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Original Article

Continuous balloon occlusion has more influence on microcirculation function assessed by the index of coronary microcirculatory resistance in a pig model

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Abstract: Objective: This study aimed to investigate the effects of balloon occlusion continuously or intermittently in establishing a microcirculation dysfunction animal model. Methods: An animal model was established by balloon occlusion of the left anterior descending artery of the pig continuously (group 1) or intermittently (group 2) under 4-8 atmospheres. Reperfusion was followed by 90 minutes of persistent occlusion in group 1 in 14 pigs. There were 3 × 30 minutes of occlusion in group 2 in 10 pigs and a 30 minute reperfusion following the first and second balloon occlusion. Continuous reperfusion began after the total time of balloon inflation, up to 90 minutes, in both groups. IMR was measured at baseline and continuous reperfusion at 0, 1, and 2 hours in all pigs. Twenty-four hours later, heparanase protein levels in the infarct area of pig hearts were detected by Western Blot. Results: Compared with group 2, IMR of group 1 was higher at reperfusion 0 hours (29.7 ± 5.9 vs 24.6 ± 4.8), 1 hour (31.6 ± 6.7 vs 26.5 ± 5.1), and 2 hours (34.1 ± 8.2 vs 25.3 ± 5.8) (all $P < 0.05$), except baseline values (11.3 ± 1.5 versus 11.8 ± 3.1, $P > 0.05$). Heparanase protein levels in group 1 were much higher than in group 2. Conclusion: Coronary occlusion and reperfusion could lead to abnormal values of IMR in the region of LAD, whereas continuous balloon inflation results in more serious microcirculation dysfunction than intermittent patterns.

Keywords: microcirculation, IMR, pig

Introduction

Coronary microvasculature dysfunction (CMD) exists in patients with ST-segment elevation myocardial infarction (STEMI) receiving percutaneous transluminal coronary angioplasty or stent implantation, with a prevalence ranging from 5% to 50% [1, 2]. Current trials have revealed the fact that CMD has a strong impact on prognosis, including a negative influence on the potential benefits of percutaneous coronary intervention, higher risk of early post-infarction complications, adverse left ventricular remodeling, and late-onset heart failure and mortality [3-5]. Unfortunately, it remains unclear which choice is the optimal treatment strategy in patients with CMD [6].

Outcomes with high diagnosis and treatment power from animal experiments are a prerequisite for translational research. It plays an important part in establishing a simple and reliable animal model mimicking CMD of patients with myocardial infarction. Because of the similarity in cardiac size, hemodynamics, coronary anatomy, and pathophysiology mechanisms to that of humans, pigs have been widely used in the diagnosis and treatment of cardiovascular diseases [7-9].

During the PCI age, balloon inflation models play an important role in animal experiments because of the significant advantages, such as providing reproducible coronary artery occlusion at a precise location and time, mimicking ischemia-reperfusion injuries, and lower mortality rates and complication rates [10, 11]. The index of microcirculatory resistance (IMR) is a specific quantitative measurement used to assess coronary microvasculature function. It shows high predictive capacity of the extent and severity of myocardial infarction in patients

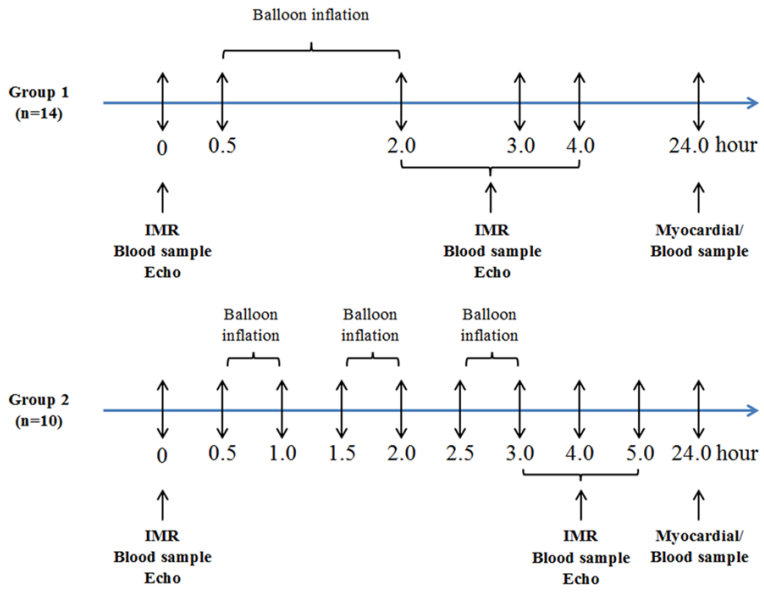

Figure 1. Outline of the animal model established protocol. IMR = index of microcirculatory resistance; Echo = echocardiography.

with myocardial infarction [12-14], but the effectiveness of producing a CMD animal model via IMR has not yet been reported. The present study compared the differences between balloon inflation continuously and intermittently in establishing a CMD model via IMR of the left anterior descending artery (LAD) of pigs.

