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Original Research Article

Citrate pre-conditioning inhibits apoptosis and protects rat myocardium from ischemia-reperfusion injury by activating PI3K/Akt signaling pathway

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Abstract

Purpose: *To study the influence of citric acid pre-exposure on apoptosis and heart tissue ischemia-reperfusion lesion, and the involvement of PI3K/Akt signal route in this process.*
Methods: *Eighty-four male Sprague-Dawley (SD) rats were randomly divided into a sham group, model group, pretreatment group, and inhibitor group, with 21 rats in each group. Changes in myocardial infarction area were determined using TCC staining, while TUNEL assay was performed to assess apoptosis of cardiomyocytes in the rats. Expression levels of p-Akt, ppi3k, Bcl-2, Bax, and caspase-3 were assayed by Western blotting.*
Results: *The levels of Bax and caspase-3 were higher in model rats than in sham group, while concentrations of Bcl-2, p-Akt, and p-pi3k were significantly reduced. In pre-treated and inhibitor-exposed rats, caspase-3 and Bax levels were significantly down-regulated, relative to corresponding levels in model rats, but Bcl-2, p-pi3k, and p-Akt were significantly up-regulated ($p < 0.05$). In inhibitor-exposed rats, Bax and caspase-3 were higher than levels in pretreatment rats, but p-pi3k, Bcl-2, and p-Akt concentrations were significantly decreased ($p < 0.05$).*
Conclusion: *Citric acid pretreatment in rats activates PI3K/Akt signal pathway, inhibits cardiomyocyte apoptosis, protects the myocardium from ischemia-reperfusion injury, and improves cardiac function. The potential of citric acid pretreatment for its protective effect against myocardial injury due to ischemia-reperfusion should be further investigated.*

Keywords: *Citric acid, Pretreatment, PI3K/Akt signal pathway, Apoptosis, Myocardial ischemia-reperfusion*

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INTRODUCTION

Myocardial ischemia-reperfusion injury (mi-ri) is regaining of blood supply within a certain period of time after partial complete obstruction of the coronary artery. At this time, although ischemic myocardium may function normally again, there is a pathology of progressive aggravation of

tissue damage [1]. The series of ischemia-induced damaging lesions in myocardial ultrastructure, energy metabolism, cardiac function, and electrophysiology are more prominent after vascular recanalization, and may even result in serious arrhythmias and sudden death [2].

Some studies have found that myocardial ischemia-reperfusion injury (mi/ri) caused related organ failures which account for its link with many serious pathophysiological events in clinics. Changes in ischemia time period form the basis for reperfusion lesion, and mi/ri is an independent pathophysiological process for the extension and deterioration of ischemia injury during reperfusion injury [3]. Therefore, early prevention, timely treatment of ischemia-reperfusion injury, and rapid elimination of ischemia are crucial in reducing the occurrence of mi/ri. Pretreatment is a traumatic stimulation that improves the resistance and adaptability of myocardial cells. Studies have found that appropriate stimulation produces ischemic preconditioning. If the ischemic time is too long, it results in irreversible damage to the myocardium [4].

Citric acid, also known as citrate, is an important organic acid. The calcium salt of citric acid reduces the concentration of plasma calcium, inhibits coagulation, and prevents blood coagulation [5]. In this study, citric acid pretreatment was used to investigate its inhibitory effect on apoptosis, and its protective effect against myocardial injury due to ischemia-reperfusion.

EXPERIMENTAL

Laboratory animals

A total of 84 healthy male SD rats were used in this study. The animals had a mean weight of 262 ± 48 g. All rats were fed adaptively for 1 week in an environment with a temperature of 26 ± 1 ℃, a humidity of 52 ± 18 %, and a 12-h light/12-h dark cycle. The study received approval from the Animal Ethics Authority of The First Affiliated Hospital of Nan Chang University in line with the National Institute of Health (NIH) guidelines [6].

