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Original Article

The protective effect of Prostaglandin E1 combined with mild hypothermia on ischemic/reperfusion injury of pulmonary micro-vascular endothelium of ROSC rat

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Abstract: This study aims to investigate Prostaglandin E1 (PGE1) combined with mild hypothermia as a therapeutic intervention on ischemic/reperfusion (I/R) injury of pulmonary micro-vascular endothelium cells (PMEC) from return of spontaneous circulation (ROSC) rat with successful cardiopulmonary resuscitation (CPR). Ventricular fibrillation (VF) in 36 rats was induced by transoesophageal cardiac pacing with alternating current. They were divided into 4 groups after CPR: ROSC group (R), Hypothermia group (H), PGE1 group (P) and PGE1/Hypothermia group (PH). Another 3 rats without VF/CPR were set as Blank control group (B). Arterial blood gas was analyzed at 0.5 h, 4 h and 8 h post ROSC and 3 rats from each group were sacrificed at each time point. I/R injury of PMEC was evaluated through tissue morphology, hematoxylin and eosin (HE) staining, vascular endothelial (VE)-cadherin RNA expression with PCR, CD34/TUNEL and vascular endothelial growth factor receptor (VEGFR)/VE-cadherin double fluorescent immunohistochemistry (IHC) staining. PGE1, mild hypothermia and PGE1/hypothermia, all 3 interventions could significantly alleviate metabolic acidosis and hypoxemia ($P<0.05$) of ROSC rats to different levels at different time points. All 3 interventions could significantly improve pulmonary interstitial edema, hemorrhagic lesion, PMEC apoptosis, and alleviate VE-cadherin protein loss, while PGE1/hypothermia showed most dramatic effect. At 0.5 h, moderate compensation tendency of VE-cadherin RNA expression of the 3 interventions may indicate the less I/R injury of PMEC of ROSC rats ($P<0.05$). Both PGE1 and mild hypothermia could alleviate I/R injury of PMEC from ROSC rats respectively, while the PGE1/hypothermia combined intervention might have synergistic better effect than either single intervention.

Keywords: CPR, PMEC, ischemic/Reperfusion Injury, PGE1, mild Hypothermia

Introduction

Vascular endothelium dysfunction is a key component in post-cardiac arrest syndrome (PCAS) study [1]. Previous studies indicate that vascular endothelium cells involve in the whole process of I/R injury of ROSC [2]. Mild hypothermia is recommended as one of the standard therapeutic strategies in post-cardiac arrest patients by American Heart Association (AHA) [3]. However, the therapeutic strategies for PCAS are integrated by multidisciplinary comprehensive strategies and mild hypothermia may lead to a series of adverse consequences, such as shivering, coagulopathy, arrhythmia and so on. Whether mild hypothermia would affect the outcome of other therapeutic treatments when they are administered together, is still unknown.

PGE1 is a natural prostaglandin with multiple functions, such as relaxing vascular smooth muscle [4], inhibiting platelet aggregation [5], decreasing blood viscosity and erythrocyte aggregation [6], improving microcirculation [7], preventing atherosclerotic plaque formation [8] and improving nerve injury [9]. It has been confirmed that PGE1 has protective effect on I/R injury in multiple organs, such as lung [10], kidney [11] and heart et al [12]. However, there is currently no study focusing on evaluating PGE1 regulatory effect on vascular endothelium dysfunction caused by I/R injury of ROSC. In the present study, we investigated the possible protective effect of PGE1, mild hypothermia and PGE1/hypothermia combined intervention on PMEC from ROSC rat. I/R injury of PMEC was evaluated among different intervention groups

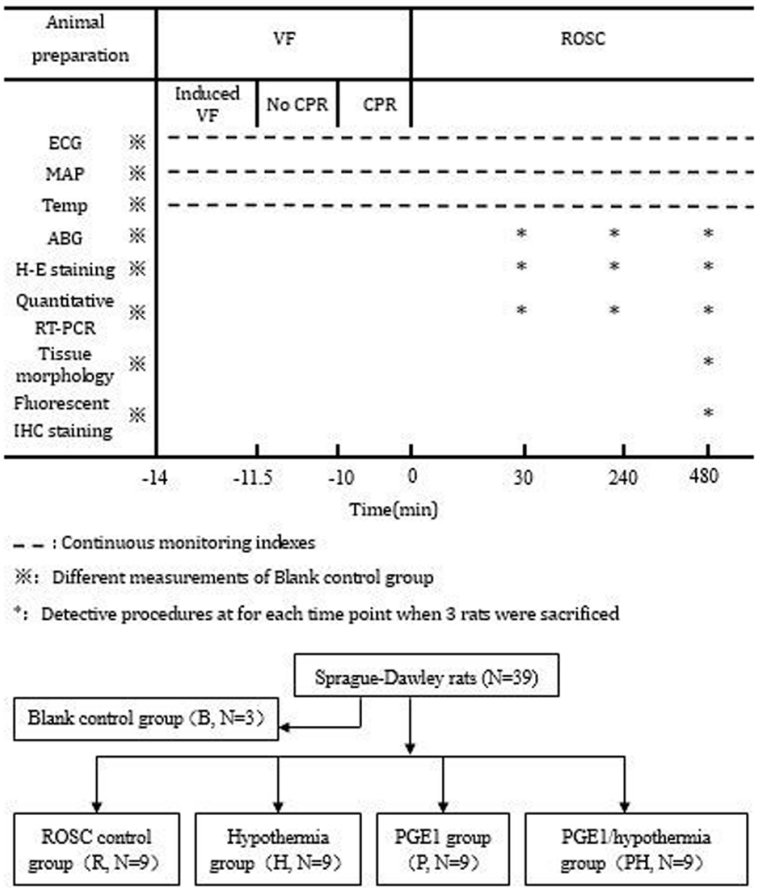

Figure 1. The experiment groups and protocol.