Materials and methods

Experimental protocol

Guangzhou General Hospital of Guangzhou Military Command's Institutional Animal Care and Use Committee approved the study protocol. In a closed chest pig model, IMR measurements were made in LAD at baseline. Next, LAD was completely occluded by a balloon of 2.0 mm × 20 mm under 4-8 atmospheric pressure. Balloons were inflated continuously in 14 pigs (group 1) and intermittently in another 10 pigs (group 2). A total of 30 minutes of reperfusion was executed after 30 minutes of balloon inflation in group 2. The second cycle of balloon inflation then began. Continuous reperfusion began after the total time of balloon inflation was 90 minutes in both groups. IMR measurements were made in the time points of continuous reperfusion 0 hours (immediately), 1 hour, and 2 hours in all pigs. Hemodynamics, echocardiography, and blood samples were recorded at the same time points as IMR measurements. Twenty-four hours later, heparanase protein levels in infarct area of pig hearts were detected by Western Blot (details of the experimental protocol are summarized in **Figure 1**).

Experimental procedure

All pigs were fed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. Five days before the measurement, pigs received aspirin (5 mg/kg, nocturnal), clopidogrel (5 mg/kg, daily), perindopril (4 mg, daily), and atorvastatin calcium (20 mg, nocturnal). General anesthesia was induced by intramuscular injections of a mixture of ketamine (200 mg), Su-mian-xin (1.5 mL, mixture of haloperidol, xylidinothiazole, and dihydroetherphine), and midazolam (10 mg), then maintained with a mixture of 8 mL of 0.9% sodium chloride, 2 mL of ketamine (100 mg), and 40 mL of propofol, continuously driven by a medical syringe pump through marginal ear veins (8-18 mL per hour). Oxygen was supplied as 2 l/min. A total of 480 U of penicillin was injected 30 minutes before the experiment. Under local injections of 10 mL lidocaine in the inguinal area, the right femoral artery was exposed and isolated after skin incision and separation of subcutaneous tissue. Next, a 6-French (6-F) guiding catheter sheath (Cordis Corporation) was placed into the artery. A 6-F JR3.5 guide catheter (Cordis Corporation) was put into the left coronary artery through the arterial sheath. Animals were heparinized (100 U/kg intracoronary and added another 2500 U after every hour). The baseline angiography was performed. After calibration, a 0.014-inch diameter coronary pressure wire (St. JUDE Medical Systems, USA) was advanced into the distal LAD. Mean aortic pressure (Pa), mean distal pressure of LAD (Pd), and mean transit time (Tmn, in seconds) of 3 × 3 mL bolus of room-temperature saline injected into the coronary were recorded at baseline and under different

Table 1. Comparison values of partial assessment indexes between two groups of establishing of animal model

	Group 1 (n = 14)	Group 2 (n = 10)	*P*-value
Baseline characteristics			
Body weight, kg	21.0 (18.9-21.1)	24.5 (20.9-27.3)	0.038
Male (%)	78.6	100	0.126
Death (%)	21.4	0	0.280
Diameter of LAD, mm	2.1 (1.9-2.2)	2.1 (1.8-2.1)	0.962
Ventricular tachycardia or fibrillation	2.6 (1.0-4.0)	1.3 (0.0-2.3)	0.024
Times of electrical defibrillation	4.6 (2.7-7.0)	1.9 (0.0-3.0)	0.013
Baseline Pa, mmHg	113 (101-123)	103 (97-106)	0.043
Baseline Pd, mmHg	109 (99-120)	101 (95-106)	0.105
Baseline HR	86 (78-96)	80 (72-86)	0.142
Hyperemic efficacy			
IMR1	11.3 (9.9-12.6)	11.8 (9.4-13.5)	0.664
IMR2	29.7 (25.9-31.3)	24.6 (20.2-28.4)	
IMR3	31.6 (26.4-36.6)	26.5 (22.8-29.5)	
IMR4	34.1 (31.6-39.2)	25.3 (22.9-29.0)	0.013
CFR1	3.5 (2.8-4.2)	3.7 (3.5-4.0)	0.632
CFR4	2.8 (2.3-3.1)	2.8 (2.4-3.2)	0.763
FFR1	0.93 (0.92-0.96)	0.93 (0.92-0.96)	0.893
FFR4	0.90 (0.86-0.97)	0.94 (0.88-0.99)	0.154
Tmn1	0.17 (0.12-0.17)	0.18 (0.14-0.20)	0.525
Tmn4	0.58 (0.51-0.63)	0.43 (0.31-0.50)	0.10

Values given are medians (inter-quartile range, 25-75th) or rates. LAD: the left anterior descending coronary artery; IMR = index of microcirculatory resistance; FFR = fractional flow reserve; CFR = coronary flow reserve; Tmn = transit mean time. IMR, CFR, FFR and Tmn were labeled at baseline (IMR1, CFR1, FFR1 and Tmn1), reperfusion 0 h (IMR2, CFR2, FFR2 and Tmn2), reperfusion 1 h (IMR3, CFR3, FFR3 and Tmn3), reperfusion 2 h (IMR4, CFR4, FFR4 and Tmn4). $P < 0.05$ means statistically significant between two groups.

