Protective effects of phosphocreatine on human vascular endothelial cells against hydrogen peroxide-induced apoptosis and in the hyperlipidemic rat model

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Abstract

Phosphocreatine (PCr) has been shown to have a cardio-protective effect during cardiopulmonary resuscitation (CPR). However, little is known about its impact on atherosclerosis. In this study, we firstly evaluated the pharmacological effects of PCr on antioxidative defenses and mitochondrial protection against hydrogen peroxide ($H_2O_2$) induced human umbilical vascular endothelial cells (HUVECs) damage. Then we investigated the hypolipidemic and antioxidative effects of PCr on hyperlipidemic rat model. Via $in vitro$ studies, $H_2O_2$ significantly reduced cell viability and increased apoptosis rate of HUVECs, while pretreatment with PCr abolished its apoptotic effect. PCr could reduce the generation of ROS induced by $H_2O_2$. Moreover, PCr could increase the activity of SOD and the content of NO, as well as decrease the activity of LDH and the content of MDA. PCr could also antagonize $H_2O_2$-induced up-regulation of Bax, cleaved-caspase3, cleaved-caspase9, and $H_2O_2$-induced down-regulation of Bcl-2 and p-Akt/Akt ratio. In addition, PCr reduced U937 cells’ adhesion to $H_2O_2$-stimulated HUVECs. Via $in vivo$ study, PCr could decrease MDA, TC, TG and LDL-C levels in hyperlipidemic rats. Finally, different-concentration PCr could increase the leaching of TC, HDL, and TG from fresh human atherosclerotic plaques. In conclusion, PCr could suppress $H_2O_2$-induced apoptosis in HUVECs and reduce hyperlipidemia through inhibiting ROS generation and modulating dysfunctional mitochondrial system, which might be an effective new therapeutic strategy to further prevent atherosclerosis.

1. Introduction

Atherosclerosis disease takes a significant toll on society and is induced by several factors, including inflammation, oxidative stress, cell adhesion, cell migration and cellular proliferation [1–3]. These factors changes can result in endothelial cell dysfunction, which is the first step of the development and progression of atherosclerosis. Endothelial cells (ECs), as the inner lining of all blood vessels, may undergo various stresses that can lead to abnormal cell proliferation and apoptosis [4], which is the central feature of atherosclerosis. Atherosclerosis is an inflammatory disease and increased levels of $H_2O_2$ are associated with inflammation. The model focuses on $H_2O_2$-induced oxidative stress under static and shear conditions. Previous studies have documented increased $O_2^-$ and cytotoxicity in smooth muscle cells exposed to $H_2O_2$. Further studies have also shown $H_2O_2$-induced apoptosis in human umbilical vascular endothelial cells (HUVECs). The cytotoxic effect occurs mainly through the induction of reactive oxygen species (ROS), although the molecular mechanism underlying this process has not to be fully understood yet.

Hyperlipidemia is a major cause of atherosclerosis and atherosclerosis-associated conditions such as coronary heart disease, ischemic cerebrovascular disease, and peripheral vascular disease [5, 6]. It has become a major threat to human health due to the increased intake of a high-fat diet in daily life [7, 8]. Clinical and experimental studies show that oxidative stress is involved in the pathogenesis of many diseases [9–12]. There is abundant evidence to show that high-fat diet-induced hyperlipidemia has tight relations with vascular damage and oxidative stress [13]. Hypercholesterolemia has been reported to
increase superoxide anion production in endothelial cells, induce endothelial dysfunction and enhance vascular reactivity to some contractile agonists [14].

Phosphocreatine (PCr) is an essential high-energy phosphate compound in the body of mammalian animals and humans [15, 16]. It plays a vital role in the normal energy metabolism by serving as an immediately available temporal energy buffer, a metabolic regulator, a spatial energy buffer or intracellular energy transport system (the CK/PCr energy shuttle or circuit) [17–19]. Now PCr can be obtained by artificial synthesis. Exogenous PCr has currently become the most extensively prescribed cardio-protective drug due to its high efficacy in protection of myocardium against ischemic injury and its excellent safety [20, 21]. Besides, some studies have shown that PCr has a potential neuro-protective effect [22]. Despite extensive research on the role of PCr in the treatment of cardio diseases [23, 24], the study of PCr on atherosclerosis has not been reported yet. However, in clinic, when ischemic disease patients associated with atherosclerosis were treated using PCr, the results demonstrated that in addition to treating ischemic disease, the condition of atherosclerosis has also been improved. Based on the discovery, we planned to explore the function and mechanism of PCr on atherosclerosis. Therefore, the present study aimed to examine whether PCr possesses a protective effect on apoptosis induced by H$_2$O$_2$ in HUVECs as well as in the hyperlipidemic rat model.

2. Materials and methods

2.1 Cell culture

PCr was purchased from Harbin Laibotong Pharmaceutical Co., Ltd. HUVECs were obtained from Institute of Biochemistry and Cell Biology, CAS. Cells were cultured in DMEM (Gibco, USA), which contained high glucose, and were supplemented with 10% fetal bovine serum (FBS) (Gibco), streptomycin (100 units/mL) and penicillin (100 units/mL) (Sigma, USA). HUVECs were placed in a humidified atmosphere at 37°C with 5% CO$_2$. Cells at passages 4–8 were used for all experiments.

2.2 Analysis of apoptosis by flow cytometry (FCM) of Annexin V-FITC/PI double staining

To determine the effects of PCr on apoptosis/necrosis induced by H$_2$O$_2$ in HUVECs, the double staining Annexin V-FITC/PI of cells was evaluated using Annexin V-FITC/PI Apoptosis Assay Kit (Nanjing KeyGen Biotech, China). Cells (5×10$^5$) were seeded onto a 6-well plate and incubated at the indicated final concentrations. After incubation, cells were collected and washed twice with ice-cold PBS. The samples were resuspended in 500µL binding buffer mixed with 5µL Annexin V-FITC and 5µL PI, and then incubated in the dark room at room temperature for 15 min. Finally, samples were analyzed by flow cytometry (FCM) within 1 h.