through arterial blood gas analysis, HE staining, double fluorescent IHC staining and VE-cadherin RNA expression analysis.

Methods

Animals and reagents

39 SPF healthy male Sprague-Dawley rats between 12-14 weeks, 370±20 g were purchased from Chengdu Dashuo Biological Technology Co. Ltd. (Chengdu, China). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan University. The rats were housed in a temperature (22±2°C) and humidity-controlled room with free access to fresh water and standard laboratory food. Bipolar pacing electrode that was used for inducing VF was Select Secure Model 3830 (Medtronic, Inc, USA). PGE1 solution (5 μg/ml) was from Beijing Tide Pharmaceutical Co. Ltd. (Beijing, China). VE-cadherin antibody was from Cell Signaling Technology Inc. (MA, USA). VEGFR antibody and CD34 antibodies were from Bioworld (OH, USA). TUNEL assay kit was from Cayman Chemical (MI, USA). RNA prep pure kit, Quant cDNA kit and Super Real PreMix kit were from TIANGEN Biotech Co. Ltd (Beijing, China).

Animal model and experimental protocol

After 1 week of conditioning in a 12-hour light/dark cycle, 3 rats without VF were set as Blank control group (B). VF was induced in other SD rats by transoesophageal cardiac pacing with alternating current (50 Hz, 6 mA, 4 ms) for 150 s [13, 14]. 4 min later, these rats with cardiac arrest were treated with 200 times/min chest compression using homemade animal cardiopulmonary resuscitator. ROSC was defined as the return of supraventricular rhythm with a mean aortic pressure of ≥60 mmHg for a minimum of 10 min [15]. 36 rats with successful cardiopulmonary resuscitation were established as ROSC model and randomly divided into 4 groups: ROSC control group (R), hypothermia group (H, body temperature was decreased to 33±1°C within 30 min by physical approach), PGE1 group (P, 1 ml PGE1 solution was given intravenously with syringe pump in 1 minute. Fast injection speed would decrease the success rate of resuscitation), PGE1/hypothermia group (PH, combined treatment with PGE1 and hypothermia). Rats were sacrificed and analyzed at 0.5 h, 4 h and 8 h post treatment with 3 rats for each time point (**Figure 1**). Procedures for animal care were approved by the Committee on the Ethics of Animal Experiments at Sichuan University.