time points of reperfusion through the 6-F JR3.5 guide catheter and pressure wire. The maximal hyperemic condition was induced by intracoronary bonus papaverine 18 mg and a bolus of IC nitroglycerine 200 ug was administrated before each IMR measurement. After baseline measurements, a balloon angioplasty catheter (Maverick, 2.0 mm × 20 mm, Boston Scientific, USA) was advanced through the location between the first and the second diagonal branch of the LAD through the 6-F guiding catheter. Coronary blood flow was interrupted totally by balloon inflation, documented through contrast angiography. Balloon deflation was performed under the protocol in all animals. Before balloon inflation, all animals were pretreated with amiodarone 0.15 g via intravenous and followed by continuous infusions driven by a medical syringe pump under the concentration of amiodarone 60 mg/h, to prevent occurrence of malignant arrhythmias. The pump could not be stopped until the balloon deflated and 30 minutes of reperfusion was performed. Left ventricular end-systolic dimension, left ventricular end-diastolic dimension, ejection fraction, and systolic wall thickening were measured using echocardiography (GE Vivid E9, GE Healthcare, USA). In all cases, a 12-lead real-time electrocardiogram (ECG) monitor system recorded the total ECG waveform, such as ventricular premature, ventricular tachycardia and ventricular fibrillation, ECG ST, and T wave changes. If ventricular tachycardia or fibrillation happened, electrical defibrillation under the energy of 200 J was performed at once. Twenty-four hours later, myocardial tissues of pig hearts were excised for H&E staining. Heparanase protein levels in the infarct area were detected by Western Blot.

Biochemical assessment

Serum levels of cTnI, BNP, and ET-1 were measured using test kits (N28016833, M25-016835, and M25016837, respectively) from the Wuhan Huamei Biological Engineering

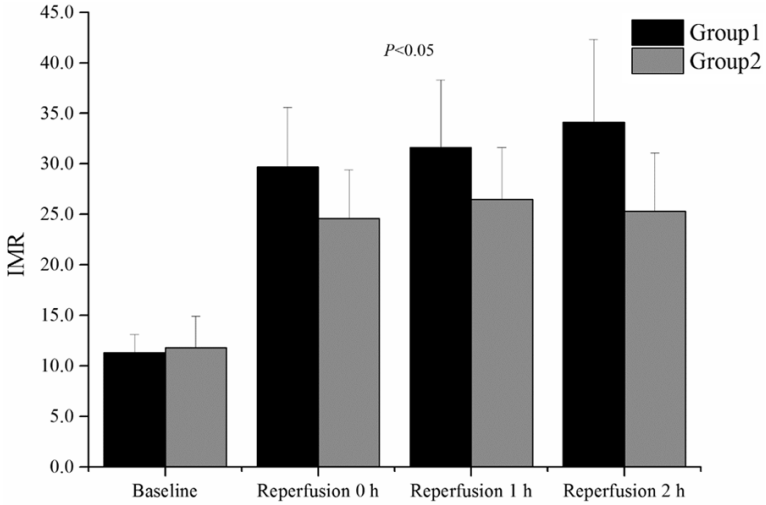

Figure 2. Comparison of IMR at different time points in both groups. IMR = index of microcirculatory resistance. P < 0.05 means statistically significant between two groups.

Research Center (Wuhan, China). Serum levels of NO were measured using test kits (20171018) from the Nanjing Jiancheng Biological Engineering Research Center (Nanjing, China). All measurements were performed in duplicate and averaged.

Statistical analyses

Continuous data are presented as medians (inter-quartile range, 25-75th) or rates. Discrete variables are expressed as frequencies and percentages. Differences in IMR under different time points were analyzed by repeated measures ANOVA. A *P* value < 0.05 (two-sided) indicates statistical significance. All statistical analyses were performed using SPSS, version 21.0.

Results

A total of 24 pigs with an average weight of 22.5 ± 4.1 kg received balloon inflation in establishing a CMD model. In group 1, 20 pigs were male and 4 were female. The mean diameter of LAD was 2.1 ± 0.3 mm in the two groups. There were no significant differences between the two groups in mean baseline Pd and HR (both *P* > 0.05), but a slight difference in baseline Pa (*P* < 0.05). During the experiment, 20 pigs survived and 4 pigs died in group 1, including one each due to cardiogenic shock, intractable ventricular fibrillation, and reperfusion arrhythmia. Baseline characteristics of all animals are presented in **Table 1**.

Baseline measurements of IMR (11.3 ± 1.5 versus 11.8 ± 3.1, *P* > 0.05), FFR (0.93 ± 0.4 versus 0.93 ± 0.4, *P* > 0.05), and CFR (3.5 ± 0.9 versus 3.7 ± 0.4, *P* > 0.05) were all without significant difference (group 1 first). Compared with IMR in group 1 at baseline (11.3 ± 1.5), IMR at reperfusion 0 hours (29.7 ± 5.9), 1 hour (31.6 ± 6.8), and 2 hours (34.1 ± 8.2) was higher (all *P* < 0.05). In group 2, IMR at reperfusion 0 hours (24.6 ± 4.8), 1 hour (26.5 ± 5.1), and 2 hours (25.3 ± 5.8) was also higher than IMR at baseline (11.8 ± 3.1) (all *P* < 0.05), but fewer changes were found in the reperfusion period. IMR in group 1, at reperfusion 0 hours, 1 hour, and 2 hours, was much higher than that of group 2, respectively (*P* < 0.05), but differences were not found in CFR or FFR between the two groups (all *P* > 0.05) (**Table 1** and **Figure 2**).

