Original Article

Post-conditioning with gradually increased reperfusion provides better cardioprotection in rats

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BACKGROUND: Rapid and complete reperfusion has been widely adopted in the treatment of patients with acute myocardial infarction (AMI), but this process sometimes can cause severe reperfusion injury. This study aimed to investigate different patterns of post-conditioning in acute myocardial ischemia-reperfusion injury, and to detect the role of mitogen activated protein kinase (MAPK) during the injury.

METHODS: Rats were randomly divided into five groups: sham group, reperfusion injury (R/I) group, gradually decreased reperfusion group (GDR group, 30/10-25/15-15/25-10/30 seconds of reperfusion/ischemia), equal reperfusion group (ER group, 20/20 seconds reperfusion/ischemia, 4 cycles), and gradually increased reperfusion group (GIR group, 10/30-15/25-25/15-30/10 seconds of reperfusion/ischemia). Acute myocardial infarction and ischemic post-conditioning models were established in the rats. Six hours after reperfusion, 3 rats from each group were sacrificed and myocardial tissues were taken to measure the expressions of phosphorylation of extracellular signal-regulated protein kinase (P-ERK), phosphorylated c-Jun N-terminal kinase (P-JNK), mitogen-activated protein kinase p38 (p38 MAPK), tumor necrosis factor- α (TNF- α), caspases-8 in the myocardial tissue, and cytochrome c in the cytosol using Western blot. Hemodynamics was measured at 24 hours after reperfusion, the blood was drawn for the determination of cardiac enzymes, and the heart tissue was collected for the measurement of apoptosis using TUNEL. One-way analysis of variance and the Q test were employed to determine differences in individual variables between the 5 groups.

RESULTS: Three post-conditioning patterns were found to provide cardioprotection ($P < 0.05$) compared with R/I without postconditioning. GIR provided the best cardioprotection effect, followed by ER and then GDR. Apoptotic index and serum marker levels were reduced more significantly in GIR than in ER ($P < 0.05$). The enhanced cardioprotection provided by GIR was accompanied with significantly increased levels of P-ERK 1/2 (1.82 ± 0.22 vs. 1.54 ± 0.32 , $P < 0.05$), and lower levels of p-JNK, p38 MAPK, TNF- α , caspase-8, caspase-9 and cytochrome c in the cytoplasm ($P < 0.05$), compared with ER. The infarct size was smaller in the GIR group than in the ER group, but this difference was not significant ($16.30\% \pm 5.22\%$ vs. $20.57\% \pm 6.32\%$, $P < 0.05$). All the measured variables were improved more significantly in the GIR group than in the GDR group ($P < 0.05$).

CONCLUSION: Gradually increased reperfusion in post-conditioning could attenuate reperfusion injury more significantly than routine method, thereby the MAPK pathway plays an important role in this process.

KEY WORDS: Ischemia-reperfusion injury; Postconditioning; Apoptosis

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INTRODUCTION

Rapid and complete reperfusion has been widely adopted in the treatment of patients with acute myocardial infarction (AMI), but this process sometimes can cause severe reperfusion injury.^[1] Since Zhao et al^[2] proposed the concept of ischemic post-conditioning in 2003, many studies have demonstrated that post-conditioning can alleviate reperfusion injuries.^[3-6]

Post-conditioning must be performed strictly according to the protocols, namely post-conditioning algorithms,^[7] or it will lose the cardioprotection effect. To the present, no "ideal" algorithm has been established.^[8,9] The aspects that have been investigated include the interval from the end of ischemia to the application of post-conditioning,^[10-12] the time of reperfusion and ischemia during the post-conditioning,^[13,14] and the number of cycles applied.^[15]

Few studies have focused on the relationship between brief reperfusion and ischemia in transient post-conditioning. Penna et al^[16] compared the effects of modified and routine post-conditioning algorithms, and found that the two algorithms reduced the infarct size and released lactate dehydrogenase. The total time from ischemia to post-conditioning differed between the two algorithms, with the routine algorithm lasting 10 seconds (5 cycles of 10 seconds reperfusion/ischemia) and the modified algorithm lasting 140 seconds (15/20-20/15-25/10-30/5 seconds). In a study of post-conditioning after brain ischemia, Wang et al^[17] discovered that when the time of brief reperfusion was too long and the ischemia time was too short (3 cycles of 60/15 seconds of reperfusion/re-occlusion), the protective effect was attenuated or even lost.

