Effect of exogenous phosphocreatine on cardiomyocytic apoptosis and expression of Bcl-2 and Bax after cardiopulmonary resuscitation in rats

Ping Yan, Shou-quan Chen, Zhang-ping Li, Jie Zhang, Ji-ke Xue, Wan-tie Wang, Wei-jia Huang, Jun-yan Cheng, Hui-ping Li

Department of Emergency Medicine, First and Second Affiliated Hospitals of Wenzhou Medical College, Wenzhou 325000, China

Corresponding Author: Shou-quan Chen, Email: csq@hosp1.ac.cn

BACKGROUND: Ischemia-reperfusion injury in the myocardium after cardiac arrest (CA) and cardiopulmonary resuscitation (CPR) is an important pathologic basis of post-cardiac arrest of syndrome (PCAS), and apoptosis is one of the major mechanisms in myocardial ischemia-reperfusion injury. To lessen myocardial ischemia-reperfusion injury after cardiac arrest and CPR, it is important to reduce energy consumption and to increase energy supply in the myocardium. This study aimed to observe changes of cell apoptosis and expression of Bcl-2 and Bax protein on the myocardium after CPR in rats, and the protective effects of different doses of exogenous phosphocreatine (creatine phosphate, CP) on them.

METHODS: A total of 32 male adult Sprague-Dawley rats were randomly divided into 4 groups: control group (group A), CPR group (group B), low-dose CP group (group C, CP 0.5 g/kg at beginning of CPR and 1.0 g/kg at 2 hours after CPR) and high-dose CP group (group D, CP 1.0 g/kg at beginning of CPR and 2.0 g/kg at 2 hours after CPR). Cardiac arrest was induced by asphyxiation and CPR started at 7 minutes after asphyxiation in groups B, C and D. Myocardium samples were taken at 24 hours after CPR. Cardiomyocytic apoptosis was detected by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method. The expression of Bcl-2 and Bax protein was measured by immunohistochemistry.

RESULTS: Cardiomyocytic apoptosis index (AI) and expression of Bcl-2 and Bax protein increased more significantly in groups B, C and D than in group A (P<0.01), but Bcl-2/Bax ratio significantly decreased (P<0.01). Cardiomyocytic AI and expression of Bcl-2 and Bax protein decreased more significantly in groups C and D than in group B (P<0.01), but Bcl-2/Bax ratio increased more significantly (P<0.01). Cardiomyocytic AI and expression of Bcl-2 and Bax protein decreased more significantly in group D than in group C (P<0.05), but Bcl-2/Bax ratio increased more significantly (P<0.05).

CONCLUSION: Exogenous phosphocreatine, especially at a large dose, could inhibit cardiomyocytic apoptosis and alleviate myocardial injury after CPR in rats.

KEY WORDS: Cardiopulmonary resuscitation; Phosphocreatine; Apoptosis; Bcl-2; Bax; TUNEL

INTRODUCTION
In the process of cardiac arrest and cardiopulmonary resuscitation (CPR), human body suffered from severe ischemia-reperfusion injury, and the heart is unable to maintain the function of circulation. This is the important pathologic basis of post-cardiac arrest syndrome (PCAS). Cell apoptosis is one of the main mechanisms in myocardial ischemia-reperfusion
injury. To lessen myocardial ischemia-reperfusion injury after cardiac arrest and CPR, it is important to reduce energy consumption while increasing energy supply in the myocardium. It has been proved that exogenous phosphocreatine (creatine phosphate, CP) has protective effect on ischemic myocardium. In this study, an asphyxial rat CA-CPR model was established to observe changes of cardiomyocytic apoptosis and expression of apoptosis gene-related protein, and to study the effect of exogenous CP after cardiac arrest and CPR in rats.

**METHODS**

**Experimental animals and grouping**

A total of 32 healthy adult male Sprague-Dawley (SD) rats at clean degree, 70-95 days old, weighing 350-400 g (373.63±10.68), were provided by the Shanghai SLAC Laboratory Animal Limited Liability Company. The animals were fasted except for water for 12 hours before operation. They were randomly divided into 4 groups: control group (group A), CPR group (group B), low-dose CP group (group C, phosphocreatine 0.5 g/kg at beginning of CPR and 1.0 g/kg at 2 hours after CPR), high-dose CP group (group D, phosphocreatine 1.0 g/kg at beginning of CPR and 2.0 g/kg at 2 hours after CPR); there were 8 rats in each group.