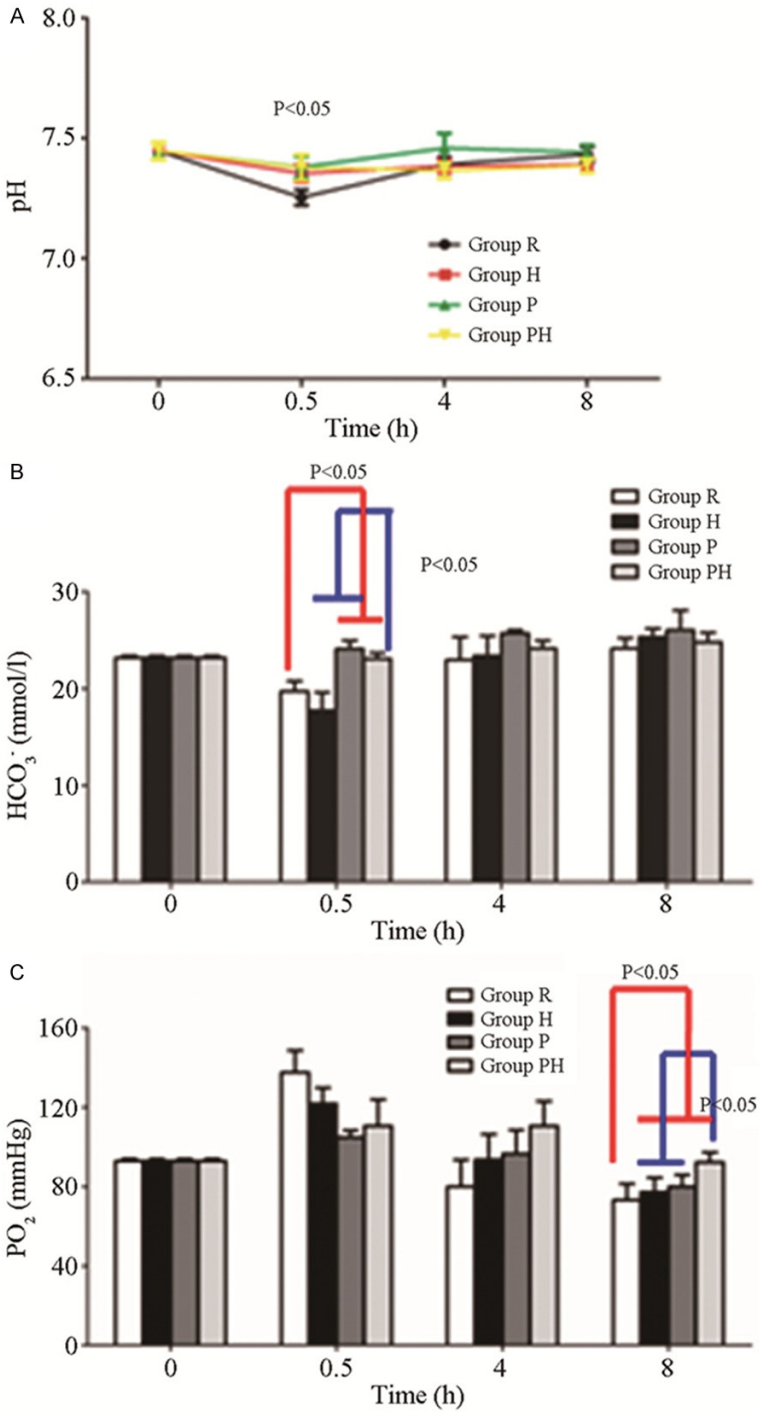

Figure 2. Arterial blood gas parameters of different groups. Compare with B group, in R group at 0.5 h, both PH (7.25±0.03) and HCO_3^- (19.70±1.05 mmol/l) significantly decreased, and then, they gradually increased at 4 h and 8 h in all groups. At 0.5 h, the improvement of metabolic acidosis was more significant in P group and PH group (P<0.05), and PH group had better effect than P and H groups (P<0.05). Hypoxemia was obviously in R group at 4 h and 8 h when air was given. While there was no obvious difference between groups in the early phase, the improvement of hypoxemia was more significant in P, H and PH groups at 8 h (P<0.05). According to the statistics, PH group showed better effect than P and H groups (P<0.05).

Tissue morphology and HE staining

Whole pulmonary tissue was taken out. Pulmonary edema and hemorrhagic foci were carefully checked. HE staining was performed on tissue slides as described before [16]. Pulmonary cell edema, inflammatory cell infiltration, necrosis and micro-thrombus formation were checked under microscope with bind method.

Fluorescent IHC staining

CD34 and TUNEL double staining was performed on pulmonary tissue slides according to manufacture procedures. VEGFR and VE-cadherin double staining was performed as described before [17]. I/R injury of PMEC were assessed.

Quantitative RT-PCR

Pulmonary total RNA was extracted using RNA prep pure kit according to manufacture procedures. The quantity of the RNA product was determined by ultraviolet-visible spectroscopy and checking the ratio of 28 s, 18 s and 5 s bands on a 2% agarose gel controlled the quality. Quantitative RT-PCR was performed using Quant cDNA kit and Super Real PreMix kit according to manufacture procedures. The amplified PCR product was visualized on a 1.5% agarose gel. RNA expression level was determined by the volume of the PCR product band. Actin was served as internal control. Forward primer (5'-3') was 5'-TGGACACCACAAGCTACGAC-3' and reverse primer (5'-3') was 5'-GGACTCTGCGATGGACTCTG-3'.

