japan) by a pathologist blinded to the experimental groups. The severity of the lung injury was scored using a 5-point scale (0 = normal histology and 5 = most severe injury) that considered alveolar congestion, haemorrhage, neutrophil accumulation in the airspace or vessel wall, alveolar wall thickness, and hyaline membrane formation. The lower lobe of the left lung was immediately harvested for double immunofluorescence staining. The lung sections (2 μm thick) were stained using the following commercial fluorescence staining kits according to the manufactures' instructions: the VEC (green) staining kit from Thermo Scientific (Rockford, II, USA) and the platelet beta3-integrin (GP IIb-IIIa, red) staning kit from Cloud-Clone (Huston, TX, USA). The stained sections were examined by a pathologist with a confocal laser scanning microscopy (Olympus CAST, Japan).

2.7. Statistical Analysis

Statistical analyses were performed using SPSS 11.01 (IBM, Chicago, USA). The data are reported as the mean ± standard error (SE). Intergroup comparisons for the same time point were performed using Student's *t*-test, and $P < 0.05$ indicated statistical significance. Comparisons of different time points in the same group were performed using two-way repeated-measures ANOVA with a threshold of $P < 0.05$.

3. Results

3.1. PRP and Platelet Count at Different Time Points during the Experiment

The PRP contained 5.58 ± 3.92 x 10^8 platelets/mL, as determined with the hematology analyzer, and the platelet harvest rate was 62.20 ± 4.6%. The concentration of mixed white blood cells was 2.5 ± 0.09 x10^5 cells/mL, and the concentration of erythrocytes was 1.75 ± 0.004 x10^7 cells/mL. The platelet harvest rate was greater than 60% and reached the standards of the American Association of Blood Banks (AABB). The platelet count was determined at different time points throughout the experiment and is shown in Fig. 2.

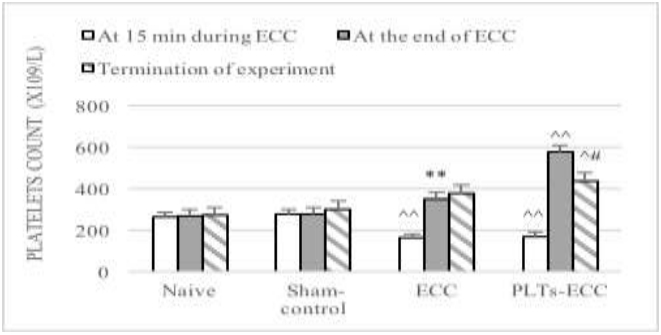

Fig. 2 The platelet count rapidly decreased to 60.43 ± 2.76% (164.52 ± 45.36 x10^9/L) of the platelet count at baseline (272.25 ± 33.74 x10^9/L) within 15 min after the beginning of ECC. However, the platelet count significantly increased 5 min after platelet transfusion (578.15 ± 43.36 x10^9/L, $P < 0.01$). At the termination of experiment, the platelet count in the PLTs-ECC and ECC mice were 436.97 ± 46.19 x10^9/L and 377.90 ± 40.04 x10^9/L, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus PLTs-ECC. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus ECC. $^{\wedge}P < 0.05$ and $^{\wedge\wedge}P < 0.01$ versus the sham-control group.

3.2. Haemodynamics during ECC

All mice in the sham-control, ECC, and PLTs-ECC groups survived the entire experimental procedure. MAP remained stable both in the ECC and PLTs-ECC groups throughout the

procedure and was not significantly different between the two groups (Table 1). MAP was only approximately 10 mmHg lower in the ECC and PLTs-ECC groups than in the sham-control group, but MAP in the ECC and PLTs-ECC groups rapidly became similar to that in the sham-control group after ECC ($P > 0.05$); however, the heart rate remained unchanged.

Hematocrit (HCT) was significantly lower in both the ECC (0.30 ± 0.02) and PLTs-ECC groups (0.31 ± 0.02) than in the naïve group (0.40 ± 0.02, $P < 0.05$) and the sham-control group (0.37 ± 0.03, $P < 0.05$). Nevertheless, the value was still within clinically acceptable limits.

TABLE 1: Haemodynamic parameters in mice subjected to extracorporeal circulation (ECC).

Parameter	Group	Baseline	During ECC	After ECC
			15min / 30min	15min / 30min
Mean arterial pressure (mmHg)	ECC	63 ± 4	54 ± 6[a]/55 ± 7[a]	62 ± 7/67 ± 5
	PLTs-ECC	63 ± 2	55 ± 4[b]/56 ± 5[b]	65 ± 3/65 ± 8
Heart rate (bpm)	ECC	410±10	420±20/411±10	413±18/425±22
	PLTs-ECC	411±14	418±16/412±15	415±12/427±13

Values reported as the mean ± SE.
[a]p <0.05 versus the PLTs-ECC group.
[b]p <0.05 versus the ECC group.