We hypothesized that protection could be increased by gradually lengthening the brief reperfusion time, shortening the ischemia time, and keeping the total reperfusion/ ischemia cycle time fixed. We refer to this new postconditioning algorithm as gradual increased reperfusion (GIR). The models of acute myocardial infarctions were established in the rats to compare the cardioprotection provided by GIR with that from routine postconditioning.

METHODS

This study conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Research Commission on Ethics of the Chinese PLA General Hospital.

Establishment of AMI models

Adult male Sprague-Dawley rats, weighing 200–250 g, were fed with normal diet prior to the experiment. The rats were anesthetized with an initial intraperitoneal injection of sodium pentobarbital (46 mg/kg), and then intubated and ventilated using a rodent respirator (ventilation rate: 52 breaths per minute, tidal volume: 4.0 mL/100 g body weight). A parasternal incision was made to open the left pleural cavity by cutting the left fourth ribs and the intercostal muscle. After pericardiotomy, a 5-0 ligature was placed under the left coronary artery (LCA) by inserting the thread into the left atrium and threading it out from the side of the pulmonary artery cone. Before tying the knot, a balloon (Grip, 3.0*12 mm, Acrostak Corp., Switzerland) connected to a pump full of water at a pressure of 1 ATM was placed into the artery. After the knot was tied, the pressure of the balloon was immediately adjusted to 12 ATM for 45 minutes. After a 45-minute occlusion period, the pressure of the balloon was quickly adjusted to zero for various periods of time, as per the protocols described below (Figure 1). When the rats recovered from anesthesia, the tracheal intubation was removed. The rats were re-anesthetized 24 hours later with an initial intraperitoneal injection of urethane (2 g/kg), and the right carotid artery was cannulated using an arterial catheter connected to a physiograph through a three-way stopcock. Finally, the hearts were excised according to the following procedures: (i) anterior wall tissue was obtained from the left ventricle after 6 hours of reperfusion and kept in a –80 °C freezer until analysis by western blot; (ii) the myocardial tissue was soaked in formalin (10%, pH=7.4) until being used for apoptotic index measurements.

Experimental protocols

Fifty rats were randomized to one of the five groups (Figure 1): (i) the sham group (control group), received a thoracotomy without ischemic treatment; (ii) the reperfusion-injury (R/I) group, received routine ischemic-reperfusion treatment; (iii) the gradually

Figure 1. Experimental protocols demonstrating that GIR was more similar to than either GDR or ER in gradual reperfusion.

decreased reperfusion (GDR) group, received 4 cycles of reperfusion and re-occlusion at the onset of reperfusion with reperfusion/occlusion times of 30/10-25/15-15/25-10/30 seconds (160 seconds for total intervention); (iv) the equal reperfusion (ER) group, received 4 cycles of 20/20 seconds reperfusion/re-occlusion beginning immediately at the onset of reperfusion (total 160 seconds); (v) the gradually increased reperfusion (GIR) group, received 4 cycles of reperfusion/re-occlusion at the onset of reperfusion that consisted of 10/30-15/25-25/15-30/10 seconds (total 160 seconds).

Measurement of hemodynamics

The heart rate and arterial pressure were physiographically monitored through an arterial catheter. Then $+dp/dt$ and $-dp/dt$ were analyzed using physiograph, and the rate-pressure product (RPP) was calculated as the product of the rate and the mean arterial pressure.

Measurement of serum marker release

The serum levels of creatine kinase (CK) and MB isoenzyme of creatine kinase (CK-MB) were analyzed using an automatic biochemistry analyzer.

Detection of apoptotic cells

Apoptotic cells were detected on transverse sections of the left ventricle using a DNA fragmentation detection kit (Roche Corp., Germany) based on the terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) method. The kit was used according to the manufacturer's instructions. The results were quantified as the "apoptotic index": the number of positively stained apoptotic cardiocytes/total number of cardiocytes counted $\times 100\%$.

Western blot analysis

Caspases-8 in the myocardial tissue and the expression of cytochrome c were observed.