Main Instruments and chemicals

The equipment and consumables employed in this study were: high-velocity centrifuge, refrigerator (Shanghai Precision Instrument Co. Ltd., model: dw-40l158), slicing instrument, electronic balance, an animal ventilator (Shanghai Yilian Science and Education Equipment Co. Ltd, sex Imam: hx-300); constant temperature water bath box (Tianjin Hengao Technology Development Co. Ltd, model: hwt-6b); phosphate-buffered saline, anti-mouse caspase-3 monoclonal antibody (Shanghai Yisheng Biotechnol.); p-Akt rabbit anti-mouse polyclonal antibody (Shanghai Xinfan Biotechnology Co. Ltd); Bax polyclonal antibody (Wuhan Aimeijie Technology Co. Ltd); Bcl-2 polyclonal antibody (Shanghai Hufeng Chemical Co. Ltd), and citric acid (Sichuan Nagel Biotechnology Co. Ltd, specification: 200 mL: 5.0 g, production batch no. 20179812).

Animal grouping and establishment of animal experimental model

The rats were anesthetized following an 8-h fast. Thereafter, the rats were fixed on the test bench and connected to the ECG monitor, and standard lead II was used for ECG monitoring. The rats were connected to organ intubation and to an animal ventilator after thoracotomy. The chest of each rat was opened between the 3rd and 4th ribs at the left edge of the sternum, to expose the heart, and surgical instruments were used to passively expose the left common carotid artery. All openings at the distal end of the common carotid artery were cut off, and the top of a cotton plug was placed about 18 cm to the junction of the carotid arteries. After 110 min, reperfusion was initiated by pulling out the blocking line. After operation, the incision sites were sutured. During the operation period, room temperature was kept constant. Rats were put back into their cages and allowed free access to drinking water after waking up. In sham operation rats, injection of physiological saline (1 mL) into the caudal vein was done 10 min prior to perfusion, but without ligation of the anterior descending coronary artery. In model rats, the anterior descending branch of the coronary artery was ligated for 35 min, after which the ligation was loosened for 2 h of reperfusion, followed by physiological saline (1 mL) injection into the caudal vein 10 min prior to reperfusion. In the citric acid pretreatment group (pretreatment group), the anterior descending coronary artery was ligated for 35 min. Then, the ligation was released for 2 h of reperfusion, and the same volume of 0.5 mol/L citric acid was injected into the caudal vein 10 min before reperfusion. In the inhibitor group, rats received p-Akt inhibitor 5 min before citric acid pretreatment. There were 21 rats in each group.

Changes in LVESP, LVEDP, injection fraction (EF), $+dp/dt_{max}$, and $-dp/dt_{max}$ were measured in each group using a cardiac function tester. Serum levels of lactate dehydrogenase (LDH)

and creative phosphate kinase (CK) were measured with enzyme-linked immunosorbent assay (ELISA). The levels of malondialdehyde (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD) in myocardial tissue of rats in each group were determined: MDA was measured with the thiobarbituric acid method; SOD was assayed with enzyme rate method, while GPx activity was assayed colorimetrically. Changes in the myocardial infarction area in each group were determined with TCC staining. The ischemic tissue is usually white, while non-ischemic tissue is red.

At the end of perfusion, the heart was quickly excised, and the ischemic heart tissue was separated. Paraffin sections were routinely made, and the apoptosis was measured with the TUNEL procedure. Protein levels of p-Akt and p-pi3k, Bcl-2, Bax, and caspase-3 in rats were measured with immunoblot assay.

Statistical analysis

The SPSS 23.0 software package was used for the statistical analysis of data. One-way ANOVA and LSD *t*-test were used for the comparison of measurement data which are presented as mean ± standard deviation (SD). Values of $p < 0.05$ indicated significant differences.