3.3. ECC-induced Inflammatory Response and Lung Injury

At 60 min after the end of ECC, the ECC group exhibited significantly higher levels of circulating leukocytes than the PLTs-ECC (13.2 versus 6.1 x10^9 cells/L, $P < 0.05$) and sham-control (13.2 versus 5.4 x10^9 cells/L, $P < 0.01$) groups. The ECC group also exhibited higher levels of neutrophils than the PLTs-ECC (7.3 ± 0.6 versus 2.8 ± 0.3 x10^9 cells/L, $P < 0.01$) and sham-control (7.3 ± 0.6 versus 2.4 ± 0.3 x10^9 cells/L, $P < 0.01$) groups. There were no differences in the levels of circulating leukocytes and neutrophils between the PLTs-ECC and sham-control (2.8 ± 0.3 versus 2.4 ± 0.3 x10^9 cells/L, $P > 0.05$) groups. Both the ECC and PLTs-ECC groups exhibited significantly higher plasma levels of TNF-a (Fig. 3(a)), IL-6 (Fig. 3(b)), and NE (Fig. 3(c)) but not sE-selectin (Fig. 3(d)), which was detected at almost the same levels in all groups, than the sham-control and naïve groups. Similarly, the lung tissue of the ECC group exhibited significantly higher levels of all three pro-inflammatory markers (Fig. 4(a)-(c)) than that of the other groups. Histopathological analysis indicated the presence of lung damage due to a SIR in the ECC group, but the lung damage was less pronounced in both the PLTs-ECC and sham-control groups.

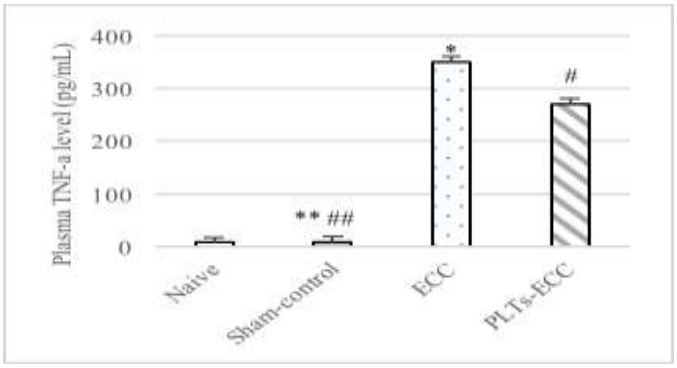

Fig. 3(a)

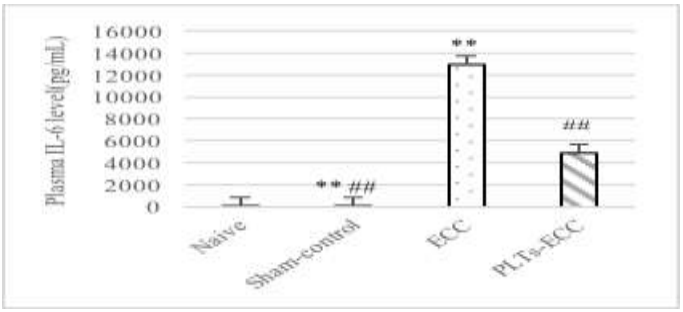

Fig. 3(b)

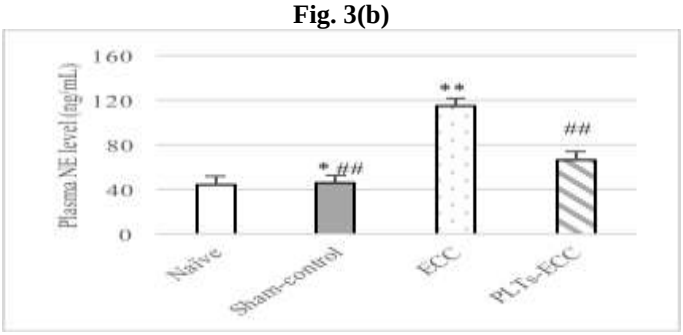

Fig. 3(c)

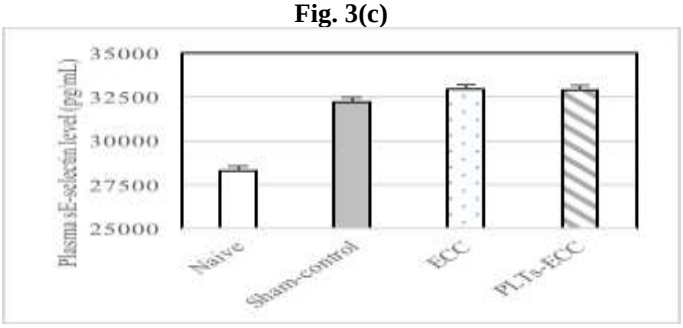

Fig. 3(d)