2.3 DAPI staining assay
HUVECs in 6-well plate were washed twice with phosphate buffer saline (PBS) after being treated with PCr for 60h and then incubated with H$_2$O$_2$ for 4 h. Cells were then fixed with 10% paraformaldehyde (Tianjin Kemiou Chemical Reagent Co., Ltd) in PBS for 10 min. The fixed cells were washed one time with PBS and stained with 1µg/mL DAPI for 10 min at 37°C in the incubator, then washed twice with PBS. At last, the stained cells in the 6-well plate were imaged by inverted fluorescence microscopy (CKX41, OLYMPUS, Japan).

2.4 Measurement of intracellular ROS

The fluorescent probe of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to examine intracellular ROS in HUVECs. DCFH-DA is a cell-permeant probe and is hydrolyzed by intracellular esterase to DCFH. The non-fluorescent DCFH is oxidized by intracellular ROS to the lipid impermeable and highly fluorescent compound dichlorofluorescein (DCF). The HUVECs were seeded onto 6-well plate until 90–100% confluence and incubated at the indicated final concentrations. After incubation, cells were trypsinized. Then HUVECs were incubated with 10 µM of DCFH-DA for 15 min. HUVECs were washed three times with PBS, then resuspended in DMEM without FBS (600µL) to a final concentration of 5×10$^5$ cells/mL. ROS generation of HUVECs was measured by FCM with excitation and emission wavelengths of 485nm and 535 nm, respectively.

2.5 Superoxide dismutase (SOD), lactate dehydrogenase (LDH) activation assay

HUVECs were cultured and treated with indicated final concentrations. After collecting the supernatant from 6-well plates, SOD and LDH activity were determined using a SOD and LDH assay kit (Nanjing KeyGen Biotech, China) according to the manufacturer’s instructions.

2.6 Malonaldehyde (MDA), NO contents assay

HUVECs were cultured and treated. Then MDA, and NO contents were determined using the relevant assay kit (Nanjing KeyGen Biotech, China) according to the manufacturer’s instructions.

2.7 Protein extraction and Western blotting

HUVECs were pre-treated with test compounds and incubated with H$_2$O$_2$ at indicated time points (1, 2, 4 h). Total cytosolic proteins were extracted with a cold lysis buffer (RIPA, 200µM PMSF) for 30 min at 4°C, then the mixtures were centrifuged at 12,000g for 5 min and the protein was obtained. And the protein concentration was determined using the BCA Protein Assay kit (Beyotime Institute of Biotechnology). Protein (5 mg/mL) was denatured by mixing with an equal volume of 2×sample loading buffer and then boiling at 100°C for 5 min. Samples (~ 50 ug) were loaded onto 10 ~ 15% SDS-PAGE, separated by gel electrophoresis, and then transferred onto PVDF membranes. After blocking with 5% skimmed milk in TBS containing 0.05% Tween 20 (TBST) at room temperature for 2 h, the membranes were incubated with primary antibody (1:1000 dilution) against caspase-3, caspase-9, Bcl-2, Bax, p-Akt and Akt proteins for overnight at 4°C. The blots were washed three times with TBST, then the membranes were incubated
with appropriate secondary antibody (1:10000 dilution) for 1 h at 37°C. After washing three times, the blots were developed with enhanced chemiluminescence (ECL) system and visualized by BioSpectrum Gel Imaging System (HR410, UVP, USA).

### 2.8 BCECF Adhesion Test

Adhesion test was conducted with BCECF (Molecular Probes, Eugene, OR, USA) using human monocytic cell lines (U-937). HUVECs were initially seeded on 24-Well Plates (0.25 million cells/well) and stimulated for 4 hours in the incubator. The following groups were studied: a. vehicle control; b. H$_2$O$_2$ (1 mM) - treated; c. H$_2$O$_2$ + PCr (15 mM); d. H$_2$O$_2$ + PCr (20 mM); e. H$_2$O$_2$ + PCr (25 mM). All measurement groups were prepared three times. Endothelial cells were loaded with I-CAM and V-CAM in the first two studies. The third study consisted of unstimulated HUVECs. Then, the fluorescent dye BCECF (50µL) was mixed with 20µl DMSO, which served as a carrier substance for the cellular uptake, frozen for 15 minutes. 15mL of RPMI was added. After confluent growth, U-937 monocytes were centrifuged, resuspended and diluted in 5mL RPMI, 5% FCS and 1% PS, until 6.5 million of U-937 cells were reached in 5mL RPMI. For each one of the 5 stimulation groups, 5 mL of the U-937 solution and respectively 1.5mL of the solution from RPMI and BCECF were transferred and incubated for 30 minutes. After incubation and following centrifugation, the supernatant was aspirated, rinsed with 5mL PBS, and then treated with 6.5mL RPMS. 250µl of the U-937 solution was added to each well and incubation of the cell suspension for 1 hour to remove non-adhered U-937 cells. The cells were then lysed by adding 500µl/well NaOH [0.1 M] and loaded with BCECF fluorescent. The fluorescence measurement was performed in the Genios plate reader at 340 nm of wavelength.

### 2.9 Animals and treatment

All animal experiments were approved by the Bioethics Committee of Dalian Medical University and the procedures of the experiment were strictly according to generally accepted International Rules and Regulations. Male SD rats (200 ± 25 g, n = 48) were housed under standard environmental conditions (23 ± 1 °C, 55 ± 5% humidity and a 12 h light/dark cycle) and free access to water and a standard diet. Animals were kept under observation for a week, and divided randomly into 6 groups of 8 rats each (males): 1) normal control group (NC group), 2) high-fat diet model group (HF group), 3) high-fat diet model group + Di-ao-xin-xue-kang group (HF + DA group), 4) high-fat diet model group + PCr group (20 mg/kg/d) (HF + IPCr group), 5) high-fat diet model group + PCr group (35 mg/kg/d) (HF + mPCr group), and 6) high-fat diet model group + PCr group (50mg/kg/d) (HF + hPCr group). The rats of the NC group were fed a standard diet and orally treated with 0.3% Tween-80 solution (10 mL/kg) [25]. The rats of HF and HF + PCr groups were fed high fat diet (pork oil, 7.5%; cholesterol, 1%; egg yolk powder, 10%; cholate, 0.3%; propylthiouracil, 0.2%; standard diet, 81%) and gavaged 0.3% Tween-80 solution or PCr solutions once per day, respectively, for 6 weeks. The animal work has been approved by the ethics committee of Dalian Medical University, and the approval number was 201,720,223.

