

Original Article

Myocardial apoptosis and injury of donor hearts kept in completely beating status with normothermic blood perfusion for transplants

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Abstract: Objective: Normothermic blood perfusion is the developing trend of donor heart preservation. Theoretically, donor hearts preserved in a beating status may be the perfect method to reduce time-dependent ischemic injury, resuscitate marginal hearts expanding the donor pool and potentially improve the function of isolated hearts. In this study, to investigate the protective effect of normothermic blood perfusion, we maintained the donor hearts in a beating status and compared the changes of myocardial apoptosis and injury with standard hypothermic and static storage. Methods: Thirty rat hearts were preserved in static cold storage (Group A, n=10, stored in 4°C histidine-tryptophan-ketoglutarate solution), or in static normothermic blood perfusion (Group B, n=10, perfused with normothermic blood) or in beating status (Group C, n=10, perfused continuously with normothermic blood) for 9 hours. Myocardial injury markers including creatine kinase-MB (CK-MB) and cardiac troponin I (cTnI), myocardial metabolic rate related indicators including Methane Dicarboxylic Aldehyde (MDA) and Adenosine Triphosphate (ATP) were investigated before and after preservation. And also TUNEL staining and mRNA and protein expression of apoptosis markers such as Bax, Bcl-2, Caspase-3 and Cleaved Caspase-3 were used to evaluate the degree of myocardial apoptosis. Results: It is found that the levels of CK-MB and cTnI in Group C were significantly lower than those of Group A and Group B ($P<0.05$). However, there was no significant statistical difference of ATP content among three groups. When compared with Group A and B, the quality of MDA in Group C was obviously lower. In addition, it showed that a remarkable reduction in TUNEL-positive nuclear staining in Group C but higher in other two groups. And inhibited apoptosis was also confirmed by the results of mRNA and protein expression of apoptosis markers including Bax and Bcl-2. Conclusions: It is an effective and appropriate approach to preserve donor hearts with continuous and normothermic blood perfusion as to keep them in beating status in heart transplantation, which could reduce myocardial ischemic damage and cardiac apoptosis in long-term preservation.

Keywords: Normothermic blood perfusion, beating status, heart transplant, myocardial injury, apoptosis

Introduction

At present, heart transplant is suitable for patients with severe heart failure which has no other optional treatment, and remains the gold standard in the treatment of end-stage heart failure [1]. However, cardiac allograft failure in heart transplant continues to be a severe problem. One cause of graft dysfunction is suboptimal preservation, resulting in cardiac ischemia, cardiac apoptosis and subsequent cardiac necrosis [2]. Method of hypothermic preservation could significantly reduce the metabolic

demands; however, it is not powerful to inhibit myocardial injury and to provide warm reperfusion [3]. This method would cause donor hearts suffered from cold ischemia phase of ischemia hypoxia injury and subsequent ischemia-reperfusion injury, which contributes to the risk of primary graft dysfunction [4]. As a result, how to extend the life of the hearts and improve the quality of donor hearts has been one of research subject in medical science. Therefore, more and more studies have been developed to solve the problem how to improve the protective effects of donor hearts storage [5-7].

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Lichtenstein et al firstly found that it was feasible that keeping the donor hearts in a beating and normothermia status in 1991, and then more and more researchers utilized this technique to continue related studies [8-10]. In recent years, it is suggested that blood perfusion solution might be the most appropriate approach to keep the donor hearts in beating status with continuous perfusion, which could extend the time of preservation and perfect the restoration of cardiac function [11-13].

In this study, we hypothesized that donor hearts perfused with normothermic blood solution and kept in completely beating status in the process of heart transplant, which may reduce the myocardial injury and bring better effectiveness. Therefore, we maintained the donor hearts in a beating status and compared the changes of myocardial apoptosis and injury with standard hypothermic and static storage.

Materials and methods

Materials and animal grouping

Primary antibodies rabbit anti-Bax (#2772), rabbit anti-Bcl-2 (#2876), rabbit anti-Caspase-3 (#9662) and rabbit anti-Cleaved Caspase-3 (#9661) were bought from Cell Signaling Technology, Inc. (CST, Danvers, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (No. MB001) was obtained from Bioworld Technology, Inc (St. Louis Park, MN, USA). The bicinchoninic acid (BCA) protein assay kit (#23227) was purchased from Thermo Scientific Pierce Biotechnology, Inc. (Rockford, IL, USA). TRIzol® Reagent (#15596018) was bought from Invitrogen Life Technologies (Carlsbad, CA, USA). This study was approved by the Ethics Committee of Renmin Hospital, Hubei University of Medicine (Shiyan, China). All procedures in the present study were conducted in accordance with the Guide for the Local Care and Use of Laboratory Animals and all surgeries and subsequent experiments were blinded. Thirty male Sprague Dawley (SD) rats weighted from 200 g to 250 g were randomly divided into three groups including Group A, B and C, which were bought from the Experimental Animal Center of Hubei Province (Wuhan, China). Group A (n=10) was stopped with and kept in 4°C histidine-tryptophan-ketoglutarate solution to preserve the donor hearts in a static status for 9

hours. Group B (n=10) was stopped with 4°C histidine-tryptophan-ketoglutarate solution and perfused with 37°C continuous oxygenation blood to preserve the donor hearts for 9 hours. Group C (n=10) was perfused with 37°C continuous oxygenation blood to preserve the donor hearts in beating status for 9 hours.

