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Original Research Article

The Cyclosporine a Post Conditioning on Mitochondrial Protective Effects in Perfused Immature Heart

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Abstract: To determine whether blocking the mitochondrial permeability transition pore (MPTP) with cyclosporine-A (CsA) can restore cardioprotection in immature rabbit heart. On the Langendorff perfusion apparatus, isolated perfused immature rabbit hearts underwent 30 minutes of normothermic ischemia, followed by 120 minutes of reperfusion. 28 isolated immature rabbit hearts were randomly divided into four groups: control group(A), infused with Krebs-Henseleit solution (KHS); ischemic reperfusion group(B), CsA group(C), receiving the reperfusion of KHS +CsA; and Atr group(D),given the reperfusion of KHS+CsA+Atr. The postischemia myocardial function were assessed by the percentage recovery of heart rate(HR),left ventricle developed pressure (LVDP), left ventricle end-diastolic pressure (LVEDP), +dp/dtmax and-dp/dtmax were also observed. Rhodamine123 Fluorescence fluorescene intensity produced by Rhodamine123 was measured. The percentage recovery of myocardial function of group C were significantly better than control groupB and group D. Compared with group A, Rhodamine123 fluorescence intensity were decreased in group B and group D(p<0.05). CsA can improve the function damage of the immature heart during reperfusion. CsA can prevent the loss of$\Delta\Psi$m. Taken together, these data can help us to better understand CsA protective effect in immature myocardial ischemia and reperfusion, thus opening new perspectives in immature myocardium protection.
Keywords: Mitochondrial permeability transition pore, Cyclosporine-A, Cardioprotection.

INTRODUCTION

In cardiac surgery, myocardial protection has become an essential adjunctive measure for many years. Some newly developed concepts and methods of cardio protection have been improved by the mechanism of myocardial ischemia/reperfusion-induced damage being unveiled through the untiring efforts of cardiac surgeon during the past 60 years. The concept of myocardial protection in cardiac surgery was proposed along with introduction of hypothermic crystalloid potassium cardioplegia in the beginning and has been diversified by temperature modulation, blood cardioplegia, controlled reperfusion, pre-and post conditioning and pharmacological additives.

Its importance regarding cell metabolism is proportional to cellular energy requirements. Cardiomyocytes, the paradigm of high energy consuming cells, have their survival tightly connected to a normal mitochondrial function. In turn, mitochondria are commonly a major target for cardiomyocyte stress factors, like ischemia/reperfusion [1]. The major contributory factors include calcium overload, oxidative stress, mitochondrial permeability transition pore (MPTP) opening, and hypercontracture Mitochondria is the main cellular energy production center. The opening of the MPTPis a critical mediator of ischemia/reperfusion injury. The influence of substrates on the role of cyclosporin A, to promote the closure of the permeability transition pore, whereas atractyloside (Atr), which opens MPTP, aborts protection of CsA. Recent evidence indicates that ischemia/reperfusion can induce apoptosis in heart myocytes both in animals and humans. But, there is a few relevant studies on immature heart.

MATERIALS AND METHODS

The rabbits (male and female, weighing 300±50g, aged 3~4 week) were purchased from the Center of Experimental Animals, Cuilin Medical University. All animals used in this study were treated in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH). The study protocol was approved by the Institutional Ethics Committee. CsA

and Atr were purchased from Sigma Aldrich, St Louis, MO, USA.

Heart Preparation

Rabbits were anesthetized with an intraperitoneal injection with 30mg/kg sodium pentobarbital and then heparinized by injecting 3 mg/ml heparin via the inferior vena cava to prevent intracoronary clot formation. After one minute, the rabbits were fully heparinized and the heart was dissected and placed in Krebs Henseleit (KH) solution containing (in per mmol) 127NaCl, 17.7NaHCO3, 5.1KCl, 1.5CaCl2, 1.26MgCl2, 11D-glucose (pH7.4). The heart was mounted on a Langendorff-perfusion apparatus and retrogradely perfused through the aorta with recirculating KH solution saturated with 95% O_2 and 5% CO_2 at 37°C. Temperatures were maintained at 37ºC throughout the experiment with a thermostatically controlled recirculating water bath.