### 2.10 Biochemical analysis of serum
Serum was separated by centrifugation at 1000×g for 15 min. TC, TG, LDL-C, GSH-PX and NOS were measured in a spectrophotometer (DU800, Beckman) using commercial kits from Jiancheng Institute of Biotechnology (Nanjing, China). Serum TC and TG levels were evaluated following the manufacturer's protocols (CHODPAP and GPO-PAP methods). The optic density of the samples was measured using a spectrophotometer 3 times at a wavelength of 546 nm. Total TC and TG in serum were calculated through absorption of the tested sample divided by standard sample and multiplied by TG and TC content in the standard sample. Serum LDL-C levels were evaluated following the manufacturer’s protocols (polyethylene sulfate precipitation method), and its concentration is the difference that cholesterol of the supernatant liquid subtracted from the general cholesterol. GSH-Px activity was measured by quantifying the rate of H$_2$O$_2$-induced oxidation of GSH to oxidized glutathione (GSSG) under the existence of GSH-Px based on the modified method of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) [26]. The absorbance was measured at 412 nm. The activities of serum NOS were based on the oxidation of oxyhemoglobin to methemoglobin by nitric oxide [27].

2.11 Biochemical analysis of liver

Liver samples were homogenized (10%, w/v) in cold saline then centrifuged at 1000 × g for 15 min. The supernatant was used for the assay of SOD, MDA, lipoprotein esterase (LPL) and hepatic lipase (HL). The protein concentrations were determined by the Bradford method using BSA as the standard [28]. The SOD activity was determined based on the serum inhibitory efficacy of nitroblue tetrazolium (NBT) produced by 0-2 and generated by the xanthine/xanthine oxidase system [29]. The absorbance at 550 nm was measured for determining SOD activity. MDA was measured using the tibituric acid (TBA) method, MDA content was assayed by analysis for the levels of thiobarbituric acid-reactive substances at a wavelength of 532 nm. The values were expressed in nmol/mg protein. The activity of LPL and HL was measured by colorimetric method. The activity of HL was determined after inhibition of LPL by preincubation with protamine sulfate. LPL activity was taken as the difference between the total lipolytic activity and the HL activity. Their activities were expressed as U/mg protein.

2.12 Liver index change

At the end of experiment, rats were kept for overnight fasting. After 1.5 h finished the last gavage, rats were weighed and anesthetized with 36 mg/kg sodium pentobarbital by intraperitoneal injection. Blood samples were collected from the aorta ventralis. The whole liver was immediately removed and weighed; the liver index (liver weight/body weight) was calculated. The diagnoses and classified histological fatty liver scores at the end of the 6-week treatment period according to the classification by as following standard: Scores: 0, no visible fat; 1), 5% of liver surface infiltrated by fat; 2), 5–25% fat; 3), 25–50% fat; 4), 50% fat.

2.13 Detect the leaching value of TG, TC, HDL from fresh atherosclerotic plaques

Take the fresh atherosclerotic plaques of thoracic aorta from the patient (73 years old, male) after surgery, then placed it in refrigerator at 4 °C for 2h in Klinefelter solution. Divided the plaque into the
same weight of five parts, then placed them in 3mL Tyrode's solution at 37 °C water bath, 95% O₂ and 5% CO₂ and taken the sample of Klinefelter solution at 0h, 1h, 2h, 4h, 6h, 8h, 10h. Then detect the related biochemical factors using a clinical diagnosis assay kit of triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). (Fig. 6A-B). Patient included in this study signed a written informed consent in accordance with the Helsinki Declaration which was approved by the ethical Committee of the Dalian Medical University.

2.14 Statistical analysis

All the data were performed using Graph Pad Prism 9 (Graph Pad Software, Inc., San Diego, CA) and expressed as means ± SD. Statistical evaluation of post hoc multiple group comparisons was performed using one-way ANOVA. Bonferroni test was used for statistical analysis and a p value < 0.05 was considered statistically significant.

3. Results

3.1 Effects of PCr on cytotoxicity and apoptosis in HUVECs induced by H₂O₂

The chemical structure of PCr is represented in Fig. 1A. The morphological changes of HUVECs with various concentrations of test compounds were observed under light microscopy. The control group exhibited a typical polygonal shape (Fig. 1B-a). H₂O₂ treatment for 4 h induced cytotoxic morphological changes (apparent reduction in cell density and loss of confluency, cell shrinkage into a rounder shape, as well as an increase in several bright objects representing floating cell fragments), while PCr could reduce those cytotoxic changes (Fig. 1Bb-e). The effect of PCr on cell morphology were also evaluated using nuclear staining dye DAPI. Compared to control (Fig. 1B-f), there were obvious nuclear chromosome condensations in cells induced by H₂O₂ shown in Fig. 1Bg. Those H₂O₂-induced nuclear chromosome condensations were reversed by PCr pre-treatment (Fig. 1Bh-j). These morphological changes were associated with decreased cell viability as assessed by MTT assay, with ~ 40% cell death at 1 mM H₂O₂ concentration in Fig. 1C. As shown in Fig. 1C, the cell viability of HUVECs was increased to 96.71%, 105.99%, 107.66% at 15 mM, 20 mM, 25 mM concentration of PCr, respectively.

FCM analysis with Annexin V-FITC/PI staining was undertaken to determine the effect of PCr on HUVECs apoptosis. Treatment of cells with H₂O₂ (1mM) for 4 h significantly increased the number of apoptotic cells by comparison to untreated control (Fig. 1D-E). The enhanced apoptosis was markedly inhibited by PCr, particularly at 25 mM concentration.

3.2 Effects of PCr on LDH, MDA, SOD contents in HUVECs and monocyte adhesion to HUVECs induced by H₂O₂
As shown in Fig. 2A, our results demonstrated that there was a significant increase in LDH release in the H$_2$O$_2$-stimulated group compared to the control group. However, upregulation of LDH was notably decreased by pretreatment of PCr. Also, in Fig. 2B-C, pretreatment with PCr significantly decreased MDA levels and increased SOD activities in HUVECs, while H$_2$O$_2$-stimulated group only markedly increased MDA levels and decreased SOD activities.