Baseline values of LV ejection fraction (60 ± 4% in group 1, 61 ± 5% in group 2) were normal in both groups (*P* > 0.05). LV ejection fraction declined violently at reperfusion 0 hours (to 37 ± 5% and 47 ± 3%, both *P* < 0.05 vs. baseline) (group 1 first) and maintained a plateau from reperfusion 0 hours to 2 hours, but LV ejection fraction in group 1 was lower than group 2 (*P* < 0.05). Regional left ventricular anterior wall thickening rose obviously from baseline to reperfusion 2 hours in group 1 (from 4.43 ± 0.64 mm to 6.71 ± 0.82 mm, *P* < 0.05) and in group 2 (from 4.20 ± 0.42 mm to 5.20 ± 0.63 mm, *P* < 0.05). Peak early filling velocity (E-wave) decreased and late diastolic filling velocity (A-wave) increased. E/A ratio < 1 was measured in reperfusion 2 hours in group 1, but E/A ratio ≥ 1 was in group 2. Left ventricular aneurysms were found in 3 pig hearts in group 1, with the mean area of 13 mm × 8 mm in reperfusion 2 hours. No ventricular aneurysms were found in group 2 (**Figures 3** and **4**).

Serum cTnI averaged 15.6 ± 2.5 pg/mL in group 1 and 15.4 ± 3.2 pg/mL in group 2 under base-

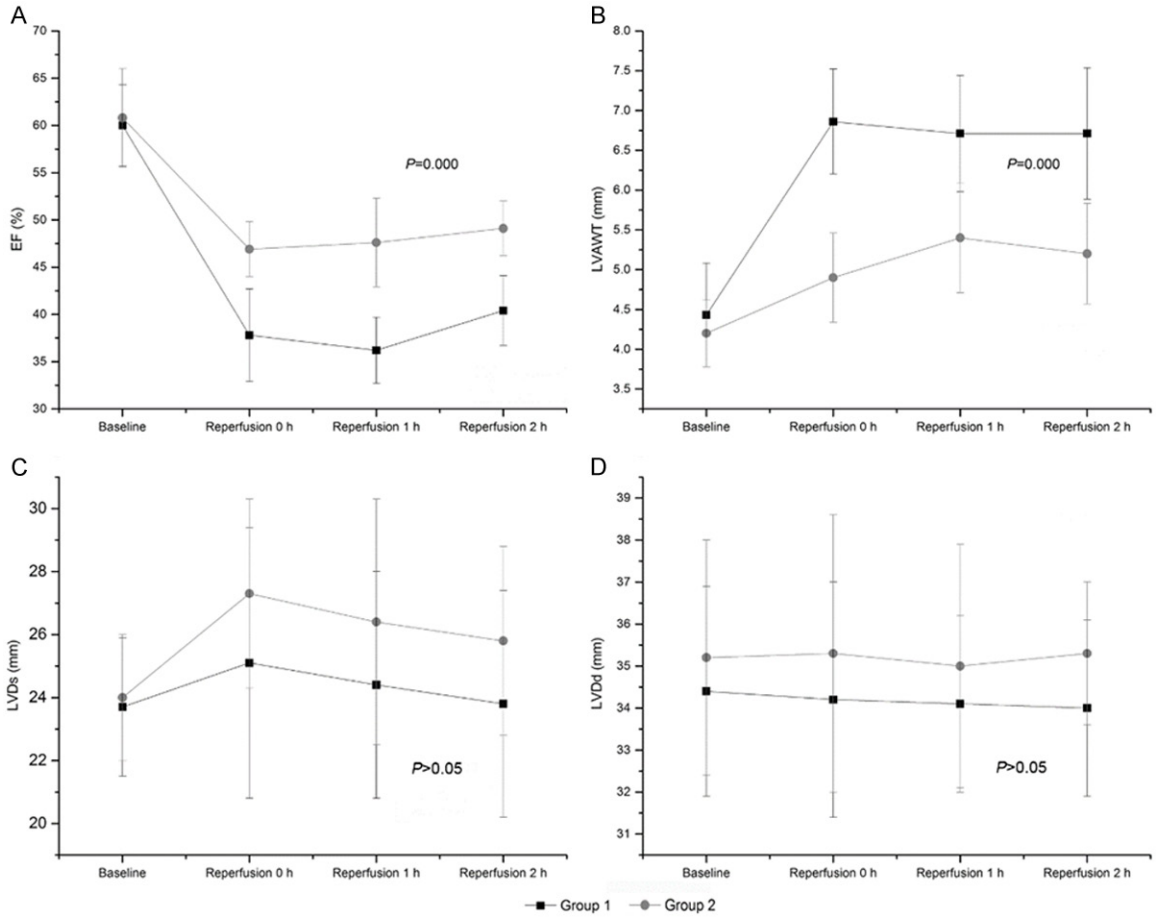

Figure 3. Comparison of EF, LVAWT, LVDs, and LVDd at different time points in both groups. EF = left ventricular ejection fraction; LVAWT = left ventricular anterior wall thickness; LVDs = left ventricular end-systolic dimension; LVDd = left ventricular end-diastolic dimension. P < 0.05 means statistically significant between two groups.