**Establishment of animal models**

A rat CA-CPR model was established with an improved asphyxial method, and the experiment parameters were set and recorded according to the Utstein-style guidelines. Under anesthesia, tracheostomy tubing and vascular puncture were performed in each group. The tracheal tube was clamped at end-expiration, CPR started at 7 minutes after injection with 100 μg/kg of adrenaline through the carotid artery, and mechanical ventilation was given in groups B, C, D, but no asphyxia and CPR in group A. The rats were injected with creatine phosphate (CP, produced by Haikou Qili Pharmaceutical Limited Company) 0.5 g/kg at beginning of CPR and 1.0 g/kg at 2 hours after CPR in group C; 1.0 g/kg at beginning of CPR and 2.0 g/kg at 2 hours after CPR in group D; and the same volume of normal saline in groups A and B at a transfusion speed of 12 mL/h through the left femoral vein. The femoral vein intubation was removed after transfusion, then ligated and sutured. That spontaneous cardiac rhythm, pulse waves and systolic blood pressure (SP) above 60 mmHg (1 mmHg=0.133 kPa) over 10 minutes was defined as the return of spontaneous circulation (ROSC). Mechanical ventilation was removed gradually at 3 hours after the ROSC. After intubation, the carotid artery was ligated and sutured. A certain length of the endotracheal tube was retained. Then the rats were put in cages and free for eating or drinking water and room temperature was controlled at 23-26 °C. The animals were anesthesized and dissected at 24 hours after the ROSC, and myocardium samples were placed on the ice for measurement.

**Cardiomyocytic apoptosis index**

Samples of cardiac apex tissue were fixed for 24 hours in 10% formalin, then embedded in paraffin and sliced in 4 μm thickness. Apoptotic cells were detected using terminal deoxynucleotidyl transferase (TdT) -mediated dUTP -biotin nick end labeling (TUNEL) method according to kit instructions. TUNEL-positive cells appeared as brown particles in the nucleus and were counted in 5 high power fields (400×) selected randomly in each slice. Apoptosis index (AI) was counted in each group according to the formula, AI=(number of apoptotic cells/total number of myocardial cells)×100%, and taking average value.

**Determination of expression of apoptosis-related gene proteins Bcl-2 and Bax**

Samples of cardiac apex tissue were fixed for 24 hours in 10% formalin, then embedded in paraffin and sliced in 4 μm thickness. Immunohistochemical stainings of Bcl-2 and Bax were processed on adjacent four slices in each sample using the two-step immunoassay method according to kit instructions (Beijing Zhongshan product), with PBS instead of primary antibody as negative controls in some slices. The semi-quantitative analysis of Bax and Bcl-2 immunohistochemical staining was made using image analysis system software. Positive cells integral optical density (IOD) was recorded in 5 high power fields (400×) selected randomly in each slice, and the average value was taken for comparison. Meanwhile, Bcl-2/Bax was counted.

**Statistical analysis**

Data were analyzed with the SPSS 13.0 package. Variables were expressed as mean ±SD. Variance analysis was used to compare multiple groups, while Levene's test was used in homogeneity analysis, LSD test in homoscedasticity, and Tamhane's test in heterogeneity. \( P<0.05 \) was considered statistically significant.
RESULTS
Comparison of basic parameters between different groups

No significant differences were observed in parameters such as weight, dosage of chloral hydrate for anesthesia, baseline blood pressure and adrenalin dosage in animals of each group.

Changes of positive expression of Bcl-2 and Bax protein and Bcl-2/Bax ratio of the myocardium at 24 hours after ROSC in rats of each group

Bax protein was mainly expressed in the myocardial cytoplasm. The positive expression of Bax protein in the myocardium significantly increased in groups B, C and D compared with group A \( (P<0.01) \). This expression significantly decreased in groups C and D compared with group B \( (P<0.01) \). It was also significantly decreased in group D compared with group C \( (P<0.05) \) (Table 1, Figure 1).