Monocyte adhesion to endothelial cells is an essential event in the initiation of endothelial injury-related diseases development. To explore the effect of PCr on H$_2$O$_2$-induced monocyte adhesion to HUVECs, a cell adhesion assay was adopted. The measurement was carried out using BCECF-AM fluorescent. The U937 monocyte adhesion studies were conducted using the tritium thymidine uptake test after an incubation period of 4 hours. It was demonstrated that treating HUVECs with H$_2$O$_2$ led to a significant increment in monocyte adhesion of 386 ± 46% compared to the control group. In the following experiment, monocyte adhesion was significantly suppressed from 386 ± 46% to 324 ± 56%, 265 ± 32%, 136 ± 15.7% of control by administration of 15mM PCr, 20 mM PCr, 25mM PCr respectively (Fig. 2D-E). This result represents that the signal transduction of H$_2$O$_2$-induced adhesion was affected by PCr.

### 3.3 Effects of PCr on intracellular ROS generation, NO production and Akt signaling pathway in HUVECs induced by H$_2$O$_2$

Intracellular ROS change responds to endothelial oxidative stress level and impairment for the stimulation of H$_2$O$_2$. To examine whether PCr could inhibit ROS production, we measured the density of probe using FCM. As shown in Fig. 3A-B, ROS was significantly increased in HUVECs treated with H$_2$O$_2$, as compared to the control group. Pre-treatment with PCr inhibited ROS production in a dose-dependent manner. Meanwhile, stimulation of cells with H$_2$O$_2$ caused a marked reduction in NO production as compared to untreated control, while NO release was dose-dependently increased after pretreatment with PCr (Fig. 3C).

To further explore the underlying mechanisms, the protein expression levels of p-Akt and Akt in HUVECs were analyzed by Western blotting. As shown in Fig. 3D, compared to control group, H$_2$O$_2$ treatment of HUVECs largely decreased the ratio of p-Akt/Akt, while pre-incubation with PCr could markedly increase its ratio. However, after adding the LY294002 (PI3K inhibitor), the promoting role of PCr has been inhibited and the ratio of intracellular p-Akt/Akt protein decreased in HUVECs. It was suggested that PCr could inhibit H$_2$O$_2$-induced apoptosis of HUVECs by regulating PI3K/Akt signaling pathway.

### 3.4 Effects of PCr on caspase activation, Bax and Bcl-2 protein expression induced by H$_2$O$_2$.

To investigate apoptotic signaling during H$_2$O$_2$-induced cell apoptotic, we analyzed the activation of caspase-3 and caspase-9, hallmark apoptotic execution enzymes, by Western blotting. H$_2$O$_2$ treatment of HUVECs markedly stimulated the activation of both caspases. Pre-incubation with PCr clearly inhibited caspase-3 and caspase-9 activation (Fig. 4A-B). As shown in Fig. 4C, stimulation of cells with H$_2$O$_2$
caused a marked reduction in Bcl-2 protein expression and a significant increase in Bax protein expression as compared with untreated control. Pre-treatment with PCr dose-dependently decreased the expression of Bax protein, while increasing the expression of Bcl-2 protein.

3.5 The effect of PCr on serum lipid, GSH-PX, NOS activities and hepatic changes of rats

The serum TC, TG and LDL-C levels of HF group rats showed a significant increase compared with NC group. PCr decreased TC, TG and LDL-C levels in a dose-dependent manner. The TC, TG and LDL-C levels in the HF + lPCr (20 mg/kg) group were higher than in the HF + DA group (Di Ao Xin Xue Kang (DiAo), a kind of total steroidal saponins, a commercial anti-hyperlipidemia agent), but they were significantly low in the HF + mPCr (35 mg/kg) and HF + hPCr (50 mg/kg) groups compared with the HF group. The activity of serum GSH-PX was found to be decreased significantly in the HF group when compared to the NC group. HF + PCr and HF + DA groups significantly increased serum GSH-PX activity compared with the HF group, and the PCr showed a dose-dependent manner. The HF + hPCr group showed 44.43% increase in GSH-PX activity and was better than DA. The serum NOS activity was decreased in the HF group. Orally treated with DA and PCr increased NOS activity and higher than the NC group. The NOS activity of HF + DA and HF + lPCr (20 mg/kg) groups significantly was higher than the HF group (Table 1).
Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>LDL-C (mg/dl)</th>
<th>GSH-PX (U/ml)</th>
<th>NOS (U/ml)</th>
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<tbody>
<tr>
<td>NC</td>
<td>Vehicle</td>
<td>53.79 ± 2.57</td>
<td>27.21 ± 1.16</td>
<td>12.37 ± 3.17</td>
<td>1063.24 ± 73.55</td>
<td>21.52 ± 1.73</td>
</tr>
<tr>
<td>HF</td>
<td>Vehicle</td>
<td>139.49 ± 8.42aa</td>
<td>77.33 ± 4.49aa</td>
<td>81.38 ± 9.96aa</td>
<td>663.10 ± 40.56a&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.10 ± 0.39</td>
</tr>
<tr>
<td>HF + DA</td>
<td>62.5</td>
<td>99.26 ± 1.69bb</td>
<td>50.91 ± 0.87bb</td>
<td>39.73 ± 2.02bb</td>
<td>872.93 ± 102.82bb</td>
<td>23.54 ± 0.68bb</td>
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<tr>
<td>HF + IPCr</td>
<td>20</td>
<td>126.99 ± 2.66cc</td>
<td>65.00 ± 1.46bc</td>
<td>72.38 ± 4.91cc</td>
<td>936.31 ± 26.22bb</td>
<td>22.38 ± 0.80b</td>
</tr>
<tr>
<td>HF + mPCr</td>
<td>35</td>
<td>111.08 ± 4.52bb</td>
<td>57.77 ± 1.97bb</td>
<td>57.85 ± 4.57</td>
<td>940.00 ± 29.65bb</td>
<td>22.21 ± 0.68</td>
</tr>
<tr>
<td>HF + hPCr</td>
<td>50</td>
<td>101.80 ± 3.68bb</td>
<td>50.11 ± 0.82bb</td>
<td>47.59 ± 3.12bb</td>
<td>957.73 ± 13.98bb,c</td>
<td>21.89 ± 0.31</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. (n = 8).