Fig. 3 Systemic inflammatory response induced by ECC. Mice in both the ECC and PLTs-ECC groups were perfused for 30 min using the ECC circuit, as shown in Fig. 3, and then observed for 60 min. Mice in the PLTs-ECC group were given 0.1 mL of platelet-rich-plasma (PRP, with 5.58 ± 3.92 x10^8 platelets/mL) 25 min after the start of ECC, and mice in the ECC group were similarly given 0.1 mL of platelet-poor-plasma (PPP) during ECC. Mice in the sham-control group were orotracheally intubated for mechanical ventilation during the 90-min observation. Mice in the naïve group were anesthetized for 90 min, and samples were harvested immediately after the observation period. The systemic inflammatory response was assessed by measuring plasma levels of (a) TNF-a, (b) IL-6, (c) NE, and (d) sE-selectin at 60 min after the end of ECC. Each treatment group contained 10 animals. Our experimental results revealed that both the ECC and PLTs-ECC groups showed significantly higher plasma levels of TNF-a, IL-6, and NE but not sE-selectin, which was detected at almost the same levels in all groups. ECC, extracorporeal circulation; TNF-a, tumor necrosis factor-a; IL-6, interleukin-6; and NE, neutrophil elastase. $^*P < 0.05$ and $^{**}P < 0.01$ versus the PLTs-ECC group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the ECC group.

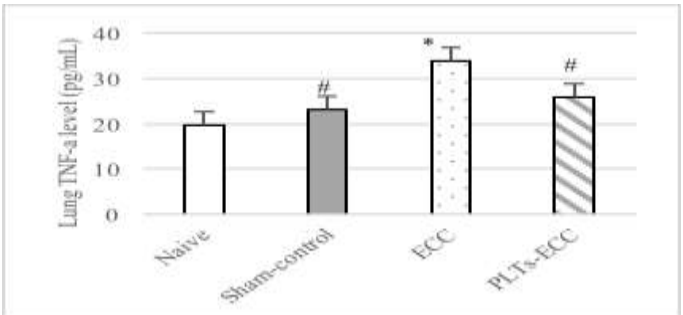

Fig. 4(a)

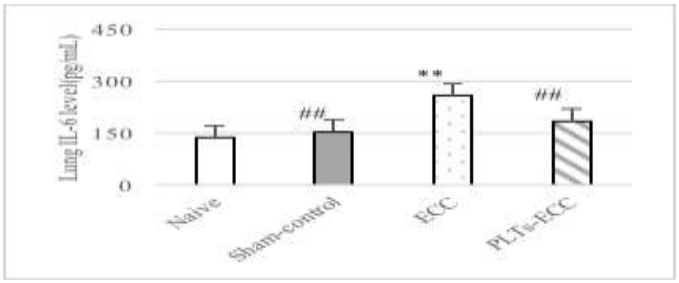

Fig. 4(b)

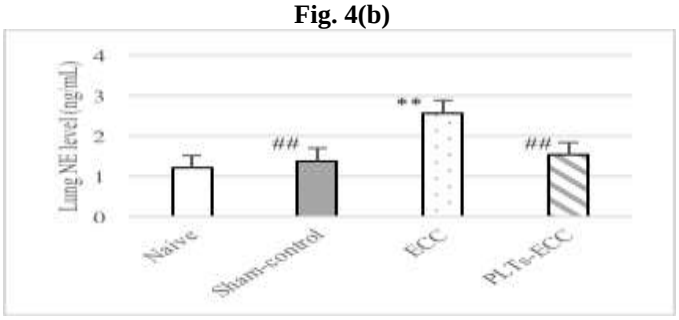

Fig. 4(c)

Fig. 4 ECC-induced inflammatory response in the lungs. Mice were treated as described in Fig. 3, and lung tissues (right lung) were harvested at the end of the experiment. The inflammatory response was assessed in lung tissue (a-c) by assessing the levels of (a) TNF-a, (b) IL-6 and, (c) NE. Our results showed that the lung tissue of the ECC group contained significantly higher levels of all three pro-inflammatory markers. ECC, extracorporeal circulation; TNF-a, tumor necrosis factor-a; IL-6, interleukin-6; and NE, neutrophil elastase. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the PLTs-ECC group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the ECC group.