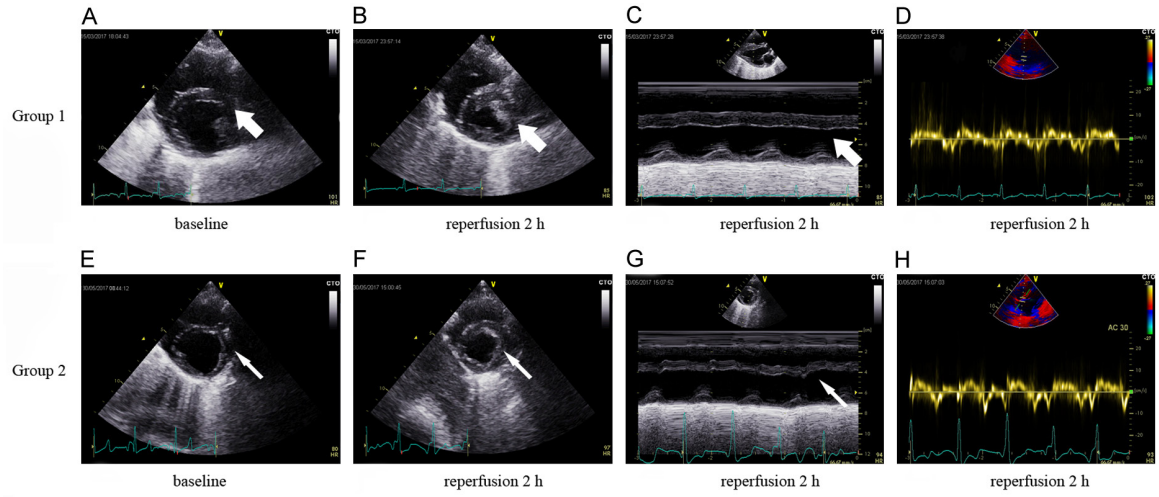

Figure 4. Photos of heart echocardiography at baseline and reperfusion 2 h in both groups. The anterior region segmental wall motion was normal (A, E). There was an aneurysm in left ventricular in group 1 (B) (thick arrow), while slight swollen and abnormal wall motion existed in the anterior region of left ventricular in group 2 (F) (thin arrow). The diastolic ability of left ventricle was abnormal in group 1 (C, D) and diastolic function of left ventricle was preserved in group 2 (G, H).

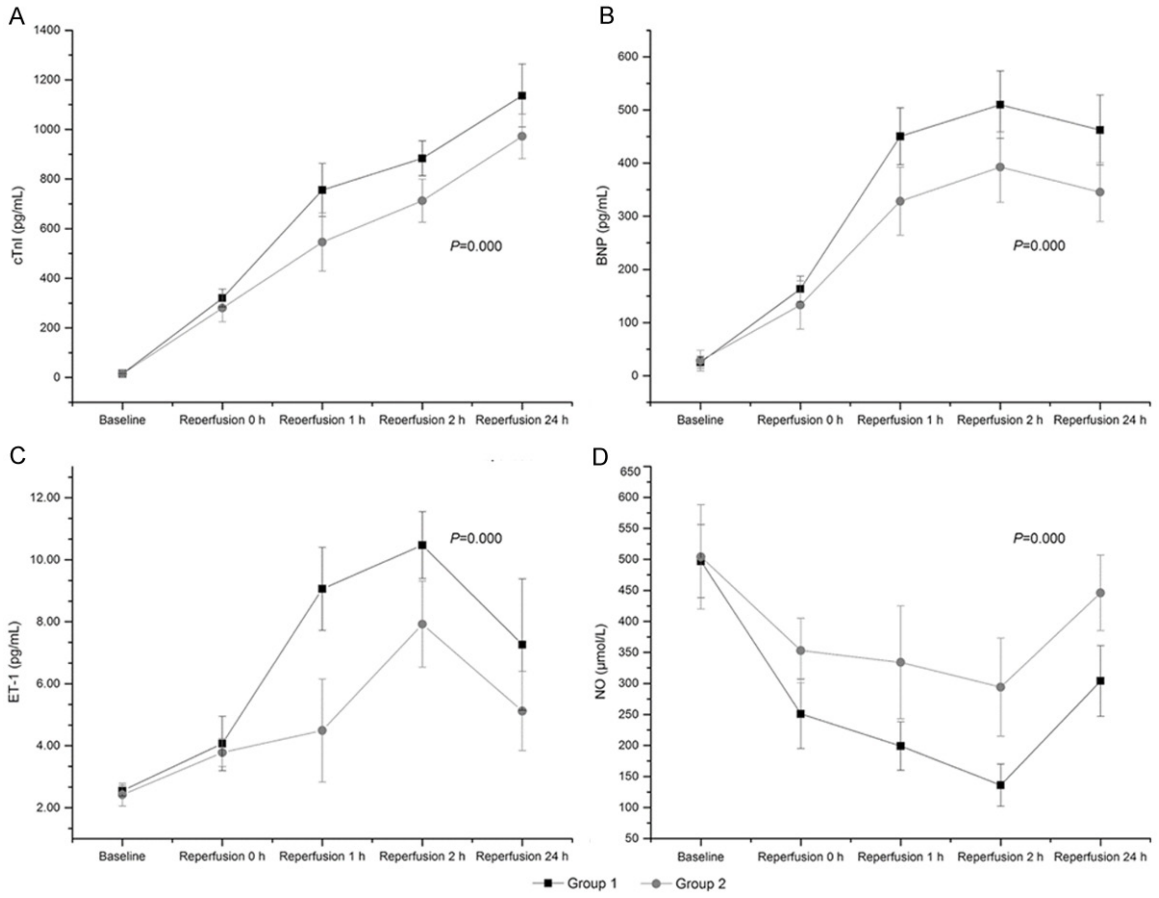

Figure 5. Comparison of concentrations of cTnI, BNP, ET-1, and NO at different time points in both groups. $P < 0.05$ means statistically significant between two groups.

line conditions ($P > 0.05$). As the mass of myocyte infarction increased, cTnI pooled from the bottom in baseline to the top in 24 hours in both two groups (nearly increasing 73 times and 63 times, respectively) ($P < 0.05$). Serum cTnI concentrations in group 1 were higher than group 2 at every time point (all $P < 0.05$). Serum BNP concentrations under baseline conditions were nearly the same (25.3 ± 10.9 pg/mL in group 1 and 28.3 ± 19.2 pg/mL in group 2) ($P > 0.05$), but the top value of BNP of group 1 at reperfusion 2 hours was higher than group 2 ($P < 0.05$). As with BNP, serum ET-1 concentrations increased gradually from baseline to the reperfusion period and the top value was observed in reperfusion 2 hours. However, serum NO concentrations began decreasing gradually from the baseline time point, nearly recovering to the baseline level in reperfusion 24 hours from the bottom time point in reperfusion 2 hours (**Figure 5**).