<sup>a</sup>P < 0.05, <sup>aa</sup>P < 0.01, vs. NC group.

<sup>b</sup>P < 0.05, <sup>bb</sup>P < 0.01, vs. HF group.

<sup>c</sup>P < 0.05, <sup>cc</sup>P < 0.01, vs. HF + DA group.

The hepatic content of MDA was significantly increased in the HF group, compared to the NC group, hepatic SOD, LPL and HL activities were significantly decreased. Compared with the HF group, PCr significantly increased hepatic SOD activity at 20 mg/kg and 50 mg/kg doses of PCr, and significantly decreased hepatic MDA in a dose-dependent manner. Hepatic LPL and HL activities were increased in the PCr groups, but significant change just occurred at 50 mg/kg dose of PCr. The effect of PCr on SOD, MDA, LPL and HL was comparable with DA. The liver index of the HF group was significantly increased than that in the NC group. The liver index was significantly decreased in PCr groups (35 mg/kg and 50 mg/kg), and better than the HF + DA group (Table 2). Figure 5 depicted the gross appearance and histological analysis of liver from five groups of rats after they had received their 6 weeks of corresponding treatments. The livers of NC group were relatively dark red, whereas those of HF, HF + IPCr, HF + mPCr, HF + hPCr groups were yellowish. It was observed that the livers of both HF + mPCr and HF + hPCr groups were less yellowish. Histological examination of the livers of NC, HF + mPCr, and HF + hPCr groups rats revealed an intact cellular architecture. In contrast, the livers of HF and HF + IPCr groups illustrated poor cellularity, with extensive lipid deposits and enlarged hepatocytes (Fig. 5). In fact, lipid deposits and hepatocyte enlargement were also observed in other groups fed with HF, but the degree of changes was smaller than that of HF and HF + IPCr groups. It showed that different doses of middle and
high dose of PCr (35 mg/kg and 50 mg/kg) could improve histological fatty liver histology in rats at the end of 6-week treatment period. There were no liver and general toxicity in the rats of the NC, HF and PCr groups.

Table 2
Effects of PCr on hepatic SOD, MDA, LPL, HL and liver index levels in hyperlipidemic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>SOD (U/mgprot)</th>
<th>MDA (nmol/mgprot)</th>
<th>LPL (U/mgprot)</th>
<th>HL (U/mgprot)</th>
<th>Liver index</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>Vehicle</td>
<td>56.07 ± 0.72</td>
<td>2.19 ± 0.21</td>
<td>2.39 ± 0.23</td>
<td>0.81 ± 0.12</td>
<td>2.61 ± 0.01</td>
</tr>
<tr>
<td>HF</td>
<td>Vehicle</td>
<td>46.02 ± 1.10aa</td>
<td>3.93 ± 0.53aa</td>
<td>1.86 ± 0.16a</td>
<td>0.48 ± 0.11a</td>
<td>3.46 ± 0.03aa</td>
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<td>HF + DA</td>
<td>62.5</td>
<td>51.00 ± 0.85bb</td>
<td>1.66 ± 0.09bb</td>
<td>2.10 ± 0.10</td>
<td>0.74 ± 0.12</td>
<td>3.41 ± 0.04</td>
</tr>
<tr>
<td>HF + lPCr</td>
<td>20</td>
<td>51.25 ± 0.34bb</td>
<td>2.02 ± 0.10bb</td>
<td>2.07 ± 0.11</td>
<td>0.57 ± 0.06</td>
<td>3.28 ± 0.06</td>
</tr>
<tr>
<td>HF + mPCr</td>
<td>35</td>
<td>49.76 ± 0.50</td>
<td>1.98 ± 0.07bb</td>
<td>2.16 ± 0.06</td>
<td>0.67 ± 0.05</td>
<td>3.16 ± 0.05bb,c</td>
</tr>
<tr>
<td>HF + hPCr</td>
<td>50</td>
<td>51.18 ± 0.93bb</td>
<td>1.75 ± 0.13bb</td>
<td>2.46 ± 0.06b</td>
<td>0.91 ± 0.09b</td>
<td>2.99 ± 0.06bb,cc</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. (n = 8).

\(aP < 0.05, \text{aa}P < 0.01, \text{vs. NC group.}\)

\(bP < 0.05, \text{bb}P < 0.01, \text{vs. HF group.}\)

\(cP < 0.05, \text{cc}P < 0.01, \text{vs. HF + DA group}\)

3.6 The effect of different concentrations of PCr on leaching of TG, TC, LDL, HDL from atherosclerosis plaque in thoracic aorta at 1h, 2h, 4h, 6h, 8h, 10h

3.6.1 The leaching value of TG

The leaching value of TG in 1µM Atorvastatin group increased by about 22.7% compared with Control group. The leaching value of TG in different concentrations of PCr was higher than Control group, and the low-dose group treated with 5mM PCr increased by 11.0% compared with Control group, and the TG level in high-dose group treated with 20mM PCr is similar to Atorvastatin group (Fig. 6C).
3.6.2 The leaching value of TC

The leaching value of TC in 1µM Atorvastatin group increased by about 35.1% compared with Control group. The leaching value of TC in different concentrations of PCr was higher than Control group in a dose-dependent manner: the low-dose group treated with 5mM PCr increased by 25.3% compared with Control group and the high-dose group treated with 20mM PCr increased by 39.1% compared with Atorvastatin group (Fig. 6D).

3.6.3 The leaching values of LDL

The leaching values of LDL in different concentrations of PCr were higher than Control group and similar to 1µM Atorvastatin group. Although the value in different concentrations of PCr was higher than Control group, the low-dose group treated with 5mM PCr increased by 3.8% compared with Control group and the high-dose group treated with 20mM PCr increased by 14% compared with positive group (Fig. 6E).

3.6.4 The leaching value of HDL

The leaching value of HDL in 1µM Atorvastatin group increased by about 31.4% compared with Control group. The leaching value of HDL in different concentrations of PCr was higher than Control group in dose-dependent manner: the low-dose group treated with 5mM PCr increased by 17.8% compared with Control group and the high-dose group treated with 20mM PCr increased by 17.5% compared with Atorvastatin group (Fig. 6F).