In contrast to the negligible tissue pathology observed in animals from the naïve group, the mice in the ECC group showed pulmonary injury typical of ECC, which was characterized by edema, haemorrhage, alveolar wall thickening, and inflammatory cell infiltration into alveolar spaces (Fig. 5(c)). However, these variations were less severe in both the PLTs-ECC and sham-control groups. Lung injury scores were significantly higher in the ECC group (4.50 ± 0.51) than in the PLTs-ECC group (1.50 ± 0.25), sham-control group (1.50 ± 0.12) or naïve group (0.05 ± 0.03, all $P < 0.01$; Fig. 5(a),5(b),5(d)).

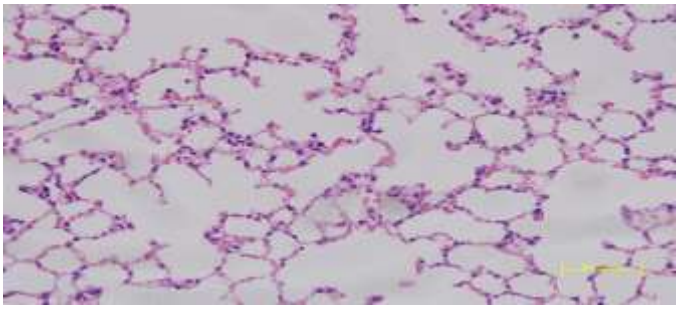

Fig. 5(a) Naïve group

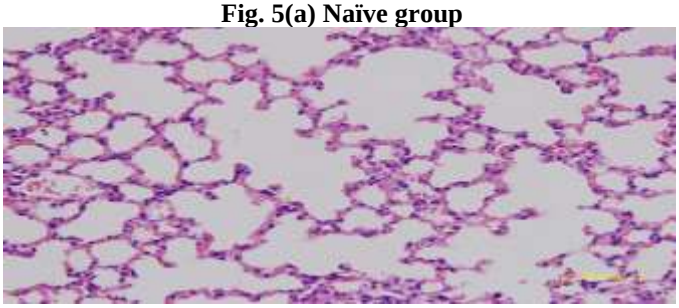

Fig. 5(b) Sham-control group

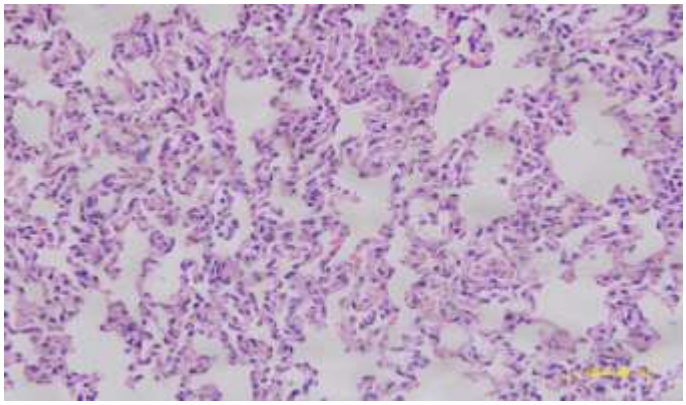

Fig. 5(c) ECC group

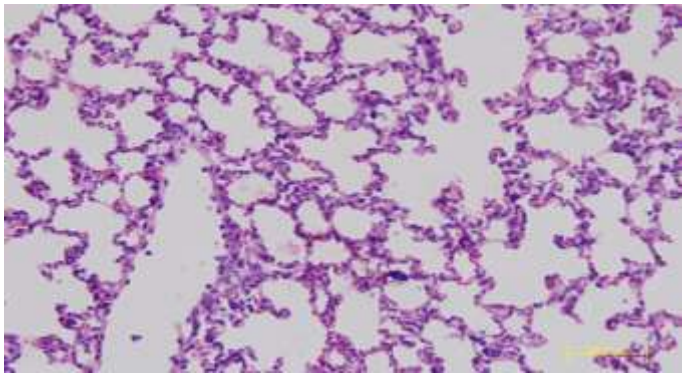

Fig. 5(d) PLTs-ECC group

Fig. 5 Tissue morphology after ECC. Mice were treated as described in Fig. 3, and histopathology was performed at 60 min after the end of ECC. The upper lobe of the left lung was fixed in para-formaldehyde, sectioned, stained with hematoxylin and eosin, and then examined by a pathologist blinded to the treatment groups ($n = 10$; magnification, 40 x). Acute lung injury was scored as described in Section 3.3.