Post-mortem analyses confirmed the presence of pathological infarction by H&E staining. Light microscopic evaluation of tissue sections collected 24 hours after reperfusion from LAD regions in both protocols showed evidence of myocyte nuclear loss and inflammatory cell infiltration, consistent with the presence of myocyte necrosis. Additionally, contraction band necrosis was observed. Myocyte necrosis was absent in non-ischemic remote areas of the left ventricle (**Figure 6**). Compared with group 2, myocyte necrosis heparanase protein levels of group 1 were much higher ($P < 0.05$) (**Figure 7**).

Discussion

The present study represents the impact of ischemic and reperfusion on coronary microvasculature function in a closed chest pig animal model with STEMI treated with balloon occlusion LAD. Coronary microvasculature

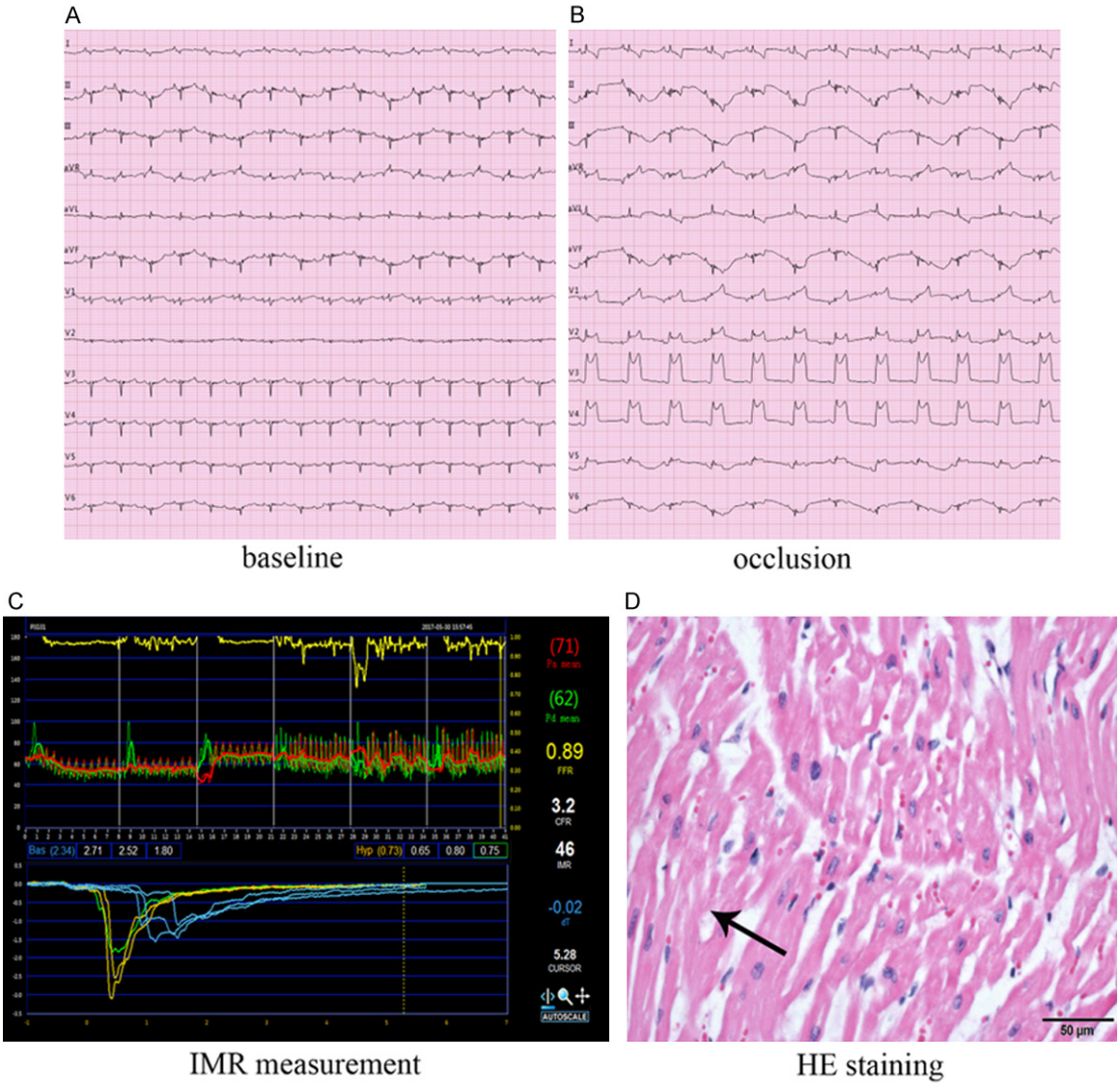

Figure 6. Photos of ECG, IMR, and heart tissue H&E staining. ST-segment was at baseline level (A) and elevated obviously several minutes after balloon occlusion (B). The value of IMR became high in reperfusion 2 h (C). Loss of myocyte nuclei, contraction bands fracture, and inflammatory cell aggradation was observed in tissue slice (D).