In general, the effect of PCr on the leaching value of TC was obvious, and the value increased by 39.1% in high-dose PCr group compared with Atorvastatin group. Then the effect of PCr on HDL was in second place, in which, the increasing degree was about 17.5%. And the effect on TG was in third place, in which, the degree was similar to Atorvastatin group. But there was no significant effect on the leaching value of LDL. Overall, the effect of different-concentration PCr increased the leaching of TC, HDL, TG, (TC > HDL > TG), which was similar to Atorvastatin. And we will explore the mechanism in the future experiment.

4. Discussion

H₂O₂ is a highly oxidative agent known to induce cellular injury and cytotoxicity. In this study, we examined the effects of PCr on H₂O₂-induced apoptosis in HUVECs. Our present data from morphological observations, MTT assay and AnnexinV-FITC/PI double staining clearly indicated that H₂O₂ was a potent inducer of cytotoxicity and apoptosis in HUVECs, and that PCr protected cells from apparent cell death in a dose-dependent manner. Consistent with the observations, pretreatment with PCr inhibited both caspase-3 and caspase-9 activation. Meanwhile, studies have shown that mitochondrial membrane permeability was regulated by the activities of the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins for initiating apoptosis. We investigated by Western blotting whether H₂O₂-induced apoptosis was associated with changes in the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins in HUVECs. It is well-established that the ratio between Bcl-2 and Bax proteins is an important factor in the
regulation of apoptosis rather than the level of each protein separately. An increase in Bax/Bcl-2 ratio is enough to promote apoptosis in mammalian cells and induce cell death by directly activating the mitochondrial apoptotic pathway. In our experiment, PCr attenuated the increase in Bax/Bcl-2 ratio. Taken together, our data confirmed the cytoprotective properties of PCr against H$_2$O$_2$-induced apoptosis.

Meanwhile, there is evidence that oxidative stress is a major stimulus in the pathogenesis of cardiovascular diseases, including atherosclerosis, hypertension, myocardial infection and heart failure [30–33]. And there is increasing attention on the relationship between oxidative stress and endothelial cell injury [34–37]. Reactive oxygen species generated primarily by mitochondria are highly reactive metabolites produced during normal cell metabolism [37–39]. It has previously been reported that increases in intracellular ROS levels can lead to apoptosis. The underlying mechanism may involve the direct interaction and destruction of cellular proteins, lipids and DNA and/or indirect interference with normal cellular signaling pathways and gene regulation [40, 41]. Our results showed that oxidative stress in the form of H$_2$O$_2$ treatment led to enhanced ROS in HUVECs, and that PCr reduced this ROS production in a dose-dependent manner. Our findings suggested that increased scavenging of ROS was involved in the mechanisms underlying the protective effect of PCr against H$_2$O$_2$-induced injury.

To clarify the signal molecular mechanism affected by PCr in H$_2$O$_2$-induced apoptosis, in this study, we focused on examining potential changes in the PI3K/Akt/eNOS pathway. Akt, the downstream of PI3K, is a key regulator of many cellular responses including protein synthesis, gene transcription, cell survival, proliferation, and differentiation [42]. And PI3K/Akt pathway regulates mechanically driven and receptor-ligand signaling [43]. The activation of eNOS can be triggered by the activation of protein kinase Akt and phosphoinositide-3-kinase (PI3K) [44]. NO, derived from the action of eNOS in endothelial cells, is one of the most important mediators in the regulation of endothelial functions [45]. Interestingly, as shown in many previous studies, H$_2$O$_2$ directly upregulated the levels of NO in endothelial cells, suggesting that overproduction of NO by endothelial cells in response to H$_2$O$_2$ stimulation was intended to protect the cells, rather than damage cells [46, 47]. In our study, Akt existed predominantly in an inactivated form in HUVECs that were not under oxidative stress but were activated and phosphorylated when cells were co-treated with H$_2$O$_2$ and PCr. PCr markedly increased the phosphorylation of Akt. This activation was significantly counteracted by treatment with the PI3K specific inhibitors wortmannin and LY294002. These results suggested that PCr may protect HUVECs from H$_2$O$_2$-induced apoptosis at least in part by activating the PI3K/Akt/eNOS signaling pathway.

BCECF adhesion assay confirmed that H$_2$O$_2$ has a significant effect on monocyte-endothelial cell adhesion. It has been reported that increased adhesion of U-937 on HUVECs after H$_2$O$_2$ stimulation [48]. H$_2$O$_2$ induces endothelial retraction accompanied by a loss of the normal spatial organization of endothelial cell adhesion molecules [49]. In our tests, a significant reduction in the adhesion assay occurred by the inhibition of PCr in a dose-dependent manner. Our findings showed that PCr reduced U937 cells adhesion to H$_2$O$_2$-stimulated HUVECs and attenuated H$_2$O$_2$-induced production of intracellular
ROS. Monocyte-HUVECs adhesion is an early step in atherosclerosis. Thus, reducing monocyte adhesion to HUVECs is a promising pharmacological target for the prevention of atherosclerosis.