3.4. Effect of ECC on Lung Function

To complement the histopathological assessment of ECC-induced lung injury, we assessed lung function using the blood OI and by detecting the levels of circulating lactate. Immediately after ECC, the OI was similar between the PLTs-ECC and ECC groups (363.50 ±16.19 versus 362.33 ±20.40 mmHg, $P > 0.05$, Fig. 6(a)), but after an additional 60 min of observation, the OI in the ECC group decreased to a level significantly lower than that in the PLTs-ECC group (162.92 ± 29.11 versus 346.33 ± 13.30, $P < 0.01$). On the other hand, there was no difference in the OI between the PLTs-ECC and sham-control (346.33 ± 13.30 versus 326.38 ± 29.72, $P > 0.05$) or naïve (346.33 ± 13.30 versus 330.32 ± 19.11, $P > 0.05$) groups at the termination of the experiment. Likewise, the outcomes of circulating lactate levels were not significantly different at the end of ECC between the PLTs-ECC and ECC groups (5.05 ± 1.67 versus 5.68 ± 1.45 mmol/L, $P > 0.05$) (Fig. 6(b)). Nevertheless, there were notable discrepancies between the ECC and PLTs-ECC (4.43 ± 1.27 versus 2.18 ± 0.65 mmol/L, $P < 0.01$) or sham-control (4.43 ± 1.27 versus 2.0 ± 1.10 mmol/L, $P < 0.01$) groups after 60 min of observation. Similarly, there was no difference in the circulating lactate levels between the PLTs-ECC and sham-control (2.18 ± 0.65 versus 2.0 ±1.10 mmol/L, $P > 0.05$) or naïve (2.18 ± 0.65 versus 2.11 ± 0.76 mmol/L, $P > 0.05$) groups at the termination of the experiment.

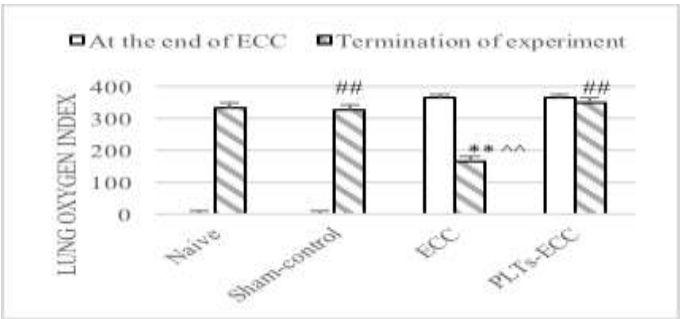

Fig. 6 (a)

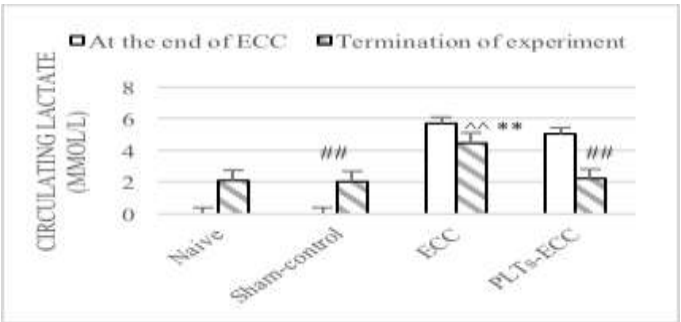

Fig. 6(b)

Fig. 6 Lung function after ECC. Mice were treated as described in Fig. 3, and arterial blood gases and circulating lactate levels were detected at 60 min after the end of ECC. (a) The oxygenation index was calculated as F_iO_2/P_aO_2. (b) The circulating lactate levels were detected in arterial blood. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the PLTs-ECC group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the ECC group. $^{\wedge}P < 0.05$ and $^{\wedge\wedge}P < 0.01$ versus the sham-control group.

3.5. Double Immunofluorescence Staining

Double immunofluorescence staining of the lung sections revealed the abundant binding of VECs and transfused platelets. Mixed fluorescence(yellow) signals were observed, which we speculate to have arisen from the tight binding of activated platelets to endothelium and sub-endothelial matrices through interactions between GP Ib/IV/X and vWF (Fig. 7).

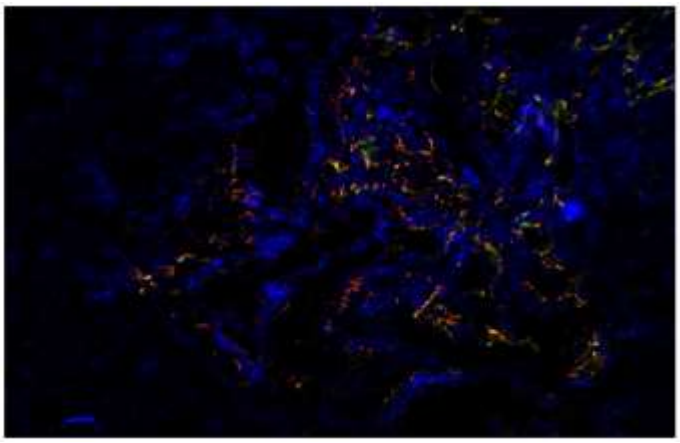

Fig 7 Double immunofluorescence staining after ECC. Mice were treated as described in Fig. 3, and double immunofluorescence staining was performed on the lung tissues collected 60 min after the end of ECC. The lower lobe of the left lung was immediately harvested, and the lung sections were stained using commercial fluorescent kits according to the manufactures' instructions, as described in Section 2.6. The stained lung sections showed abundant binding of vascular endothelial cells (green) to transfused platelets (red) (magnification, 40 x). There was a "mixed" yellow fluorescence signal between the vascular endothelial cells and platelets, which was speculated to be from the tight binding of activated platelets with endothelial cells and sub-endothelial matrices.