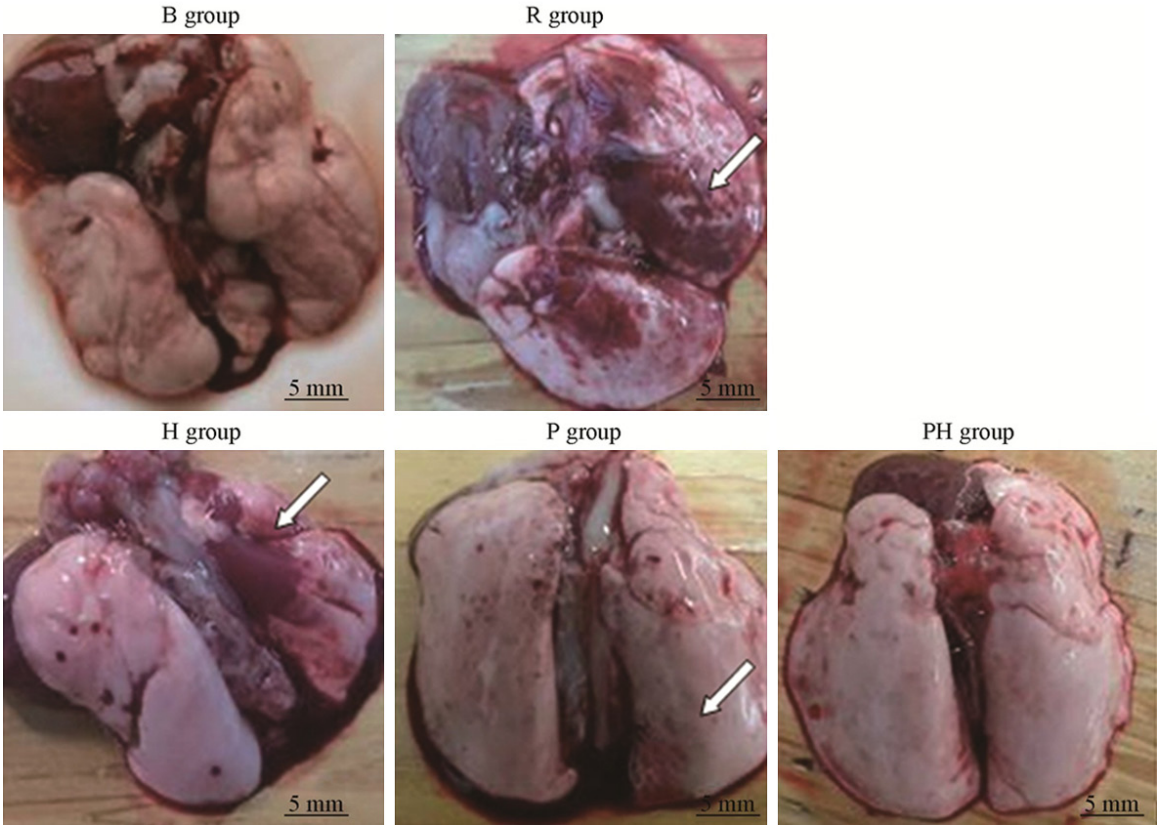

Figure 3. Whole pulmonary tissue morphology. The pulmonary tissue from B group showed normal structure without obvious edema and local hemorrhagic foci. However the tissue from R group showed diffuse hemorrhagic lesion and obvious pulmonary edema. The hemorrhagic foci were less and smaller in H and P groups than R group while hemorrhagic foci were least in PH group.

Statistical analysis

Data are presented as mean ± SD. Comparison between groups were tested by one-way ANOVA analysis. Comparison between 2 groups with $P<0.05$ were further tested by SNK analysis. $P<0.05$ was considered as statistically significant.

All statistical analysis was performed using SPSS19.0 software (SPSS Inc., Chicago, IL, USA).

Results

For experimental groups, the 36 SPF SD rats were healthy and their physiological index were within normal ranges (Weight: 372.3±17.8 g, Heart rate: 389.9±28.4 beats/min, Mean arterial pressure: 89.4±7.6 mmHg, Temperature: 37.1±0.5°C).

PGE1, hypothermia and PGE1/hypothermia combined interventions improved arterial blood gas parameters of ROSC rat

The normal range for arterial blood PH is 7.29-7.38 and HCO_3^- is 22.80-26.75 mmol/l in rats [18]. Obvious metabolic acidosis was observed in R group at 0.5 h, and gradually relieved while time went by. The improvement of metabolic acidosis was significant between different groups only at 0.5 h ($P<0.05$). But this effect had become not so obvious along with time (**Figure 2**).

As the previous study reported, the normal value for arterial pressure of oxygen (PO_2) is 87-106 mmHg in rats [18]. According to the experiment procedure, rats were given 100% oxygen within 0.5 h after successful CPR, and then they were given air with 21% oxygen. R group initially showed higher PO_2 (137.66±