Western blot was used as previously described.^[18] In brief, the left ventricular myocardium was homogenized in lysis buffer. After sodium dodecyl sulfate polyacrylamide

gel electrophoresis (SDS-PAGE), the proteins were transferred to a nitrocellulose membrane and incubated with antibodies against phosphorylation of extracellular signal-regulated protein kinase (P-ERK), phosphorylated c-Jun N-terminal kinase (P-JNK), mitogen-activated protein kinase p38 (p38 MAPK), phospho-JNK, tumor necrosis factor- α (TNF- α), caspase-8, and β -actin (all were mouse polyclonal antibodies diluted 1:200, obtained from Santa Cruz Biotechnology, USA) for 6 hours followed by a peroxidase-conjugated secondary antibody. Antigen-antibody complexes were visualized using enhanced chemiluminescence.

Cytosolic and mitochondrial fractions were isolated as described by Li et al.^[18] The tissues were homogenized on ice using a tight-fitting Dounce homogenizer. The homogenates were centrifuged at 2 500 r/min for 10 minutes at 4 °C. Then the supernatants were centrifuged at 14 000 r/min for 25 minutes at 4 °C to obtain the cytosolic fractions (supernatants) and mitochondrial fractions (pellets). After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane and incubated with anti-cytochrome c and β -actin antibodies (mouse polyclonal antibodies diluted 1:200, Santa Cruz Biotechnology, USA) for 6 hours followed by a peroxidase-conjugated secondary antibody. Antigen-antibody complexes were visualized by enhanced chemiluminescence as described above.

Statistical analysis

All values were expressed as mean $\pm$ SE. Data were analyzed using the statistical software package SPSS 13.0 for Windows. One-way analysis of variance and the q test were employed to determine whether there was any significant difference in individual parameters between the groups. In all tests, $P < 0.05$ was considered statistically significant.

RESULTS

Hemodynamic parameters

The hemodynamic data were listed in Table 1.

Table 1. Hemodynamic data obtained 24 hours after reperfusion (mean $\pm$ SD, $n=12$).

Groups	HR (beats/min)	MAP (mmHg)	RPP (1000*mmHg*beats/min)	$+dp/dt$ (mmHg/sec)	$-dp/dt$ (mmHg/sec)
Sham	418.00 $\pm$ 65.93	119.33 $\pm$ 11.21	49.35 $\pm$ 0.74 ^{*Δ}	1019.09 $\pm$ 318.65 ^{*Δ}	752.31 $\pm$ 231.05 ^{*Δ}
I/R	396.17 $\pm$ 62.21	105.95 $\pm$ 31.68	41.97 $\pm$ 1.98 ^{#Δ}	526.37 $\pm$ 123.99 ^{#Δ}	445.44 $\pm$ 79.58 ^{#Δ}
GDR	423.21 $\pm$ 50.00	108.00 $\pm$ 14.32	45.70 $\pm$ 0.72 [*]	721.45 $\pm$ 89.97 [*]	546.23 $\pm$ 100.12 [*]
ER	427.21 $\pm$ 36.03	109.57 $\pm$ 10.36	46.81 $\pm$ 0.37 [*]	855.42 $\pm$ 150.54 [*]	645.90 $\pm$ 132.24 [*]
GIR	396.57 $\pm$ 61.23	123.00 $\pm$ 15.42	48.78 $\pm$ 0.94 ^{*#Δ}	913.24 $\pm$ 63.25 ^{*Δ}	721.45 $\pm$ 110.23 ^{*Δ}

Compared with the R/I group, ^{*} $P < 0.05$; compared with the ER group, [#] $P < 0.05$; compared with the GDR group, ^Δ $P < 0.05$.

Figure 2. Detection of apoptotic cells using TUNEL staining (A), with TUNEL-positive cells dyed green. Apoptotic indexes (B) were lower in the GIR group than those in the R/I, GDR and ER groups. Compared with the R/I group, * $P<0.05$; compared with the ER group, # $P<0.05$; compared with the GDR group, Δ $P<0.05$.

Twenty-four hours after myocardial infarction, no significant differences were observed in heart rate (HR) and mean arterial pressure (MAP) between the groups. Compared with the GDR and ER groups, the GIR group exhibited a significantly higher rate-pressure product (RPP) value ($P<0.05$). In addition, the $+dp/dt$ and $-dp/dt$ levels were much higher in the GIR group than in the GDR group ($P<0.05$).