RESULTS

Changes in cardiac function indices

The level of LVEDP was significantly higher in model rats, while LVESP, EF and +dp/dt$_{max}$, and -dp/dt$_{max}$ were significantly reduced, relative to corresponding levels in sham operation rats, but LVESP, EF, and +dp/dt$_{max}$ were significantly increased in the pretreatment group and the inhibitor group, when compared with the model group ($p < 0.05$). Compared with the model group, the levels of LVEDP decreased significantly, while the levels of LVESP, EF, -dp/dt$_{max}$, and +dp/dt$_{max}$ increased significantly ($p < 0.05$). Relative to pretreatment rats, LVEDP and -dp/dt$_{max}$ in inhibitor-exposed rats were significantly increased, while LVESP, EF, and +dp/dt$_{max}$ decreased significantly ($p < 0.05$). These results are shown in Table 1.

LDH and CK levels

As presented in Table 2, the levels of LDH and CK were increased in the model rats, relative to sham rats. However, pretreatment rat levels of LDH and CK were significantly lower than the corresponding model rat values. Relative to the pretreatment group, levels of LDH and CK in the inhibitor group were higher ($p < 0.05$).

Changes in myocardial infarction area

As shown in Table 3, model rats had a significant increase in MDA, while activities of GPx and SOD were decreased significantly, when compared to sham operation rats. However, MDA levels in the pretreatment group were significantly reduced, while GPx and SOD activities were significantly increased, relative to model rats.

Compared with pretreatment rats, the MDA level in inhibitor-exposed rats was increased significantly, while the activities of GPx and SOD were decreased significantly.

Cardiomyocyte apoptosis

There were higher percentages of apoptosis in myocardial cells in the model group, pretreatment group, and inhibitor-treated rats than in sham-operation rats. Moreover, compared with the model rats, the percentage of myocardial apoptosis in the pretreatment group was significantly reduced, while the apoptosis of myocardial cells was significantly raised in inhibitor-exposed animals, relative to pretreatment rats. These data are presented in Table 4.

Table 1: Changes in cardiac function indices of rats (n = 21)

Group	LVESP (mmHg)	LVEDP (mmHg)	EF (%)	+dp/dt$_{max}$ (mmHg/sec)	-dp/dt$_{max}$ (mmHg/sec)
Sham	133.66±8.42	4.71±0.53	92.66±4.74	5479.62±251.33	-3876.81±229.57
Model	63.16±9.48^{a}	10.97±2.05^{a}	68.35±6.72^{a}	3542.19±208.46^{a}	-2108.84±128.28^{a}
Pretreatment	94.09±9.37^{ab}	7.49±1.46^{ab}	81.27±7.11^{ab}	4722.61±153.49^{ab}	-2982.27±218.86^{ab}
Inhibitor	78.39±8.95 ^{abc}	8.85±1.15 ^{abc}	75.82±4.39 ^{abc}	4015.11±179.85 ^{abc}	-2537.48±169.26 ^{abc}
F	234.83	72.76	64.01	369.93	329.77
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a,b,c}P < 0.05$, ^{a}vs, sham operation; ^{b}vs model rats; ^{c}vs pretreatment

Table 2: Comparison of LDH and CK levels in rats (mean ± SD, n = 21)

Group	LDH (U/L)	CK (U/L)
Sham operation	885.39±92.62	1244.32±117.39
Model	2106.68±297.22[a]	3389.62±195.29[a]
Pretreatment	1226.47±162.49[ab]	2735.35±148.34[ab]
Inhibitor	1775.67±206.25 [abc]	3035.73±188.33 [abc]
F	151.53	683.31
P-value	<0.001	<0.001

[a,b,c]*P* < 0.05, [a]vs, sham operation; [b]vs model; [c]vs pretreatment

Table 3: Changes in myocardial infarction area in rats (n = 21)

Group	Myocardial infarction area (%)
Sham operation	0
Model	30.01±2.65[a]
Pretreatment	18.11±2.49[ab]
Inhibitor	24.93±1.97 [abc]
F	131.33
P-value	<0.001

[a,b,c]*P* < 0.05, [a]vs, sham operation; [b]vs model; [c]vs pretreatment

Changes in oxidative indices

In model rats, the MDA level was increased significantly, while the activities of GPx and SOD were significantly reduced, relative to sham operation rats. However, MDA levels in the pretreatment group were significantly reduced, while the activities of GPx and SOD were significantly increased, when compared with the model group ($p < 0.05$). Compared with the pretreatment group, the MDA level in the inhibitor group was increased significantly, while the activities of GPx and SOD were decreased significantly ($p < 0.05$). These data are presented in Table 5.