function was measured using angiographic and IMR criteria that are considered gold standard techniques for diagnosis of CMD in a clinical setting. Main findings of this study can be summarized as follows: 1) In an animal model with STEMI, the value of IMR after ischemia 90 minutes and reperfusion 1 hour and 2 hours increased gradually under balloon inflation continuously or intermittently; 2) IMR induced by balloon inflation continuously resulted in higher values than balloon inflation intermittently at every same measurement time point; and 3) Myocyte necrosis heparanase protein levels of pigs induced by balloon inflation continuously were much higher than balloon inflation intermittently. Additionally, left ventricular aneurysms were induced by ischemia continuously.

Ischemia-related injuries have significant influence on coronary microcirculation function [15]. Without blood flow for only a short time, myocardial cells began losing activity [16]. In the present study, ST segment elevated sharply several minutes later after coronary blood flow was interrupted via the 12-lead real-time electrocardiogram monitor system. Along with the number of myocardial infarction cells increasing, Pa decreased gradually via the 6-F JR3.5

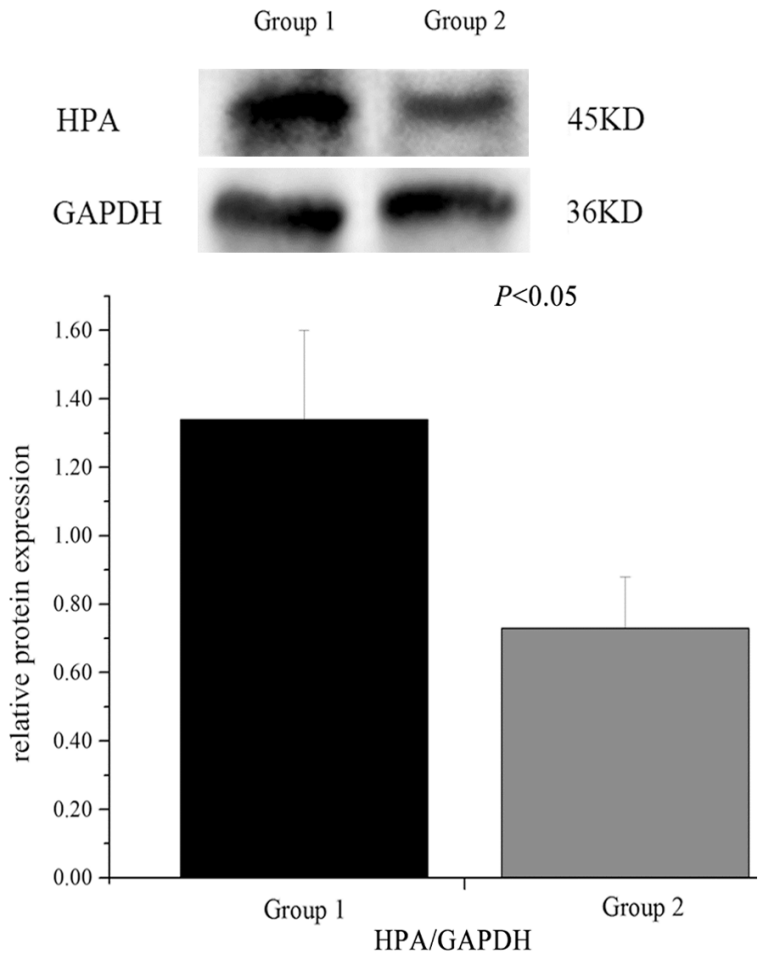

Figure 7. Heparanase (HPA) protein levels of infarct area of both groups. P < 0.05 means statistically significant between two groups.

guide catheter and LV ejection fraction declined sharply at reperfusion time points. Serious myocyte nuclear loss, mass inflammatory cell infiltration, and contraction band necrosis were evaluated by light microscopic in the tissue sections from LAD regions by H&E staining in each group of pig hearts. Myocardial cell swelling was associated with an obvious gap between cardiocytes fibrinolysis. Thus, under the ischemia related injury, IMR rose notably at reperfusion 0 hours in both groups of animals.

Reperfusion-related injuries have an important impact on microvasculature function [17, 18]. After reperfusion, LV ejection fraction did not rise and maintained a platform in a low level. Compared with reperfusion 0 hours, regional left ventricular anterior wall thickening rose obviously at reperfusion 2 hours. The anterior region segmental wall motion became serious abnormal during the reperfusion period. Peak early filling velocity (E-wave) decreased and late diastolic filling velocity (A-wave) increased. Serum concentrations of ET-1 continued rising at reperfusion 1 hour and 2 hours, but NO decreased gradually. Therefore, as the result, coronary microcirculation function deteriorated continuously as reflected in the value of IMR rising step by step in the reperfusion time points in group 1. However, the trend of IMR in group 2 was not like group 1 in the reperfusion period. IMR did not have significantly changes since reperfusion in group 2.