Serum markers of cardiac damage

The levels of creatine kinase (CK) and creatine kinase-MB (CK-MB) release were significantly reduced in all three post-conditioning groups ($P<0.01$), compared with the R/I group. CK and CK-MB were released less significantly in the GIR group than in the ER group (CK: 251.00 ± 45.16 μL vs. 388.56 ± 75.01 μL , $P<0.05$; CK-MB: 146.00 ± 60.12 μL vs. 291.16 ± 52.41 μL , $P<0.05$) and the GDR group (CK: 251.00 ± 45.16 μL vs. 599.41 ± 63.00 μL , $P<0.05$; CK-MB: 146.00 ± 60.12 μL vs. 406.76 ± 90.01 μL , $P<0.05$).

Cardiocyte apoptosis

In this study, R/I caused a significant increase in the number of TUNEL-positive cells (apoptotic index, $P<0.01$) similar to that in the sham group (Figure 2). The apoptotic index was significantly reduced in the three post-conditioning groups compared with the R/I group ($P<0.01$). Furthermore, the apoptotic index in the GIR group was much lower than that in the ER ($4.32\%\pm1.16\%$ vs. $8.58\%\pm1.12\%$, $P<0.05$) and GDR groups ($4.32\%\pm1.16\%$ vs. $11.34\%\pm2.34\%$, $P<0.05$).

Expression of phosphorylated ERK1/2, p38 and JNK MAPK (P-ERK, P-p38 and P-JNK)

The MAPK family is an important group of signaling molecules including ERK that can inhibit apoptosis

Figure 3. Expression of phosphorylated ERK, p38 and JNK MAPK in all groups. The GIR group had a higher expression of P-ERK and a lower level of P-p38/JNK compared with the R/I, GDR and ER groups. Compared with the R/I group, * $P<0.05$; compared with the ER group, # $P<0.05$; compared with the GDR group, Δ $P<0.05$.

and necrosis as well as the apoptosis promoter p38/JNK. As shown in Figure 3, a significant increase in the expression of P-ERK and a marked decrease in the expression of both P-p38 and P-JNK were detected in all post-conditioning groups compared with the R/I group ($P<0.01$). GIR further increased the expression of P-ERK to a greater extent than the ER (1.82 ± 0.22 vs. 1.54 ± 0.32 , $P<0.05$) and the GDR groups (1.82 ± 0.22 vs. 1.22 ± 0.21 , $P<0.01$). The levels of P-p38 and P-JNK were decreased more significantly in the GIR group than in the ER (P-p38: 0.82 ± 0.26 vs. 1.63 ± 0.24 , $P<0.05$; P-JNK: 0.76 ± 0.28 vs. 1.33 ± 0.21 , $P<0.05$) and GDR groups (P-p38: 0.82 ± 0.26 vs. 1.98 ± 0.21 , $P<0.05$; P-JNK: 0.76 ± 0.28 vs. 1.72 ± 0.24 , $P<0.05$).

Figure 4. Expression of TNF- α and caspase-8 in all groups, with a lower expression of TNF- α and caspase-8 in the GIR group than in the R/I, ER and GDR groups ($P<0.05$); compared the with R/I group, $^*P<0.05$; compared with the ER group, $^{\#}P<0.05$; compared with the GDR group, $^{\Delta}P<0.05$.

Figure 5. Cytosolic cytochrome c expression in all groups. There was a significantly lower expression in the GIR group than in the R/I, GDR and ER groups ($P<0.05$). Compared with the R/I group, $^*P<0.05$; compared with the ER group, $^{\#}P<0.05$; compared with the GDR group, $^{\Delta}P<0.05$.

TNF- α and caspase-8 expression

TNF- α and caspase-8 are essential components of the death receptor apoptotic pathway, so their expression was measured in all groups. The expression of TNF- α and caspase-8 was significantly lower in all post-conditioning groups than in the R/I group ($P<0.01$) (Figure 4). The expression of the two components was significantly lower in the GIR group than in the ER group (TNF- α : 0.62 ± 0.20 vs. 1.00 ± 0.12 , $P<0.05$; caspase-8: 0.61 ± 0.21 vs. 1.00 ± 0.21 , $P<0.05$) and the GDR group (TNF- α : 0.62 ± 0.20 vs. 1.72 ± 0.47 , $P<0.05$; caspase-8: 0.86 ± 0.21 vs. 1.62 ± 0.21 , $P<0.05$).