Bcl-2 protein was mainly expressed in the cytoplasm or membrane of cardiomyocyte. The positive expression of Bcl-2 protein in the myocardium increased significantly in groups B, C and D compared with group A \( (P<0.01) \). The positive expression decreased significantly in groups C and D compared with group B \( (P<0.01) \). The expression of Bcl-2 protein decreased significantly in group D compared with group C \( (P<0.05) \) (Table 1, Figure 2).

Bcl-2/Bax ratio in the myocardium decreased significantly in groups B, C and D compared with group A \( (P<0.01) \). Bcl-2/Bax ratio in the myocardium increased significantly in groups C and D compared with group B \( (P<0.01) \). Bcl-2/Bax ratio in the myocardium increased significantly in group D compared with group C \( (P<0.05) \) (Table 1).

Changes of cardiomyocytic Al at 24 hours after ROSC in rats of each group

Cardiomyocytic Al increased significantly in groups B, C and D compared with group A \( (P<0.01) \), but it was decreased significantly in groups C and D compared with group B \( (P<0.01) \). Also it was decreased significantly in group D compared with group C \( (P<0.05) \) (Table 2, Figure 3).

Table 1. Changes of expression Bcl-2, Bax protein and Bcl-2/Bax ratio in the myocardium in rats of each group (mean±SD, n=8)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bax</th>
<th>Bcl-2</th>
<th>Bcl-2/Bax</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>44.01±13.34</td>
<td>32.89±16.20</td>
<td>0.742±0.157</td>
</tr>
<tr>
<td>B</td>
<td>316.19±32.59</td>
<td>177.15±30.00</td>
<td>0.564±0.103</td>
</tr>
<tr>
<td>C</td>
<td>206.98±31.07</td>
<td>145.59±15.18</td>
<td>0.709±0.062</td>
</tr>
<tr>
<td>D</td>
<td>154.10±22.41</td>
<td>121.22±10.23</td>
<td>0.725±0.095</td>
</tr>
</tbody>
</table>

Compared with group A, \( ^{\#}P<0.01 \); compared with group B, \( ^{\#\#}P<0.01 \); compared with group C, \( ^{\#\#\#}P<0.05 \)

Figure 1. Expression of Bax protein in groups A, B, C, and D (400×)

Figure 2. Expression of Bcl-2 protein in groups A, B, C, and D (400×)
DISCUSSION

Ischemia-reperfusion injury is an important factor for apoptosis, and cells poptosis is one of the pathologic characteristics of myocardial ischemia-reperfusion injury. Apoptosis-related proteins play an important role in modulation of cell apoptosis induced by ischemia-reperfusion injury. Among them, both inhibiting apoptosis protein Bcl-2 and promoting apoptosis protein Bax have been extensively studied at present, but their function is still unclear. Brocheriou et al\(^5\) reported a significant reduction of infarct size and cardiomyocyte apoptosis in transgenic mice after myocardial ischemia-reperfusion, overexpressing anti-apoptotic human Bcl-2 in cardiomyocyte. In transgenic mice with human Bcl-2 transcripts other than in nontransgenic mice with myocardial ischemia-reperfusion, Chen et al\(^6\) found a significant functional recovery of the heart, small infarct sizes (52.4%), and few apoptotic positive myocytes, accompanied by a three-fold decrease in lactate dehydrogenase (LDH). These findings indicate that Bcl-2 plays an anti-apoptotic role in myocardial ischemia-reperfusion. Hochhauser et al\(^7\) reported that the number of apoptotic TUNEL positive cardiomyocytes decreased more significantly in the ischemia-reperfusion isolated hearts of knockout Bax transgenic mice than in controls, and Bax could stimulate apoptosis in ischemia-reperfusion. Homologous or heterologous dimmers from the Bcl-2 family members through competition can regulate mitochondrial changes, modulate indirectly the activities of protease and nuclease, and thereby regulate apoptosis. Apoptosis is closely related to expression of promoting apoptosis proteins or inhibiting apoptosis proteins; more importantly, it is dependent on the ratio of inhibiting apoptosis proteins to promoting apoptosis proteins. Therefore, the Bcl-2/Bax ratio is more closely related to apoptosis.\(^8\)