4. Discussion

Our experimental results show that platelet transfusion during ECC can inhibit systemic and pulmonary inflammatory responses and protect lung function.

Previous studies have confirmed that contact between the blood and foreign surfaces of the ECC apparatus as well as systemic heparinization and low temperatures during ECC can activate platelets [1]- [2]. Activated platelets may directly adhere to the surface of the circuits and ECs, which occurs via the vWF present in the sub-endothelial matrices [10]- [12]. Meanwhile, activated platelets can also release pro-inflammatory and other inflammatory cytokines, thus activating ECs to release IL-8 and other chemokines, which further promote the adhesion and invasion of neutrophils and exacerbate vessel wall damage [15]- [18]. Therefore, it was thought that platelet activity should be inhibited to reduce its activation and adhesion during ECC; consequently, tirofiban, abciximab, and FK633 (short-acting GP IIb/IIIa receptor inhibitors) are currently commonly used during ECC [19]. Platelet consumption in the ECC group could be significantly reduced compared with that in the sham-control group by inhibiting the GP IIb/IIIa receptor. However, nearly all the articles focus on postoperative bleeding control and have reported conflicting or negative conclusions regarding whether reduced platelet consumption can reduce postoperative bleeding. Additionally, the application of GP IIb/IIIa receptor antagonists did not ameliorate pulmonary edema or reduce lung injury [19]- [20].

Previous research has also confirmed that the main component involved in the repair of damaged VECs is platelets [21]- [25]. Platelet activation is triggered by GP Ib/IX/V binding to the vWF within the endothelium and sub-endothelial matrices [9]- [10]. Activated platelets can express both beta1 and beta3 integrin (alphaIIbbeta3 and GP IIb/IIIa), beta1 integrin binds with exposed sub-endothelial collagen (alpha2beta1), fibronectin (alpha5beta1), and laminin (alpha6beta1), and beta3 integrin binds to fibronectin and vWF, thus leading to the tight adhesion of platelets to ECs and sub-endothelial matrices and promoting the emergency repair of damaged vessel walls [9]- [12]. Likewise, many articles have suggested that the proteins and lipids released from activated platelets can maintain the integrity of microcirculation and that a reduction in circulating platelets will cause increased liquid exudation and pulmonary edema [10]- [12].

ECC can activate VECs and a SIR, resulting in the contraction and deformation of ECs or even loss of ECs, which can lead to increased capillary permeability [1]- [2]. The exposed sub-endothelial collagen may further promote the adhesion and infiltration of neutrophils and other inflammatory cells, and the released cytokines can cause tissue damage and exacerbate tissue edema [3]- [4]. There is evidence that capillary leakage caused by an ECC-induced inflammatory response is the primary reason for pulmonary dysfunction after surgery [1], [2]- [4], and reports have shown that the platelet count can be reduced to below 20% of normal within 5 min after ECC begins [13], [22]- [23]. Impaired adhesion and aggregation of platelets impede their ability to repair damaged VECs and thus exacerbate the severity of ECC-related organ damage.

Based on the above evidence, an increase in fresh platelets may promote their role in the urgent repair of VECs. To explore the impact of platelets on the inflammatory response and respiratory function in mice during ECC, we increased the platelet count by transfusing fresh platelets (PRP) into mice. The platelet count rapidly decreased to 60.43 ± 2.76% of baseline within 15 min after the beginning of ECC, indicating that the contact between blood components and the non-physical surfaces of circuits during ECC leads to the loss of more than 2/3 of platelets. However, the platelet count was significantly increased 5 min after platelet transfusion, indicating that the platelet concentrates used in our study can notably increase the amount of circulating platelets. Significant respiratory function protection and significant mitigation of inflammatory responses in both the blood and lungs were observed in this experiment.

ECC-induced systemic inflammation involves increases in the levels of circulating leukocytes and the plasma levels of pro-inflammatory cytokines [15]- [16]; therefore, the present study focused on the effects of ECC on the levels of leukocytes, especially neutrophils, as well as the plasma levels of TNF-a, IL-6, and NE in the ECC group compared with the PLTs-ECC group to better explore the effects of platelet transfusion during ECC. Our study detected decreased inflammatory responses in both the blood and lungs in the PLTs-ECC group.