Expression of cytochrome c (Cyt-c) in the cytosol

Cytochrome c is an important pro-apoptotic factor activating caspase-9. In our study, the post-conditioning groups exhibited a significantly lower Cyt-c expression in the cytosol than the R/I group ($P<0.01$) (Figure 5). Meanwhile, the cytosolic Cyt-c level was lower in the GIR group than in the ER (0.66 ± 0.16 vs. 1.68 ± 0.22 , $P<0.05$) and GDR groups (0.66 ± 0.16 vs. 2.97 ± 1.23 , $P<0.05$).

DISCUSSION

This study showed that GIR can provide better cardioprotection than the other two postconditioning algorithms. Apoptosis and serum marker release reduced to a far greater extent in the GIR group than in the ER group, while P-ERK was present at a higher level and TNF- α , caspase-8 and cytochrome c were expressed at lower levels. GIR provided better protection than GDR for all variables measured.

The better cardioprotection provided by GIR may be attributed to the following reasons. Many studies^[12,19] have shown that ERK1/2 is one of the reperfusion injury survival kinases (RISK) or components of an important post-conditioning pathway. In this study, ERK1/2 was phosphorylated at higher levels in the GIR group than in the ER and GDR groups. Myocardial ischemia/reperfusion has been shown to activate p38/JNK MAPK, resulting in cardiac injury and cell death, most prominently via apoptosis. In their study using neonatal rat cardiocytes, Sun et al^[20] found that hypoxic post-conditioning could inhibit the expression of p38/JNK MAPK, which reduced the expression of TNF- α and Bax, and conversely that anisomycin, an activator of p38/JNK MAPK, could offset this protection. The level of phosphorylation of p38/JNK was lower in the GIR group than in the R/I, ER and GDR groups.

Apoptosis is an important part of reperfusion injury. The death receptor pathway and the mitochondrial pathway are the main pathways leading to cell apoptosis. The death receptor pathway begins with death receptors located in the cell membrane, such as TNF- α receptor-1 and Fas receptor. In this study, the expression of TNF- α and caspase-8 was reduced more significantly in the GIR group than in the ER group. These changes could cause the opening of the mitochondrial permeability transition pore (mPTP), which is associated with apoptotic cell death as it controls the release of many pro-apoptotic factors like cytochrome-c to the cytoplasm.^[21-24] If this occurs, these pro-apoptotic factors could activate many proenzymes,

and this could start the apoptotic cascade and cause the release of molecules such as caspase-9 and caspase-3.

The data showed some differences in the expression of downstream mediators among the GIR, ER and GDR post-conditioning algorithms. The next question is how these different algorithms acted to influence these mediators? At this point, we cannot clearly answer this question, but the gradual change of reperfusion time during post-conditioning is clearly important. In some respects, post-conditioning is a logical extension of gradual reperfusion, which is known to be cardioprotective for reperfusion injury.^[25–27] If a sudden change can cause injury, a gradual change will attenuate the injury by making the process milder. When myocardial cells remain viable after severe ischemia, they need a "warm-up" period to restart their metabolic activity, and post-conditioning leads to a gradual change in the metabolic state. Following this logic, we modified the post-conditioning process so that it mimicked gradual reperfusion. In the ischemia-post-conditioning reperfusion sequences tested in our study, GIR was similar to gradual reperfusion. The gradual change used in this algorithm resulted in better cardioprotection than routine postconditioning as described above.

In the present study, no significant difference was observed in infarct size between GIR and ER. This may be due to the limited sample size or the small improvement of infarct size in the rat model which was difficult to observe. Nevertheless, we still consider that GIR provides better cardioprotection than ER. We conclude that compared with ER, GIR could significantly reduce the apoptosis and necrosis, and markedly improve the function of the left ventricle, and that GIR could improve all the variables as compared with GDR. These findings indicate that the gradual increase of reperfusion time during post-conditioning is associated with greater cardioprotection. Although the results of our study are encouraging it has several limitations including a small sample size and the lack of comparison between GIR and gradual reperfusion.

In summary, the duration of reperfusion and ischemia in post-conditioning algorithms is important. Gradually increased reperfusion is helpful to improve cardioprotection. Further studies are needed to determine the mechanisms of this method.

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Contributors: Zhang GM proposed the review and wrote the paper. All authors contributed to the design and interpretation of the study and to further drafts.

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