Perfusion was maintained at a constant pressure of 75 mmHg. For measurement of left ventricular pressure, a fluid-filled latex balloon was inserted in the left ventricle (LV) via the left atrium for pressure measurement. The balloon was connected to a pressure transducer and inflated to an initial LV end-diastolic pressure between 8 and 10 mmHg. Cardiac mechanical function was estimated as heart rate (HR), left ventricular developed pressure (LVDP) and peak rate of increase or decrease of left ventricular pressure ($\pm dp/dt_{max}$). The HR, LVDP, $+dp/dt_{max}$and $-dp/dt_{max}$were sampled and digitally processed via a hemodynamic system (Pclab,China)

Experimental Protocol

On the Langendorff perfusion apparatus, isolated perfused immature rabbit hearts underwent 30 minutes of normothermic ischemia, followed by 120 minutes of reperfusion. 28 isolated immature rabbit hearts were divided into four groups randomly: control group(A) (n=7), infused with Krebs-Henseleit solution (KHS); ischemic reperfusion group(B)(n=7), CsA group(C)(n=7), receiving the reperfusion of KHS +CsA(0.2μmol/l); and Atr group(D)(n=7),given the reperfusion of KHS+CsA+Atr(20μmol/l). The postischemia myocardial function were assessed by the percentage recovery of LVDP, LVEDP, $+dp/dt_{max}$ and $-dp/dt_{max}$.

To investigate the effects of CsA on immature myocardial cell apoptosis, we observed the change of mitochondrial membrane potential (ΔΨm) in immature perfused rabbit's heart. Press 200mg myocardium extracted mitochondria (diluted 5 × Assays Buffer washed with water 5 times) with an appropriate amount of 1 × Assays Buffer and resuspend; with 1 × Assays Buffer formulated into mitochondria solution of 3mg/mL, 2mL 1 × Assays Buffer and 1mL mitochondria solution, adding an appropriate amount of test compound (and blank control group), 25 ℃heated for3 min. Mitochondrial inner membrane potential was monitored by incorporating fluorescence dye Rh123. For this purpose ,cardiomyocytes were loaded with Rh123 (10uL)at 25℃ after the cells had been placed inthe Rh123-free medium. Rh123 fluorescence was measured by KeyGENE® diagnostic kit (Keygene, China). Andloaded cells were excited at 490 nm and the emitted fluorescence was collected at 530 nm.

Statistical Analysis

The data were expressed as mean ± SD values. Statistical analysis was performed by using SPSS17.0 statistical software. The data normal distribution was checked using the Kolmogorov-Smirnov goodness-of-fittest. Comparisons between groups were performed using repeated measurement ANOVA or one way ANOVA followed by LSD multiple comparison tests. A p values < 0.05 was considered statistically significant.

RESULTS

Hemodynamic analysis

The baseline values of LVDP, HR and$\pm dp/dt_{max}$ of immature hearts in experiment were recorded respectively. No differences were found either among the baseline values of the hearts in the four groups(Table 1).Compared with group B, HR recovery rate improved of group C (p<0.05).After adding Atr, group DHR recovery was lower than group C (p<0.05). See Table 2.

Both $+dp/dt_{max}$ and $-dp/dt_{max}$ can reflect myocardial contractility and diastolic function changes. In group B,$+dp/dt_{max}$ and $-dp/dt_{max}$ recovery rateis significantly lower than in group A (p<0.01). Compared with group B, Group C improved significantly, the difference was statistically significant (p<0.01). After blocking by Atr, in D group, + dp / dt_{max} and -dp / dtmax recovery rate was significantly lower than that in group A and C (p<0.01), no significant difference (p> 0.05), compared with group B(See Table 3,4).

LVDP was significantly lower in group B than group A (p<0.01).After using CsA protection, group C compared with group B has improved significantly, the difference was statistically significant (p<0.01). Group D significantly reduced compared with group C (p<0.01), see Table 5.

ΔΨm

Mitochendrial Permeability Fluorescent was used to assess ΔΨm which was found to be decreased in A group and C(p<0.05). Administration of CsA can recover the declined ΔΨm during reperfusion, indicating its role in inhibiting MPTP opening (Table-6).