Several factors may explain the phenomenon of change trend of IMR between the two groups. Mass cardiocytes and lots of endothelial cells of coronary circulation system from LAD region were necrosis under the circumstance of continuous balloon occlusion, for up to 90 minutes in group 1. Lots of animal models of occlusion and reperfusion have been adopted to investigate the sequence of events that culminate in microcirculation dysfunction [19, 20]. Previous animal experiments have revealed that myocardial cell injuries occur ahead of endothelial cells under coronary occlusion [21]. Through electron microscopes, without blood flow over 20 minutes, myocardial cells began necrosis and deteriorated continuously in severity with longer durations of ischemia [22]. After 40 minutes of continuous ischemia, red blood cell stasis was occasionally observed. At 60-90 minutes of occlusion, the phenomenon of red blood cell stasis became common [21]. Nonetheless, ultrastructure destruction of endothelial cells was not observed until 60 minutes of coronary ischemia and with a large loss of pinocytotic vesicles at over 90 minutes of ischemia. This conclusion was supported by other studies as well [23]. Using an acetylcholine test, researchers investigated the differences of endothelial-dependent vasodilation between 15 minutes and 60 minutes of occlusion and reperfusion

on normal animal hearts. Results showed intact endothelial function after 15 minutes of occlusion in contrast to a sharply reduced vasodilatory ability after 60 minutes of occlusion and reperfusion in reversibly and especially in irreversibly injured myocardium. The above studies suggest that microvasculature damage was behind ultrastructure damage within the myocardial cells. In experimental studies, after endothelial cells are damaged, intraluminal endothelial protrusions and intraluminal membrane-bound bodies, which observed by electron microscopy, obstruct capillary blood flow and cause microcirculation dysfunction [24, 25]. In the present study, myocardial cells and intact endothelial cells were damaged in group 1. In group 2, the total ischemia time was up to 90 minutes in all animal hearts, but continuous ischemia time was only 30 minutes in one circle, which could lead to myocardial cell necrosis except the structure of vessel endothelial cells. Because of significant influence on ischemia related injuries in each group, myocardial cell necrosis and endothelial cell dysfunction resulted in obvious changes of IMR at different reperfusion time points.

A pathologic role of oxygen radicals during reperfusion related injury was also noted. Oxygen radicals contributed to direct endothelial cell and myocardial tissue damage and abnormal cardiac action potential duration, arrhythmogenesis, and contractile dysfunction [26, 27]. Loss balance of NO/ET-1 concentrations released by damaged endothelial cells contributed to sustained vasoconstriction of coronary microcirculation, which in turn blocked regional blood flow [28, 29]. Moreover, ET-1 was a mediator of CMD and predicted angiographic CMD during myocardial infarction [30].

Heparanase protein levels of the infarct area of pig hearts, induced by balloon inflation, continuously were much higher than balloon inflation intermittently. Thus, heparanase might play a vital role in the pathogenesis of CMD after STEMI in pigs. Heparan sulfate is a major component of glycocalyx, a linear proteoglycan compound coating luminal surface of the vascular endothelial cells [31]. Heparanase is the sole β-endo-glucuronidase that specifically cleaves heparan sulfate in the mammalian [32, 33]. When levels of heparanase become higher, more heparan sulfate is cleaved and function barriers of endothelial cells are damaged. As a result, because of ischemia and reperfusion related injuries, the method of balloon occlusion and deflation could lead to microcirculation dysfunction in an animal model. However, significant differences in ejection fraction, loss balance of NO/ET-1 concentration, and ultrastructure damage of glycocalyx and endothelial cells, especially the value of IMR, revealed the fact that continuous ischemia occlusion causes more serious microvasculature dysfunction than intermittent ischemia. It should be noted that higher incidence rates of ventricular tachycardia, more times of electrical defibrillation, and higher mortality have occurred during animal experiments in continuous occlusion groups than intermittent occlusion groups.

In the past 40 years, adopting thoracotomy and ligating left anterior descending coronary artery or left circumflex coronary artery has become a well-known model of MI induction [7, 34-36]. Intracoronary $FeCl_3$ has become quite popular for producing physiological and biochemical changes like human coronary arterial thrombus formation in an open chest animal model [37]. Up into the PCI age, it is common that ischemia and reperfusion achieve via balloon occlusion and deflation in large animal experiments [9, 36]. The present study adopted a closed-chest catheter-based technique. Compared with thoracotomy, this intervention technique had the significant advantages and avoided shortcomings of requirement of highly skilled surgeon, high mortality rates, more chances of surgical trauma, and post-surgical infection [38].

There were several limitations to the present study. First, this study did not evaluate IMR at time points between 3 hours and 24 hours after reperfusion. Thus, this study could not determine variation tendencies of IMR. Likewise, although regional LAD returned to reperfusion, the highest concentration of cTnI emerged at 24 hours after reperfusion. This study cannot exclude the possibility that it could have increased further or decreased gradually between 3 hours and 24 hours after reperfusion. Second, this study used a cardiac ultrasonography, not cardiac magnetic resonance, evaluation effect of animal models. Thus, the sensitivity of assessment coronary microvasculature dysfunction was poor. Lastly, various factors contribute to CMD in clinical setting, such as distal embolization, ischemia-related injury, reperfusion-related injury, indi-

vidual predisposition of coronary microcirculation to injury, and so forth. In this study, the obvious change of IMR remaindered CMD in animal model, which only mimicked the factors of ischemia and reperfusion.

In conclusion, coronary occlusion and reperfusion could lead to abnormal values of IMR in region of LAD, whereas continuous balloon inflation results in more serious microcirculation dysfunction than intermittent patterns.

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Disclosure of conflict of interest

None.

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