Table 4: Cardiomyocyte apoptosis of rats (n = 21)

Group	Cardiomyocyte apoptosis (%)
Sham operation	0
Model	10.24±1.65[a]
Pretreatment	2.67±1.14[ab]
Inhibitor	6.29±1.33 [abc]
F	144.11
P-value	<0.001

[a,b,c]*P* < 0.05, [a]vs, sham operation; [b]vs, model; [c]vs pretreatment

Expression levels of p-Akt, p-pi3k, and anti-apoptotic and pro-apoptotic protein

In model rats, relative concentrations of Caspase-3 and Bax were significantly raised, while Bcl-2, p-Akt, and p-pi3k levels were significantly reduced, relative to sham operation rats (Table 6). The levels of Bax and Caspase-3 in the pretreatment group and inhibitor group were significantly reduced, while there was a significant up-regulation of Bcl-2, and phosphorylated Akt and pi3k, relative to model rats ($p < 0.05$). In inhibitor-treated rats, Bax and caspase-3 levels were significantly increased, while Bcl-2 and phosphorylated forms of p-Akt and p-pi3k were significantly decreased when compared to the pretreatment rats.

Table 5: Comparison of changes in oxidation indices of rats (n = 21)

Group	MDA (μmol/L)	GPx (μmol/L)	SOD (U/mL)
Sham operation	4.11±1.54	1616.22±95.83	158.16±22.07
Model	8.78±2.69[a]	1161.31±72.68[a]	72.48±12.53[a]
Pretreatment	5.57±1.66[ab]	1401.44±66.48[ab]	129.36±15.49[ab]
Inhibitor	7.21±1.09[abc]	1299.21±60.59[abc]	93.72±16.46[abc]
F	25.32	136.76	104.73
P-value	<0.001	<0.001	<0.001

[a,b,c]*P* < 0.05, [a]vs, sham; [b]vs, model; [c]vs pretreatment

Table 6: Expression levels of p-Akt, p-pi3k, anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax and caspase-3 in rats

Group	p-Akt	p-PI3K	Bcl-2	Bax	Caspase-3
Sham operation	1.11±0.27	2.45±0.24	1.45±0.21	0.85±0.17	0.35±0.08
Model	0.38±0.09[a]	0.38±0.08[a]	0.64±0.16[a]	1.53±10.23[a]	1.24±0.17[a]
Pretreatment	0.81±0.24[ab]	1.26±0.14[ab]	1.18±0.17[ab]	1.14±0.18[ab]	0.58±0.19[ab]
Inhibitor	0.45±0.11[abc]	0.67±0.17 [abc]	0.84±0.20 [abc]	1.32±0.27 [abc]	0.77±0.21 [abc]
F	16.78	42.64	21.87	34.74	24.52
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

[a,b,c]*P* < 0.05, [a]vs, sham operation; [b]vs, model; [c]vs pretreatment

DISCUSSION

In recent years, although the development of percutaneous coronary angioplasty, intracoronary stenting, and coronary artery bypass grafting have significantly increased the cure rate and survival rate of cardiovascular disease patients, the incidence of mi/ri has also increased significantly [7]. Therefore, strategies for reducing myocardial damage caused by mi/ri and enhancing recovery from it have become the focus of medical research. Lin *et al* [8] have reported that ischemic myocardial tissue after ischemia preconditioning significantly reduced the resultant long-term mi/ri and played a myocardial protective role. Citric acid is an anticoagulant. Calcium ions promote the activation of platelets during blood coagulation. Citric acid combines with free Ca^{2+} to form soluble calcium citrate, thereby reducing the concentration of Ca^{2+}, and enhancing myocardial remodeling [9].