Table-1: The baseline values of immature hearts（$\overline{X}$ ±S）

group	HR（bmp）	CF(ml/min)	LDVP	+dp/dt(mmHg/s)	-dp/dt(mmHg/s)
A	172.67±23.10	14.63±1.83	102.18±25.23	1678.12±323.37	1575.70±327.83
B	174.88±9.17	14.05±1.40	109.31±40.57	1563.73±615.37	1412.46±645.39
C	181.78±31.12	16.79±1.54	108.43±13.03	1685.31±557.47	1272.43±546.37
D	184.34±22.37	15.12±1.34	99.40±18.19	1704.15±317.49	1411.20±309.29

Table-2: HR recovery percentage after reperfusion, (%， $\overline{X}$ ±S)

group	15m	30m	60m	90m	120m
A	84.37±4.32	88.33±9.46	89.40±5.47	89.90±6.48	83.47±9.48
B	72.32±7.91*	79.31±4.57*	80.31±4.98*	81.41±10.47*	80.47±9.46*
C	81.32±6.18△	82.72±4.19*	84.37±6.45*	88.47±6.45*	87.62±10.40*
D	70.50±9.40*	78.33±4.48*	80.75±4.19*	81.17±10.70*	81.83±8.28*

Compared with group A, △p<0.05, Compared with group C, ＊p<0.05

Table-3: +dp/dt recovery percentage after reperfusion (%，$\overline{X}$ ±S)

group	15m	30m	60m	90m	120m
A	81.47±15.00	83.50±12.14	88.17±10.55	90.40±14.98	89.61±12.17
B	64.25±20.15*	68.13±13.78*	75.70±12.13*	80.16±7.32*	81.50±6.13*
C	73.78±12.14	85.45±12.45	89.75±9.50	88.23±7.70	90.41±13.98
D	54.50±17.67*△	65.74±18.65*△	66.37±12.16*△	67.25±12.38*△	66.50±16.35*△

Compared with group A, ＊p<0.01; Compared with group C, △p<0.01,

Table-4:-dp/dt recovery percentage after reperfusion（%， $\overline{X}$ ±S）

group	15m	30m	60m	90m	120m
A	69.47±9.44	79.31±10.08	78.98±8.13	86.07±6.76	88.95±7.65
B	46.83±12.16*	47.18±11.79*	52.31±12.19*	61.20±11.90*	65.32±5.98*
C	59.40±15.58	70.40±16.34	86.76±7.12	90.20±12.49	90.43±6.96
D	48.32±6.31*	53.17±12.13*	62.25±13.51*	65.53±13.96*	68.71±3.23*

Compared with group A, ＊p<0.01; Compared with group C, △p<0.01

Table-5: LVDP recovery percentage after reperfusion（%， $\overline{X}$ ±S）

group	15m	30m	60m	90m	120m
A	65.25±9.22	79.17±12.15	86.12±8.16	85.77±9.72	83.12±9.32
B	56.33±15.75*	59.50±12.31*	64.59±13.00*	69.50±9.38*	70.40±9.17*
C	65.80±9.32	86.40±13.17	87.61±7.36	88.20±7.38	86.81±5.47
D	59.20±11.19*△	65.80±9.13*△	67.71±9.13*△	68.93±12.18*△	69.12±12.69*△

Compared with group A, ＊p<0.01; Compared with group C, △p<0.01

Table-6: The Rh123 fluorescence values of immature hearts

Group	fluorescence
A	21.7±3.7
B	29.1±4.2＊
C	23.5±2.8
D	32.3+3.7＊△

Compared with group A, ＊p<0.05；Compared with group C, △p<0.05。

DISCUSSION

Myocardial ischemia-reperfusion may have many causes. In the past, the researchers always concentrated on the mechanisms causing cellular injury during ischemia period and on protective procedures designed to reduce development of ischemic injury. During reperfusion period, potential injury causes have been difficult to analyze, as these must be clearly differentiated from ischemic causes [2]. Recent studies showed that a period of ischemia-reperfusion of heart to the opening of a non-specific pore in the inner mitochondrial membrane, known as the MPTP [3]. The MPTP has emerged as a critical mediator of acute ischemia–reperfusion injury, thereby making it an

important target for cardio protection. Experimental animal studies have shown that pharmacologically inhibiting MPTP opening can reduce myocardial infarct size, a therapeutic strategy which has been reported in initial proof-of-concept clinical studies to be beneficial [4-7]. The mitochondrial channel is a non-selective, high-conductance channel located in the inner mitochondrial membrane, mediates necrosis and/or apoptosis in the early phase of ischemia-reperfusion injury. The opening of the MPTP renders the inner mitochondrial membrane non-selectively permeable to molecules less than 1.5kDa, inducing mitochondrial membrane potential collapse, cytochrome c efflux, ATP breakdown, and ultimately, cardiomyocyte death [7, 8].