We first investigated the possible hypolipidemic and antioxidative effects of PCr on rats fed with a high-fat diet. In the present study, PCr not only decreased serum TC but also decreased serum TG. The liver acted in metabolizing and hastening the process of excretion of excess lipids thereby producing hypolipidemic condition. Hepatic LPL and HL are essential in the hydrolysis of lipoproteins [50]. Thus, we investigated the LPL and HL activities in liver. The result showed that PCr enhanced LPL and HL activities in a dose-dependent manner, and PCr at the dose of 50 mg/kg significantly enhanced LPL and HL activity, when compared with HF group. It suggested that PCr could accelerate lipids metabolism through increasing LPL and HL activities, thereby producing hypolipidemic conditions. Recent studies have demonstrated that lipid peroxidation even has an important relationship with hyperlipidemia [51]. ROS affects lipids and leads to lipid peroxidation which result in the formation of aldehyde by-products such as MDA to damage liver [52]. In our current study, PCr decreased hepatic MDA and the liver index, and increased hepatic SOD activity and serum GSH-PX activity. It is well known that SOD and GSH-PX are among the main defensive antioxidant agents, which scavenge oxygen free radicals. Taken together with the above results, it may be explained that PCr increased in SOD and GSH-PX activities, thus attenuating oxygen free radicals and decreased MDA generation, which is favorable to protecting liver metabolism functions. This is helpful to metabolize lipid in liver. Hyperlipidemia is a major cause of atherosclerosis. Endothelium-derived NO plays an important protective role during the early phases of atherosclerosis, and NO is generated via oxidation of L-arginine that is catalyzed by NOS [53, 54]. The result showed PCr increased NOS activity in serum to comparative level with the NC group. It indicated that PCr has a potential protective effect to postpone the process of atherosclerosis. Oxidative stress in the arterial wall plays a major role in the initiation and progression of the cardiovascular dysfunction associated with hyperlipidemia [55]. SOD and GSH-PX through dismutating superoxide could decrease oxidative stress and induce vascular endothelial cell apoptosis [56]. In the current study, PCr significantly increased SOD and GSH-PX activities in hyperlipidemia rats. It is reasonable for us to have a hypothesis that PCr may protect endothelial cells against oxidative stress-induced apoptosis in vitro, which further inhibits pathological process of cardiovascular disease [57, 58]. To confirm the hypothesis, we investigated the protective effects of PCr on H₂O₂-induced human vascular endothelial apoptosis. Oxidative stress alters diverse functional responses of endothelial cells and thereby is regarded as a critical pathogenic factor in the development of cardiovascular diseases. For example, in pathophysiological situations blood vessels can be exposed to 100µM concentrations of endogenous H₂O₂ [59], suggesting a link between oxidative stress and endothelial cell injury. H₂O₂ has been suggested as an inducer of apoptosis in several types of cells [60, 61]. So H₂O₂ was selected to induce HUVECs apoptosis.

It has been reported that cholesterol crystals perforating the intima are shown to be associated with plaque disruption. Crystal content is significantly associated with clinical events, suggesting that cholesterol crystallization may have a role in plaque disruption [62]. Our study showed that PCr could increase the leaching of TC, HDL, TG, (TC > HDL > TG), which is similar to Atorvastatin. It has been
reported that the evolution of plaque is characterized by inflammation. Thus, inhibition of inflammation can dissolve or prevent cholesterol crystal formation as a result of stabilizing vulnerable plaques. Cholesteryl ester hydrolases (CEHs) can convert esterified cholesterol (ESC) into free cholesterol (FRC) [63]. Therefore, we hypothesized that the mechanism is likely related to the activity of anti-inflammatory or cholesterol ester related enzymes such as cholesteryl ester hydrolases. However, if cholesterol transport is enhanced, cholesteryl ester hydrolases can rapidly dissolve and promote reversal of atherosclerotic lesions [64]. Hence, its detailed mechanism needs to be further explored in the future.

5. Conclusion

In summary, the major findings of this study are that (a) PCr could inhibit oxidative reaction by reducing H$_2$O$_2$-induced ROS overexpression and modulating the PI3K/Akt signaling pathway in HUVECs. (b) PCr could prevent H$_2$O$_2$-induced apoptosis of HUVECs through regulating Bcl2 family expression and caspase activation in mitochondrial system. (c) PCr could be a very useful compound to control hypercholesterolemia by both reducing cell adhesion as well as improving the lipid profile and modulating oxidative stress (Fig. 7).

Collectively, PCr may provide a novel therapeutic avenue for the treatment of atherosclerosis. It may also provide new insight into the cellular protective mechanisms of PCr. However, for the limitations of this study, the H$_2$O$_2$ concentrations and shorter incubation times used in our study may represent a likely difference to in vivo conditions, where the endothelium would be expected to be exposed to a continual external flux of H$_2$O$_2$ over an extended period. Therefore, further research of atherosclerosis protective roles of PCr in tissue-, animal- and patient-specific studies still need to be explored in the future.

Abbreviations

CA, low cytometry; CEHs, Cholesteryl ester hydrolases; TC, Total cholesterol; DA group (Di Ao Xin Xue Kang (DiAo), a kind of total steroidal saponins, a commercial anti-hyperlipidemia agent); DCF, Dichlorofluorescein; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; ECL Chemiluminescence; ECs, Endothelial cells; ESC, Esterified cholesterol; FCM, Flow cytometry; FBS, Fetal bovine serum; FRC, Free cholesterol; GSSG, Oxidized glutathione; H$_2$O$_2$, Hydrogen peroxide; HDL, High-density lipoprotein; HL, Hepatic lipase; HUVECs, Human umbilical vascular endothelial cells; LDH, Lactate dehydrogenase; LDL, Low-density lipoprotein; LPL, Lipoprotein esterase; MDA, Malonaldehyde; NO, Nitric oxide; CPR, Cardiac arrest and cardiopulmonary resuscitation; PCr, Phosphocreatine; PI3K, Phosphoinositide-3-kinase; ROS, Reactive oxygen species; SOD, Superoxide dismutase; TBA, Tbituric acid; TBST, Tween 20; TG, Triglycerides;

Declarations

Ethical Approval
The animal work has been approved by the ethics committee of Dalian Medical University, and the approval number was 201,720,223. All animal housing and experiments were conducted in strict accordance with the institutional guidelines for care and use of laboratory animals.

**Consent to participate**

Patient in this study signed a written informed consent to participate in accordance with the Helsinki Declaration which was approved by the ethical Committee of the Dalian Medical University.

**Consent to publish**

Written informed consent was obtained from the patient for the publication of this study.

**Competing interests**

All the authors declared that no competing interests.

**Authors’ contributions**

Zhongyuan Tang, Zonghui Zhang, Jiaqi Wang share the first authorship. Zhongyuan Tang, Zhaohong Geng, Zeyao Tang, Qiying Yao studied and designed the experimental studies and revised, polished manuscript. Zonghui Zhang performed cell line-based studies. Zhongyuan Tang repeated and supplemented relevant experiment, and wrote manuscript, Jiaqi Wang and Zhongyuan Tang performed the statistical analysis and interpreted and drawn the data. Zhaohong Geng, Zeyao Tang, Qiying Yao directed the overall project, who are equally corresponding authors. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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**Availability of data and materials**

The authors declare that all data supporting the findings of this study are available within the article.