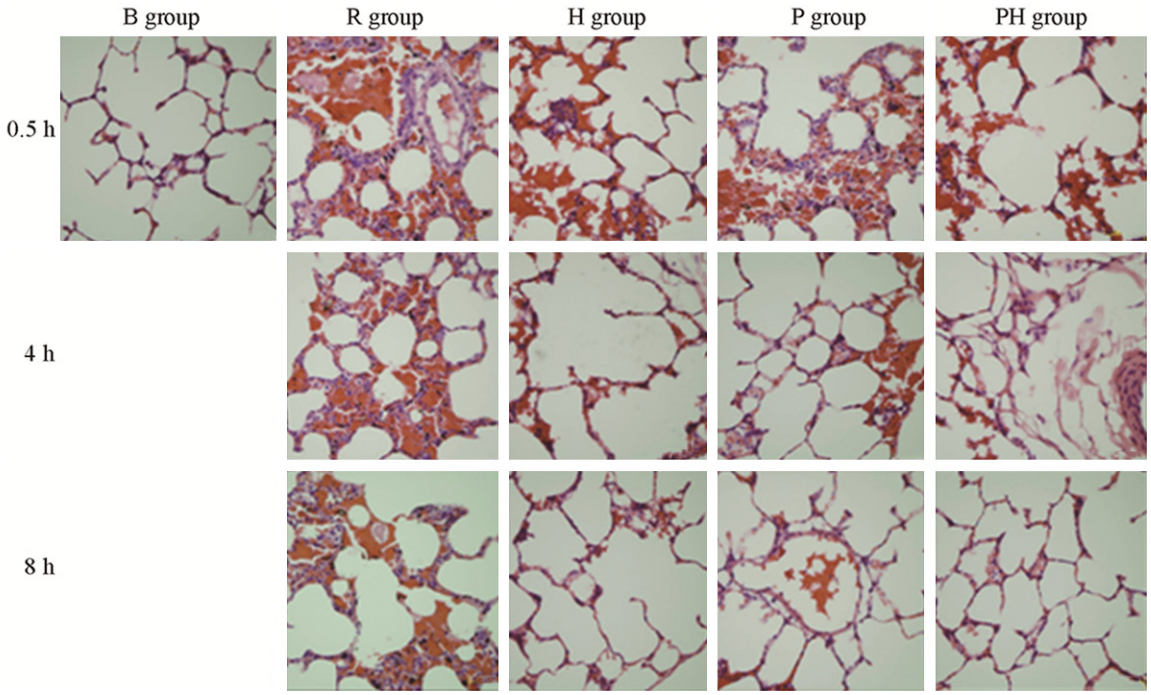

Figure 4. HE staining. HE stained histological evaluation of rat lungs in B, R, H, P and PH groups at 0.5 h, 4 h and 8 h (×200 magnification). At every time spot, Compared with B group, R group showed interstitial edema and inflammatory cell infiltration. In H, P and PH groups, the alveolar and pulmonary interstitial edema, hemorrhagic lesion and inflammatory cells infiltration were remarkably alleviated, especially the PH group.

11.2 mmHg) than normal value at 0.5 h, and gradually decreased with hypoxemia observed later. When hypoxemia became severe, the three different interventions all showed significantly improvement at 8 h, among them, PH group had better effect than P and H groups (**Figure 2**).

PGE1, hypothermia and PGE1/hypothermia combined interventions had protective histopathology effects on ROSC rat

Whole pulmonary tissues were taken out from sacrificed rats at the end of experiment procedure. From the comparison of the amount and range of local hemorrhagic foci, it is easy to find out that the damage of the pulmonary tissue after ROSC was obviously reduced in H, P and PH groups, while hemorrhagic foci were least in PH group (**Figure 3**).

HE staining of normal pulmonary tissue from B group showed clear and healthy structure. Cells arranged closely and orderly and the alveolar wall was thin. R group showed obvious alveolar inflammation, with alveolar and pulmonary interstitial edema, pink colored edema fluid filled in alveolar space, injury of PMEC, diffuse hemorrhagic lesion, alveolar space collapse and infiltration of inflammatory cells into alveolar and pulmonary interstitial spaces. As time went by, infiltration of inflammatory cells decreased, however, blood vessel structure destruction and micro blood vessel interruption were not improved. In H and P groups, the alveolar and pulmonary interstitial edema, hemorrhagic lesion and inflammatory cells infiltration were alleviated compared to R group and as time went by, edema fluid in alveolar space and inflammatory cells infiltration were gradually improved. In PH group, the alveolar and pulmonary interstitial edema, hemorrhagic lesion and inflammatory cells infiltration were most alleviated and as time went by, the improvement was most dramatic (**Figure 4**).

CD34/TUNEL double fluorescent IHC staining CD34 is a vascular endothelial cell surface marker that is commonly used. IHC staining showed that in normal tissue of B group, healthy PMEC (CD34 positive, green) scattered in the image and no obvious apoptosis observed.