TNF-a is secreted by activated macrophages, monocytes, and neutrophils at the earliest phase of ECC and is the most important endogenous mediator of the inflammatory response. NE is mainly secreted by neutrophils granulocytes and is a member of the serine protease family, which participates in the body's defense system under physiological conditions by killing pathogenic bacteria and preventing infection [15]- [16]. However, in pathological conditions, such as during ECC, NE secretion undermines the tight junctions between ECs and the basement membrane, resulting in increased vascular permeability and edema in the lung and causing advanced inflammatory injuries [22]- [25]. There is evidence that lung injury is closely associated with NE activation [16]. Moreover, the transfusion of fresh platelets during ECC can significantly reduce the infiltration and activation of neutrophils and other inflammatory cells in both the blood and lungs. Histopathological biopsies of lung tissue also suggest that platelet transfusion during ECC can significantly reduce pulmonary edema and the retention of neutrophils in the lungs. Double immunofluorescence- -stained sections showed a "mixed" yellow fluorescence signal, which is speculated to reflect the tight binding of activated platelets with endothelium and sub-endothelial matrices for the emergency repair of damaged VECs. To our knowledge, platelet transfusion for the prevention of systemic and pulmonary inflammatory responses in ECC has not yet been reported, and the potential platelet-pulmonary protective mechanisms have not been proposed or practiced before.

Here, we proposed two possible platelet-pulmonary protective mechanisms: (1) platelets reduce the release of inflammatory cytokines in the lung to prevent an inflammatory response and (2) platelets tightly bind to the endothelium and sub-endothelial matrices via the association of GP Ib/IX/V with vWF to repair damaged VECs and secrete sphingosine-1-phosphate (S1P), which helps maintain the integrity and promote the growth of vessel walls to reduce exudation and the retention of neutrophils in the lungs.

Our study provides a new perspective on the role of platelets during ECC and shows that platelets are inflammatory cells and may cause inflammatory responses under certain conditions. Platelets can be pro-inflammatory at the early phase of ECC but may be anti-inflammatory in the later phase of ECC; therefore, it is important to determine the appropriate time to use platelets to improve clinical outcomes.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81170234, 81370349, and 81370350).

Declaration of Interest Statement

The authors declare that there are no conflicts of interests regarding the publication of this paper.

References

[1] Boyle EM Jr, Pohlman TH, Johnson MC, Verrier ED. Endothelial cell injury in cardiovascular surgery: the systemic inflammatory response. Ann Thorac Surg. 1997 Jan; 63(1): 277-84. Review.

[2] Misoph M, Babin-Ebell J. Interindividual variations in cytokine levels following cardiopulmonary bypass. Heart Vessels. 1997;12(3):119-27.

[3] Markovic N, McCaig LA, Stephen J, Mizuguchi S, Veldhuizen RA, Lewis JF, Cepinskas G. Mediators released from LPS-challenged lungs induce inflammatory responses in liver vascular endothelial cells and neutrophilic leukocytes. Am J Physiol Gastrointest Liver Physiol. 2009 ,Dec; 297(6): G1066-76.

[4] Noris M, Remuzzi G. New insights into circulating cell-endothelium interactions and their significance for glomerular pathophysiology. Am J Kidney Dis. 1995 Sep; 26(3): 541-8. Review.

[5] Luo S, Tang M, Du L, Gong L, Xu J, Chen Y, Wang Y, Lin K, An Q. A novel minimal invasive mouse model of extracorporeal circulation. Mediators Inflamm. 2015; 2015: 412319.

[6] Lam CF, Roan JN, Lee CH, Chang PJ, Huang CC, Liu YC, Jiang MJ, Tsai YC. Transplantation of endothelial progenitor cells improves pulmonary endothelial function and gas exchange in rabbits with endotoxin-induced acute lung injury. Anesth Analg. 2011 Mar; 112(3): 620-7.

[7] Gao X, Chen W, Liang Z, Chen L. Autotransplantation of circulating endothelial progenitor cells protects against lipopolysaccharide-induced acute lung injury in rabbit. Int Immunopharmacol. 2011 Oct; 11(10): 1584-90.

[8] de Boer HC, Verseyden C, Ulfman LH, Zwaginga JJ, Bot I, Biessen EA, Rabelink TJ, van Zonneveld AJ. Fibrin and activated platelets cooperatively guide stem cells to a vascular injury and promote differentiation towards an endothelial cell phenotype. Arterioscler Thromb Vasc Biol. 2006 Jul; 26(7): 1653-9.

[9] Wu G, Essex DW, Meloni FJ, Takafuta T, Fujimura K, Konkle BA, Shapiro SS. Human endothelial cells in culture and in vivo express on their surface all four components of

the glycoprotein Ib/IX/V complex. Blood. 1997 Oct 1;90(7): 2660-9.