In 1988, Crompton's group found that MPTP opening could be inhibited by the immunosuppressant, CsA, which has facilitated the investigation of the MPTP as a target of cardio protection [7]. Crompton and colleagues were the first to use CsA to investigate the MPTP as a target for cardio protection. They found that pre-treatment of adult rat ventricular cardiomyocytes with CsA protected against cell death induced by simulated acute ischemia/reperfusion injury [9]. Griffiths later reported the effects of CsA pre-treatment in isolated perfuse rat hearts, observing improved heart functional recovery and preserved myocardial ATP content following acute ischemia/reperfusion injury [10].

The MPTP opening primarily occurred at the onset of myocardial reperfusion has positioned the CsA as an important therapeutic target for reducing myocardial infarct size, which is readily manageable, to intervention in patients presenting with an acute myocardial infarction being treated with reperfusion therapies. The perfusion of isolated perfused rat hearts with CsA administered solely at the onset of myocardial reperfusion could limit myocardial infarct size, confirming that MPTP opening primarily occurred at the onset of myocardial reperfusion(11].In this study, the cardioprotection of CsA in immature rabbits heart is blocked by Atr. The post conditioning effect of CsA inhibits MPTP opening in the first minutes of reperfusion. CsA treatments significantly increased the amount of Ca^{2+} required to open the MPTP in vitro [12].

Because inner membrane polarization modulates MPTP opening, we addressed whether CsA might modify membrane potential during reperfusion, as measured by ΔΨm[13]. A moderate uncoupling could play a protective role after ischemia by decreasing ROS production and the mitochondrial calcium uptake [14]. Usually, the respiratory chain, while oxidizing NADH or $FADH_2$ in order to reduce O_2 into H_2O, actively pumps hydrogen ions out of the mitochondrial matrix into the intermembranespace. The MPTP remains closed during myocardial ischemia but opens due to Ca2+ overload and excessive production of reactive oxygen species during the onset of reperfusion which in turn, leads to disturbance in mitochondrial integrity and its function [15]. Opening of the MPTP results in collapse of the mitochondrial membrane potential, and uncoupling of the respiratory chain. In addition, pore opening leads to an influx of solutes and water that causes mitochondrial matrix swelling and loss of critical electrochemical gradients. Long-lasting MPTP opening is followed by profound alterations of cellular bioenergetics that are considered irreversible [16].

Compared with preconditioning which must be applied before an ischemic event, CsA' post conditiong effect has the advantage that it may be applied after sustained ischemia. It has much more extensive clinical applicability. Our findings found that cyclosporine A, a specific MPTP inhibitor, can restore the protective effect in immature animal heart [17]. It will provide a new drug to restore the cardio protective effect of immature animal heart.

In this work we demonstrated that CsA, a MPTP opening inhibitor, could restore the cardio protection in immature heart. Therefore, we speculated that ischemic CsA is blocked by Atr may due to the excessive the opening of MPTP and inhibiting of the MPTP is able to reverse this loss of cardio protection. The identification of a cause of true reperfusion injury requires that a therapeutic intervene at the reperfusion period attenuates the injury [18]. Many attempts in myocardial protection could made in cardiac surgery, since operations using cardiopulmonary bypass easily allow varying myocardial reperfusion. In general, research on the principles of reperfusion injury opens an entirely new approach to clinical myocardial protection.

CONCLUSION

CsA can improve the function damage of the immature heart. During reperfusion. CsA can prevent the loss ofΔΨm. These data can help us to better explain CsA cytoprotective effect in immature myocardial ischemia and reperfusion, thus opening new perspectives in immature myocardium.

CONFLICT OF INTERESTS

None declared.

AUTHOR'S CONTRIBUTION

Zhenzong Du and Haiyong Wang wrote the paper. Tianci Qian, FuguiRuan, Jiangbin Sun, Jianfei Song, Donghua Pan and Xiaolin Sun supervised the composition of the paper. All authors read and approved the final paper.

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