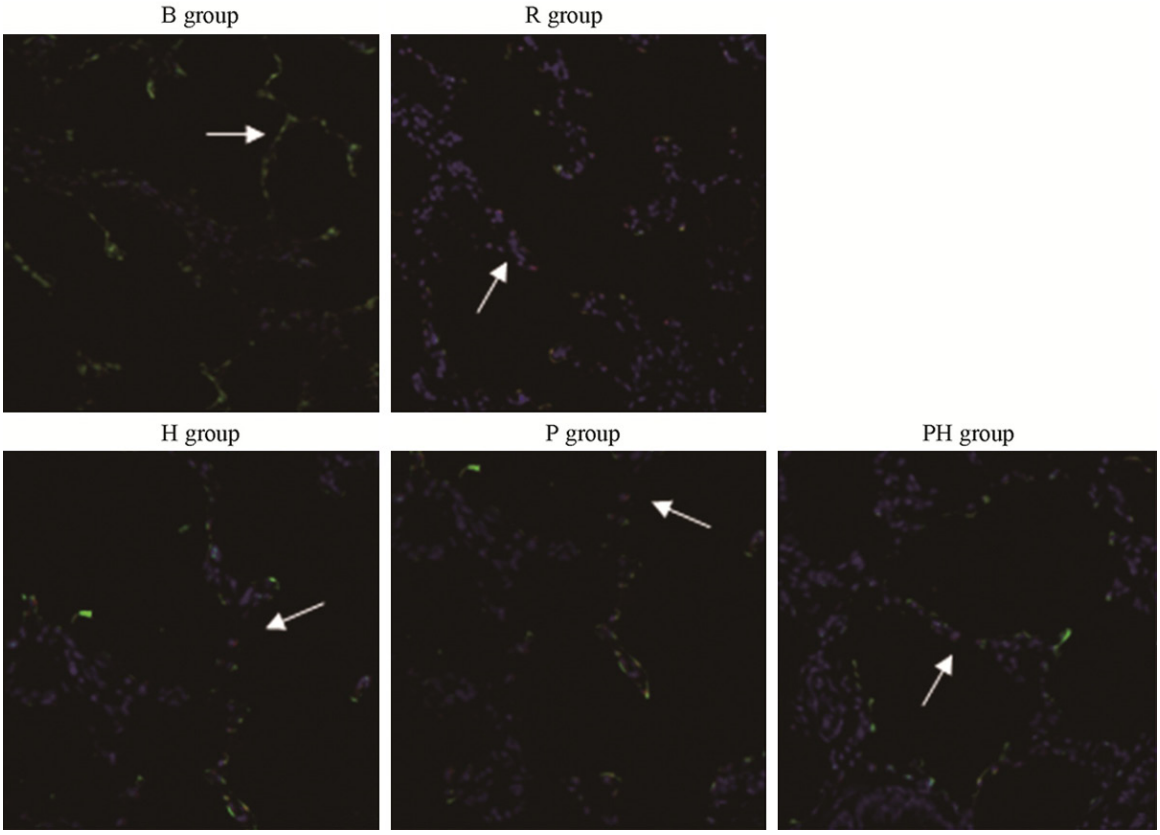

Figure 5. CD34/TUNEL double fluorescent IHC staining. In B group, the normal PMEC was showed (CD34 positive, green) without apoptosis. But In R group, healthy PMEC was hard to find, instead of which, apoptotic PMEC increased (CD34/TUNEL positive, pink). Compare with R group, apoptosis was significantly decreased in H, P and PH groups. PH group seemed to have the greater improvement than H and P groups.

TUNEL staining would be red of cell apoptosis. In R group, healthy PMEC significantly decreased and apoptotic PMEC increased (CD34/TUNEL positive, pink). However, apoptosis was significantly improved in P, H and PH groups with much less apoptotic PMEC (**Figure 5**).

VEGFR/VE-cadherin double fluorescent IHC staining VEGFR (VEGFR positive, green) was used to label the PMEC. In B group, VE-cadherin protein (VE-cadherin positive, red) expressed normally in PMEC junctions (VEGFR/VE-cadherin positive, pink). In R group, the expression significantly decreased while in H and P groups, it was improved. PH group showed the highest expression among interventions (**Figure 6**).

VE-cadherin RNA analysis in PMEC showed that the compensatory RNA expression increase after ROSC and the expression gradually decreased as time went by. There was significantly difference between R group and H, P or HP groups at 0.5 h ($P<0.05$). There was no significant difference at 4 h and 8 h ($P>0.05$) (**Figure 6**).

Discussion

Animal model

The most common initial arrhythmia encountered in cases of sudden cardiac death is VF. This arrhythmia is well simulated by electric simulation of the heart in experimental animals. Compare with alternating current delivered to the right ventricular endocardium [19], VF induced by transoesophageal cardiac pacing in the present study was easier to perform, less harmful and more reproducible [13, 14].

I/R injury of PMEC from ROSC rat

Sudden cardiac arrest (CA) remains a major public health issue. Even though the significant