The myocardium is subjected to the process of ischemia and reperfusion during CPR and ROSC after cardiac arrest. A large number of studies have shown that mechanisms of myocardial injury in cardiac arrest and CPR are diversified, including free radicals oxidative injury, calcium overload, inflammatory cytokine release, cells apoptosis, and so on.\(^9\) Among them, apoptosis is a characteristic change of ischemia-reperfusion injury, and apoptotic severity is related to prognosis of ischemic reperfusion injury.\(^10\)

Li et al\(^11\) observed myocardial injury, apoptosis and expression of Bcl-2 and Bax protein by transmission electron microscopy, TUNEL and immunohistochemical staining in rats with cardiac arrest induced by asphyxiation, ice-cold potassium chloride and CPR in 5 minutes. They found that myocardial TUNEL-positive cells and expression of Bcl-2 and Bax proteins increased markedly in CPR rats. Shi et al\(^12\) reported that myocardial apoptosis index increased at 3 hours following CPR, and peaked at 48 hours. The expression of Bcl-2 and Bax protein increased significantly after CPR, and Bcl-2/Bax ratio was unbalanced or inverted at early stage in CPR rats. Jiang et al\(^13\) also found that myocardial apoptosis index (TUNEL method) increased significantly at 6 hours after CPR in dogs. In this study, cardiomyocytic AI and the expression of Bcl-2 and Bax proteins increased significantly, but Bcl-2/Bax ratio decreased significantly in the CPR, low-dose CP group and high-dose CP group, compared with the control group at 24 hours after ROSC in rats. This finding indicates that cardiac arrest or CPR could stimulate apoptosis of myocardial cells.

Myocardial dysfunction is an important pathological

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**Table 2. Changes of cardiomyocytic AI in rats of each group (mean±SD, %, n=8)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>AI (TUNEL method)</th>
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<tr>
<td>A</td>
<td>4.75±1.99</td>
</tr>
<tr>
<td>B</td>
<td>45.54±2.43*</td>
</tr>
<tr>
<td>C</td>
<td>31.21±4.12</td>
</tr>
<tr>
<td>D</td>
<td>21.85±1.53*</td>
</tr>
</tbody>
</table>

Compared with group A, \(^*P<0.01\); compared with group B, \(^*P<0.01\); compared with group C, \(^*P<0.01\)

**Figure 3. TUNEL in groups A, B, C, and D (magnification×400)**

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**Table 2. Changes of cardiomyocytic AI in rats of each group (mean±SD, %, n=8)**

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Compared with group A, \(^*P<0.01\); compared with group B, \(^*P<0.01\); compared with group C, \(^*P<0.01\)
basis of post-cardiac arrest syndrome, and the important way to alleviate myocardial ischemia-reperfusion injury after cardiac arrest and CPR is to reduce myocardial energy consumption and increase energy supply. In energy metabolism, CP acts as one of the main substances of energy supply. Studies have shown that inhibition of energy metabolism at the level of glucose uptake, glycolysis, tricarboxylic acid cycle and oxidative phosphorylation could induce cell apoptosis, and the exogenous CP could alleviate myocardial ischemia injury. Feng et al reported that treatment with exogenous CP could significantly reduce apoptosis index of cardiomyocytes in rats with myocardial infarction, and improve the contraction and dilation of the heart. Exogenous CP significantly reduced the serum levels of CK-MB and cTnI and alleviated histopathological changes of the myocardium, indicating that treatment with exogenous CP could alleviate myocardial injury. In this study, cardiomyocytic apoptosis index (AI) and the expression of Bcl-2 and Bax proteins decreased significantly but Bcl-2/Bax ratio increased significantly in the low-dose CP group and high-dose CP group compared with the CPR group. Cardiomyocytic AI and the expression of Bcl-2 and Bax proteins decreased significantly but Bcl-2/Bax ratio increased significantly in the high-dose CP group compared with the low-dose CP group. This shows that treatment with exogenous CP could inhibit cardiomyocytic apoptosis, and the effect is more significant in the high dose CP group than in the low-dose CP group. The anti-apoptosis action may be one of the main mechanisms of exogenous CP in preventing the myocardium from injury after cardiac arrest.

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Ethical approval: Not needed.

Conflicts of interest: The authors declare that there is no conflict of interest.

Contributors: Yan P and Chen SQ composed and wrote the paper. All authors read and approved the final version of the manuscript.

REFERENCES