[10] Chen J, López JA. Interactions of platelets with subendothelium and endothelium. Microcirculation. 2005 Apr-May;12(3): 235-46. Review.

[11] Devi S, Kuligowski MP, Kwan RY, Westein E, Jackson SP, Kitching AR, Hickey MJ. Platelet recruitment to the inflamed glomerulus occurs via an alphaIIbbeta3 /GPVI-dependent pathway. Am J Pathol. 2010 Sep; 177(3): 1131-42.

[12] Devi S, Kuligowski MP, Kwan RY, Westein E, Jackson SP, Kitching AR, Hickey MJ. Platelet recruitment to the inflamed glomerulus occurs via an alphaIIbbeta3 /GPVI-dependent pathway. Am J Pathol. 2010 Sep;177(3): 1131-42.

[13] Li S, Ji H, Lin J, Lenehan E, Ji B, Liu J, Liu J, Long C, Crane TA. Combination of acute preoperative plateletpheresis, cell salvage, and aprotinin minimizes blood loss and requirement during cardiac surgery. J Extra Corpor Technol. 2005 Mar; 37(1): 9-14.

[14] Guay J, Ruest P, Lortie L. Cardiopulmonary bypass induces significant platelet activation in children undergoing open-heart surgery. Eur J Anaesthesiol. 2004 Dec; 21(12): 953-6.

[15] Kotani N, Hashimoto H, Sessler DI, Muraoka M, Wang JS, O'Connor MF, Matsuki A. Neutrophil number and interleukin-8 and elastase concentrations in bronchoalveolar lavage fluid correlate with decreased arterial oxygenation after cardiopulmonary bypass. Anesth Analg. 2000 May; 90(5): 1046-51.

[16] Celik JB, Gormus N, Okesli S, Gormus ZI, Solak H. Methylprednisolone prevents inflammatory reaction occurring during cardiopulmonary bypass: effects onTNF-alpha, IL-6, IL-8, IL-10. Perfusion. 2004 May; 19(3): 185-91.

[17] McGregor L, Martin J, McGregor JL. Platelet-leukocyte aggregates and derived microparticles in inflammation, vascular remodelling and thrombosis. Front Biosci. 2006 Jan 1; 11: 830-7. Review.

[18] Giannini S, Falcinelli E, Bury L, Guglielmini G, Rossi R, Momi S, Gresele P. Interaction with damaged vessel wall resulting in endothelial activation with no effect of aspirin intake. Am J Physiol Heart Circ Physiol. 2011 Jun; 300(6): H2072-9.

[19] Straub A, Wendel HP, Azevedo R, Ziemer G. The GP IIb/IIIa inhibitor abciximab (ReoPro) decreases activation and interaction of platelets and leukocytes during in vitro cardiopulmonary bypass simulation. Eur J Cardiothorac Surg. 2005 Apr; 27(4): 617-21.

[20] Uthoff K, Zehr KJ, Geerling R, Herskowitz A, Cameron DE, Reitz BA. Inhibition of platelet adhesion during cardiopulmonary bypass reduces postoperative bleeding. Circulation. 1994 Nov; 90(5 Pt 2): II269-74.

[21] May AE, Kälsch T, Massberg S, Herouy Y, Schmidt R, Gawaz M. Engagement of glycoprotein IIb/IIIa (alpha(IIb)beta3) on platelets upregulates CD40L and triggers CD40L-dependent matrix degradation by endothelial cells. Circulation. 2002 Oct 15; 106(16): 2111-7.

[22] Wahba A, Black G, Koksch M, Rothe G, Preuner J, Schmitz G, Birnbaum DE. Cardiopulmonary bypass leads to a preferential loss of activated platelets. A flow cytometric assay of platelet surface antigens. Eur J Cardiothorac Surg.1996; 10(9): 768-73.

[23] Menges T, Welters I, Wagner RM, Boldt J, Dapper F, Hempelmann G. The influence of acute preoperative plasmapheresis on coagulation tests, fibrinolysis, blood loss and transfusion requirements in cardiac surgery. Eur J Cardiothorac Surg. 1997 Mar; 11(3): 557-63.

[24] Slotta JE, Braun OO, Menger MD, Thorlacius H. Capture of platelets to the endothelium of the femoral vein is mediated by CD62P and CD162. Platelets. 2009 Nov;20(7): 505-12.

[25] Braun OO, Slotta JE, Menger MD, Erlinge D, Thorlacius H. Primary and secondary capture of platelets onto inflamed femoral artery endothelium is dependent on P-selectin and PSGL-1. Eur J Pharmacol. 2008 Sep 11;592(1-3):128-32.