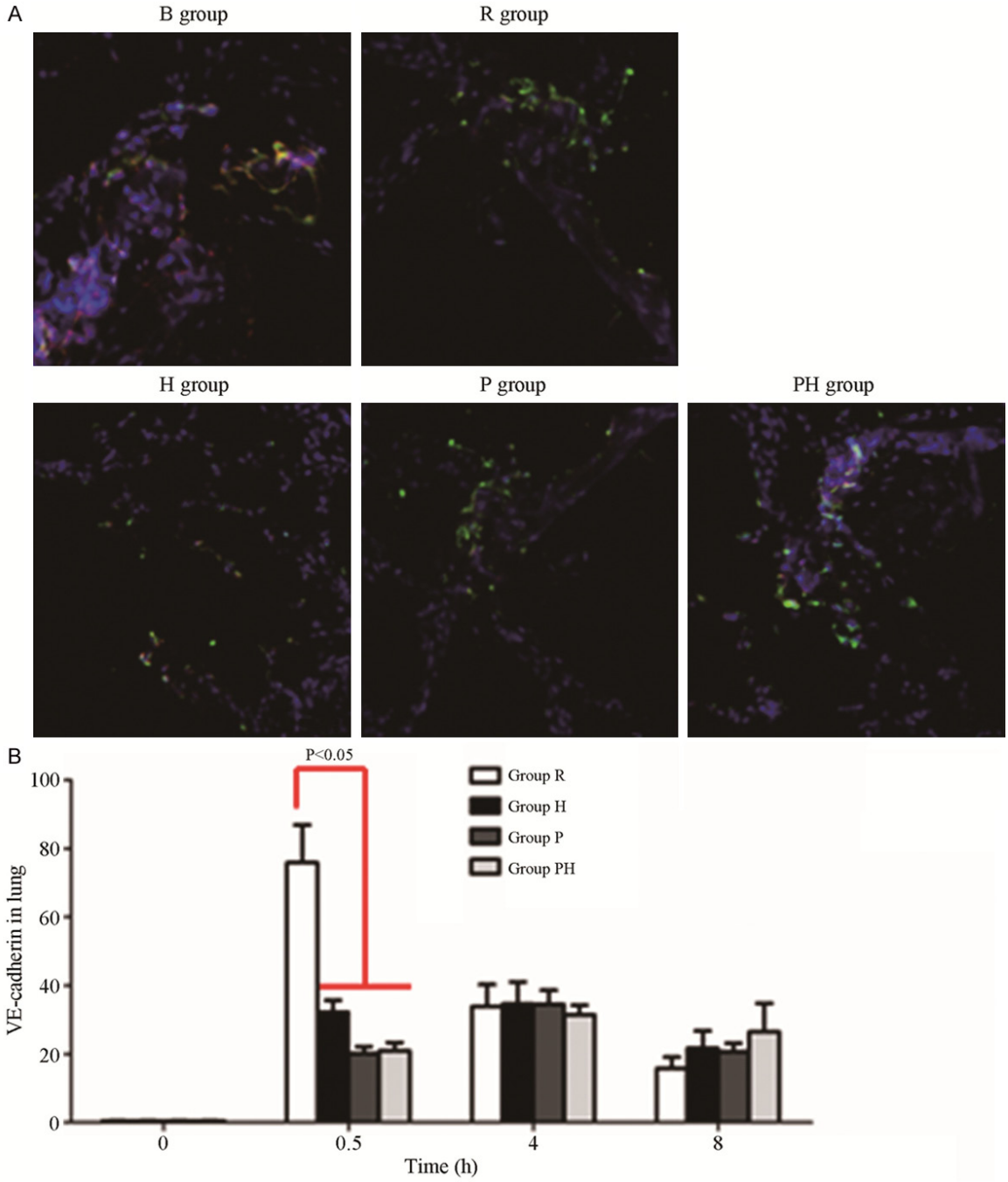

Figure 6. VEGFR/VE-cadherin double fluorescent IHC staining and VE-cadherin RNA analysis. The staining showed that in normal tissue of B group, VE-cadherin protein obviously expressed (VEGFR/VE-cadherin positive, pink). In R group, the expression significantly reduced. Compare with R group, VE-cadherin expression was increased in both H and P groups, while PH group had the remarkable improvement. 0.5 h after ROSC, all groups PMEC showed that the compensatory RNA expression increase of VE-cadherin and the expression. There was significantly difference between R group and H, P or HP groups ($P<0.05$). But as time went by, these increasing were gradually reduced. There was no significant difference at 4 h and 8 h ($P>0.05$).

progress in initial pulmonary resuscitation has improved the restoration of spontaneous circulation (ROSC) in CA victims, the overall survival to discharge remains dismally low at 9.6-17% for out-of-hospital cardiac arrest (OHCA) and in-hospital cardiac arrest (IHCA) [20]. The patho-

physiology of CA represents stages of cell injury that may be attributed initially to ischemia and then amplified following the ROSC [21]. Prognosis after CA is primarily determined by the degree of I/R injury. Reperfusion injury denotes a potentially avoidable pathological process wherein potentially viable cells die not entirely because of the cellular derangements of ischemia [22]. During CPR, whole body oxygen consumption compensatory increases while perfusion pressure is limited, thus shortage of tissue oxygen supply occurs. The result of our study showed that though the circulation was returned, the oxygen debt accumulation. During the 8 hours, arterial blood gas analysis of ROSC rat indicated that there was obvious metabolic acidosis of body system during the initial stage of CPR (0.5 h). Though it was getting better as time went by, hypoxia condition continued and developed.

CPR is a classic model of whole-body I/R injury. The cessation of blood flow followed by a period of restoration of flow is the hallmark of engendering I/R injury. Along with the continuous development of research, the researchers found that, during the period of ischemia, a series of metabolic and biochemical changes occur. Tissue hypoxia, followed by anaerobic metabolism, acidosis, increased intracellular Ca^{2+}, and decreased adenosine triphosphate, comprise the classic phenomena. Restoration of blood flow produces reperfusion injury, which consists of molecular phases [2]. In our study, pulmonary histopathology analysis indicated that pulmonary tissue from ROSC rat developed obvious edema and local hemorrhagic foci. HE staining showed alveolar and pulmonary interstitial edema, structure disorders, infiltration of inflammatory cells, local diffuse hemorrhagic lesion and micro-thrombus formation. It is consistent with previous study that marked activation of blood coagulation and fibrin formation would occur after prolonged cardiac arrest and it would induce fibrin deposition and micro-thrombus formation. Endogenous fibrinolysis is not adequate during reperfusion, leading to diffuse micro-thrombus formation in blood vessels and microcirculation compromise [23].

Endothelial involvement in CPR as a primary or secondary target has been described in both animal models and human patients [24, 25]. Because of its vast extension and active metabolic status of producing mediators for vasomotor tone, coagulation, and inflammation, it is a key target for therapy during I/R injury. It has become apparent that the endothelium participates in a host of responses elicited by I/R. According to the results, endothelial injury is central to activation of the coagulation cascade, leukocyte rolling and interaction with transmigration of leukocytes, and burst of reactive oxygen species. Large amounts of inducible nitric oxide synthase, primarily produced via leukocytes, macrophages, and inflammatory cells, lead to activation of the transcriptional factor nuclear factor-κβ [2]. Our TUNEL apoptosis analysis of PMEC indicated that PMEC went through significant apoptosis in micro-vessels enriched pulmonary tissue after CPR.