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Figures
Figure 1

Effects of PCr on H$_2$O$_2$-induced cytotoxicity and apoptosis in HUVECs. (A). Chemical structure of PCr; (B). Representative images of H$_2$O$_2$-treated cells with and without PCr. a, f. vehicle control; b, g. H$_2$O$_2$-treated; c, h. H$_2$O$_2$+PCr (15 mM); d, i. H$_2$O$_2$+PCr (20 mM); e, j. H$_2$O$_2$+PCr (25 mM). Original magnification: 20×; (C). Cell viability was analyzed by MTT assay after 4 h exposure to H$_2$O$_2$ in the presence of various
concentrations of PCr. (D). FCM analysis with Annexin V-FITC/PI staining to determine the effect of PCr on HUVECs apoptosis. a. vehicle control; b. H$_2$O$_2$-treated; c. H$_2$O$_2$ + PCr (15mM); d. H$_2$O$_2$ + PCr (20mM); e. H$_2$O$_2$ + PCr (25mM). (E). The different early apoptosis and late apoptosis rates of cells treated with 1mM H$_2$O$_2$ in the presence of different concentrations of PCr. Data on the graph were from three independent experiments (n=6) and analyzed by ANOVA followed by Bonferroni’s post hoc test (###p<0.001 vs. vehicle control and *p<0.05, **p<0.01, ***p<0.001 vs. 1mM H$_2$O$_2$ treatment only).
Figure 2

Effects of PCr on LDH, MDA, SOD contents in HUVECs and U937 adhesion to HUVECs induced by H$_2$O$_2$.

(A). HUVECs were pre-treated with PCr for 60h followed by co-incubation with H$_2$O$_2$ (1mM) for 4h. The LDH release was determined by microplate reader; (B-C). MDA production and SOD activity were determined by spectrophotometer. (D-E). Effects of PCr on U937 adhesion to H$_2$O$_2$-induced HUVECs.
Representative images of H$_2$O$_2$-treated cells with and without PCr. **a.** vehicle control; **b.** H$_2$O$_2$ (1 mM) - treated; **c.** H$_2$O$_2$+PCr (15 mM); **d.** H$_2$O$_2$+PCr (20 mM); **e.** H$_2$O$_2$+PCr (25 mM). Original magnification: 20×. Data were from three independent experiments (n=6) and were analyzed by ANOVA followed by Bonferroni's post hoc test (##p<0.01, ###p<0.001 vs. vehicle control and *p<0.05, **p<0.01, ***p<0.001 vs. 1mM H$_2$O$_2$ treatment only).
Effects of PCr on intracellular ROS generation, NO production and Akt signaling pathway in HUVECs induced by H$_2$O$_2$. HUVECs were pre-treated with PCr for 60h followed by co-incubation with H$_2$O$_2$ (1mM) for 4h. (A). FCM analysis for ROS generation. a. vehicle control; b. H$_2$O$_2$-treated; c. H$_2$O$_2$+PCr (15mM); d. H$_2$O$_2$+PCr (20mM); e. H$_2$O$_2$+PCr (25mM); (B). Mean intensity of DCFH-DA fluorescence; (C). The production of NO; (D). Representative Western blots of phosphorylated and total Akt. The intensity of each band was quantified by densitometry analysis. Data were from three independent experiments (n=6) and analyzed by ANOVA followed by Bonferroni’s post hoc test (#p<0.05, ##p<0.01 vs. vehicle control and *p<0.05, **p<0.01 vs. 1mM H$_2$O$_2$ treatment only).

![Figure 4](image1)

**Figure 4**

Effects of PCr on caspase activation and Bax and Bcl-2 protein expression in HUVECs induced by H$_2$O$_2$. HUVECs were pre-treated with PCr for 60h followed by co-incubation with H$_2$O$_2$ (1mM) for 4h. (A). Representative Western blotting of cleaved-caspase3 and cleaved-caspase9 protein expression. (B). Representative Western blotting of Bax and Bcl-2 protein expression. Data were from three independent experiments (n=6) and were analyzed by ANOVA followed by Bonferroni’s post hoc test (#p<0.05, ##p<0.01 vs. vehicle control and *p<0.05, **p<0.01, vs. 1mM H$_2$O$_2$ treatment only).
Figure 5

**Histological examination of the liver obtained from rats from different groups.** The gross appearances of the entire livers (top) and photographs of the cross-section (×100 magnification) of liver (bottom) are illustrated (n =8). a, NC group; b, HF group; c, HF + IPCr group (20 mg/kg/d); d. HF + mPCr group (35 mg/kg/d); e, HF + hPCr group (50 mg/kg/d). Lipid dispositions in the livers were indicated by arrows.
Figure 6

The morphology of atherosclerosis plaque and the random same weight of 5 parts. (A). The fresh atherosclerotic plaque of thoracic aorta from a patient after surgery; (B). Evenly divided same weight plaque of five parts into different groups (Control, 1µM Atorvastatin, 5 mM, 10 mM, 20 mM PCr); (C-F). The dissolved TG, TC, LDL and HDL values of the above different groups at 0h, 2h, 4h, 6h, 8h, 10h were measured using a clinical diagnosis assay kit respectively.
Figure 7

Schematic diagram showing cytoprotective signaling of PCr in H$_2$O$_2$-induced HUVECs apoptosis. As depicted, PCr inhibited the apoptotic signaling cascades initiated by H$_2$O$_2$-generated ROS. The details are as follows. After entering the cell from outside the cell, H$_2$O$_2$ produces oxidative stress reactions, causing mitochondria to produce excessive ROS, thereby inhibiting the normal PI3K/Akt/eNOS/NO pathway, while exogenous PCr, after entering the cell, prevents this process by inhibiting excessive ROS production and meanwhile promotes PI3K production; On the other hand, exogenous PCr inhibits the BAX/Bcl-2 ratio on the mitochondrial cell membrane, thereby reducing the release of cytochrome, further decreasing the production of caspase-9/caspase-3 reactions, leading to preventing cell apoptosis, under the cooperation through inhibiting the PI3K/Akt/eNOS/NO pathway. Abbreviations: H$_2$O$_2$, Hydrogen peroxide; ROS, Reactive oxygen species; PI3K, Phosphoinositide-3-kinase. The “↑” arrowhead indicates activation or induction. The“↓” arrowhead indicates inhibition.