Changes in cardiac function are amongst the vital manifestations of mi/ri, and myocardial enzyme levels in serum are closely related to the extent of myocardial infarction and prognosis [10]. Lactate dehydrogenase (LDH) catalyzes the interconversion of lactic acid and pyruvic acid during glycolysis and gluconeogenesis, and it is most abundant in the heart and skeletal muscle. Skeletal muscle and myocardium are rich in creatine kinase (CK) which is also known as creatine phosphokinase. The enzyme is a specific and sensitive indicator of myocardial injury, and it is essential in the diagnosis of early cardiopulmonary arrest and estimation of the size of myocardial infarction. Some studies have demonstrated that the measurement of the myocardial infarction area serves as an effective index for the evaluation of myocardial injury [11].

Apoptosis is the most significant feature in mi/ri. It has been reported that the severity of myocardial infarction is a function of the extent of cardiomyocyte apoptosis [12]. This research demonstrated that citric acid pretreatment significantly reduced the levels of LDH and CK, improved the level of cardiac function, inhibited cardiomyocyte apoptosis, and reduced the area of myocardial infarction.

The myocardium is the tissue with the highest oxygen supply and demand in the body. Some studies have found that the production of oxygen free radicals and enhanced lipid peroxidation reactions are the major mechanisms that underlie myocardial injury [13]. During ischemia-reperfusion, a large number of oxygen free radicals are generated, leading to significant increases in the permeability of the membrane. At the same time, ischemia-reperfusion causes intracellular Ca^{2+} overload and induces the production of inflammatory mediators, thereby leading to vasoconstriction or microcirculation disorders [14]. The results of this study showed that, compared with model group, the MDA level of pretreatment group was significantly reduced, while the activities of GPx and SOD were significantly increased. These results suggest that citric acid pretreatment significantly increased the level of antioxidant enzymes, reduced lipid peroxidation, and decreased myocardial injury in mi/ri.

Phosphatidylinositol 3 kinase (PI3K) is a phosphatidylinositol kinase which phosphorylates the third hydroxyl group in the inositol ring. It is an important signal transduction molecule in cells. Studies have found that PI3K activity is usually strictly regulated by a variety of mechanisms in normal cells [15]. When PI3K is stimulated and activated, it binds to PIP3 and signals protein kinase (Akt) on the cell membrane, leading to the activation of Akt, a serine/threonine kinase and an important target downstream of PI3K. Wang *et al* [16] have demonstrated the importance of the PI3K/AKT signal route in promoting cell proliferation, inhibiting apoptosis, and promoting cell migration. In this study, Bax and Caspase-3 levels in model rats were significantly increased, while Bcl-2, p-Akt, and p-pi3k were significantly decreased, relative to sham operation rats. However, in pretreatment rats and the inhibitor group, Bax and Caspase-3 were significantly decreased, while Bcl-2 and phosphorylated forms of pi3k and Akt were significantly up-regulated when compared with model group. In addition, there were significant increases in Bax and Caspase-3 in inhibitor-exposed rats, while Bcl-2 and phosphorylated forms of pi3k and Akt were decreased, relative to pretreatment rats. These data suggest that citric acid pretreatment significantly activated the PI3K/AKT signaling pathway, reduced the expressions of Bax and caspase-3, and up-regulated the expression of Bcl-2.

CONCLUSION

Citric acid pretreatment activates the PI3K/AKT signal route, inhibits cardiomyocyte apoptosis, protects rat myocardium from ischemia-reperfusion lesions, and improves cardiac function. However, the potential of citric acid pretreatment in protecting against myocardial

injury caused by ischemia-reperfusion requires further investigation.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Wenfeng Duan and Zhiyun Zhu designed the study, supervised the data collection, and analyzed the data. Fugen Nie, Bo Qiu, and Diqin Wan interpreted the data and prepared the manuscript for publication. Zhiyun Zhu supervised the data collection, analyzed the data, and reviewed the draft of the manuscript.

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