Cell adhesion molecules (CAMs) are proteins located on the cell surface involved in binding with other cells or with the extracellular matrix in the process called cell adhesion. Currently a lot of researchers use vascular endothelial adhesion molecules as an indicator of vascular endothelial injury of cardiac arrest [1]. When endothelial injury happens, CAMs on cell surface decrease. It could be recovered through CAM transportation from storage pool inside cell or induction of de novo synthesis. Transportation happens very quickly, which only needs seconds, however it couldn't last long. De novo synthesis needs hours, which is slow but sustainable.

VE-cadherin belongs to cadherin family. It is the major and specific adhesion molecule located at junctions between vascular endothelial cells and of vital importance for the maintenance of vascular endothelial cells polarity and integrity. Previous studies show that VE-cadherin plays vital roles in vascular permeability, angiogenesis and endothelial cell proliferation [26]. In the present study, VEGFR/VE-cadherin double fluorescent IHC staining showed that VE-cadherin expression in PMEC from ROSC rat significantly decreased after pulmonary resuscitation, indicating the destruction of adherent junctions between vascular endothelial cells. Meanwhile, quantitative RT-PCR analysis in tissue lysate from different groups showed that there was compensatory large increase of VE-cadherin RNA expression during the initial stage (0.5 h) after CPR, due to VE-cadherin loss at cell surface, and then the expression gradually decreased as time went by. These data

support the idea that I/R injury of PMEC after CPR was remarkable. As the injury occurred, body system would motivate self-recovery potential to prevent the injury from developing. So the VE-cadherin RNA level was driven up compared to B group even though the protein level significantly decreased.

Protective effect of PGE1 and hypothermia on I/R injury of PMEC from ROSC rat

More and more studies have confirmed the protective effect of therapeutic mild hypothermia on nervous and circulating systems after CA. Early studies of hypothermia therapeutic mechanism focus on decreased metabolic rate caused by lower temperature. Recent studies give more attention to inhibited cell apoptosis and inflammation caused by lower temperature [27]. The possible mechanism of protective effect of hypothermia on PMEC may involve inhibiting expression of cytokines and CAMs, which is very complicated. Essentially, hypothermia decreases protein biological activities through changing advanced structures and optimum status of protein molecules, including enzymes, cytokines et al.

During the initial stage of ROSC after CA, whole body I/R injury often superimposes on the disease or injury that caused the CA. Therapies that focus on individual organs may compromise other injured organ systems [28]. PGE1 is a biological active substance having broad effect on vascular endothelial system. Applied research of PGE1 has currently extended to brain [29], lung [10], kidney [11], liver [30], heart, major blood vessels [12] and peripheral blood vessels [31] et al Considering the pharmaceutical effect of PGE1 without organ selection, our study aims to investigate the possible protective effect of PGE1 on PMEC injury after CPR.

Our results indicated that PGE1 and mild hypothermia were both able to improve ischemia of ROSC rat. Pulmonary histopathology and TUNEL fluorescent IHC staining analysis of different intervention groups showed that PGE1 and mild hypothermia were both able to alleviate alveolar and pulmonary interstitial edema, structure disorder, inflammatory cells infiltration, PMEC apoptosis/destruction and microthrombus formation. VEGFR/VE-cadherin double fluorescent staining indicated that PGE1 and hypothermia were both able to improve VE-cadherin loss on PMEC surface caused by I/R injury after CPR, to some extent. VE-cadherin RNA expression in pulmonary tissue lysate analysis indicated that VE-cadherin RNA level in PMEC within 0.5 h after CPR of those groups with the interventions of PGE1 and hypothermia could significantly lower. Moderate compensation tendency may indicate the less injury of PMEC.

In our study, we compared PGE1/hypothermia combined intervention with either single intervention, and found that the combined intervention had better effect than either single intervention on metabolic acidosis at early stage of ROSC (0.5 h) or hypoxemia at late stage (8 h). It could also strongly improve pulmonary edema, inflammatory cells infiltration, PMEC apoptosis and VE-cadherin rapid loss on PMEC surface, so as to protect PMEC from injury. However, for the VE-cadherin RNA expression, there was no difference.

Limitations

First, our study was performed in a rat model. Anatomic and phsiological differences between human and rat may limit the applicability of these results to humans. Moreover, due to the limitation of the experimental facilities, we used alternating current delivered through the transoesophageal cardiac pacing instead of to the right ventricular endocardium to induce the VF. That would be different from the real clinical etiology of cardiac arrest. Finally, because the research funding was not so sufficient to meet all the requirements of the primary project, further research would be needed to determine the long-term effect and potential hazard, so that we may find a new reasonable and practicable way for the ROSC patient.

Conclusions

In summary, we arrive at following conclusions based on the results: 1. PMEC structure and function disorder is a key component of I/R injury of ROSC rat after CPR. 2. PGE1 and mild hypothermia had protective effect on I/R injury of PMEC from ROSC rat separately, while the PGE1/hypothermia combined intervention might have synergistic better effect than either single intervention.

Prior presentations

We investigated PGE1 combined with mild hypothermia as a therapeutic intervention on ischemic/reperfusion injury of pulmonary micro-vascular endothelium cells from ROSC rat with successful CPR, and found the PGE1/hypothermia combined intervention might have synergistic better effect than either single intervention.

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Disclosure of conflict of interest

None.

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