Donor heart procurement and preservation

Animal experiments in the present study were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. Briefly, SD rats were anaesthetized with 1% pentobarbital sodium (30 mg/kg) by intraperitoneal injection and 1ml of 1% lidocaine by local injection, and then tracheal intubation was performed gently. Thoracotomy was performed to isolate the great vessels via median sternotomy. Perfusion solution of autologous blood was harvested before the donor hearts were harvested in Group B and C. Leukocyte-depleting filters were used to deplete blood leukocytes and reduce leukocyte-related myocardial injury. Filtered blood which was added with insulin (All Medicine, Xuzhou, China), glucose (Jingxi Pharmaceutical Group, Xian, China), penicillin (Lukang Drugs Group, Jining, China), fructose diphosphate (Yangtze River Pharmaceutical Group), methylprednisolone (Tiandi Pharmaceutical Group, Xingyi, China) and heparin (Science Sun Pharmaceutical Group, Beijing, China) was poured into the venous reservoir for oxygenated perfusion. To keep perfusion continuous in Group B and C, a tube was used as the perfusion cannula of the aorta with one inlet and one outlet. The inlet was connected to arterial blood outlet of membrane oxygenator, the outlet was connected to the aortic cannula with right brachiocephalic artery for perfusion. The distal aorta arch was cross-clamped and then blood perfusion began once the inferior vena cava and the right pulmonary veins were cut off. And Donor hearts of Group B were stopped by pouring with 4°C histidine-tryptophan-ketoglutarate solution and then cut off promptly to be perfused with 37°C continuous oxygenation blood for 9 hours. The hearts in Group C were cut off promptly to be perfused with 37°C continuous oxygenation blood to preserve them in beating status for 9 hours. However, the donor hearts of Group A were stopped by pouring cold histidine-tryptophan-

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ketoglutarate solution and cut off promptly to storage in 4°C histidine-tryptophan-ketoglutarate solution for 9 hours once the aorta arch was cross-clamped.

Serum creatine kinase-MB (CK-MB) and Cardiac Troponin I (cTnI) levels

According the instructions of manufacturer (Sigma-Aldrich, St. Louis, USA), serum CK-MB and cTnI among three groups were investigated after the hearts preserved 3 hours, 6 hours and 9 hours.

Myocardial malondialdehyde (MDA) and adenosine triphosphate (ATP) content

Myocardial tissue homogenate was harvested from 100 mg myocardial tissue and cracked fully by adding with collagenase. According the manufacturer's instructions of detection kits (Beyotime Institute of Biotechnology, Wuhan, China), MDA and ATP concentration were detected.

Terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL)

Rat hearts among three groups were harvested and then perfused with 0.1 ml phosphate-buffered saline (PBS) via left coronary artery. After that, all samples were fixed by 4% paraformaldehyde in PBS (pH 7.4) at room temperature. When fixed for 24 hours, the heart tissues were embedded in paraffin. TUNEL staining was followed with the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Five 6mm thickness sections were selected from each sample. Cardiac tissue was specifically labeled with the mouse monoclonal antibody anti- α -actinin (ab9465, Abcam, Cambridge, MA, USA); in addition, the nuclei were stained in blue by DAPI (Vector Laboratories Inc., Burlingame, CA, USA). Apoptotic index was estimated in a blinded manner, which was measured by the ratio of the number of positively stained nuclei in total number of nuclei counted.

Reverse transcription-polymerase chain reaction

According to the manufacturer's instructions of TRIzol® Reagent, total mRNA was extracted from myocardium tissue. The cDNA was synthesized with oligo (dT) primers according to the

instructions of the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). SYBR green (Roche, Basel, Switzerland) was used to confirm selected gene differences and the results were normalized against gene expression of GAPDH. The sequences of primers used in the present study were shown below: Bax forward: 5'-GACACCTGAGCTGACCTTGG-3'; Bax reverse: 5'-GAGGAAGTCCAGTGTCCAGC-3' and Bcl-2 forward: 5'-CTGGTGGACAACATCGCTCTG-3'; Bcl-2 reverse: 5'-GGTCTGCTGACCTCACTTGTG-3'.

Western blot

Protein extracted from rat heart tissues which was lysed in radioimmunoprecipitation lysis buffer (Cell Signaling Technology, Danvers, MA, USA) was used for SDS-PAGE (Life Technologies, California, USA). The proteins were subsequently transferred to nitrocellulose membranes in the cold room and blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST; Cell Signaling Technology, Inc.) for 90 min at room temperature. Membranes were respectively incubated with primary antibodies anti-Bax, anti-Bcl-2, anti-Caspase-3 and anti-Cleaved Caspase-3 overnight in the cold room. The next day, the membranes were washed with 1×TBS with Tween-20 (TBST) for 15 min, and then incubated with horseradish peroxidase labeled polyclonal mouse anti-rabbit antibody (1:2000) and anti-avidin antibodies (1:1000) (Jackson ImmunoResearch, West Grove, PA, USA) for 60 min at room temperature. After washed for 3 times with TBST, the film was incubated in 10mL LumiGLO solution (Cell Signaling Technology, Danvers, MA, USA) for 1 min. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody was used as a loading control. And an automatic image analyzer was used to measure gray scale values of the purpose of proteins.

Statistical analysis

Values are expressed as the mean \pm standard error of the mean (SE). Comparisons between three groups were performed using one-way analysis of variance using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). However, comparisons between two groups were performed by using t-test. $P < 0.05$ was considered to indicate a statistically significant difference between values.

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Table 1. Serum CK-MB and cTnI levels among three groups

Parameters	Time points	Group A	Group B	Group C
CK-MB (ng/ml)	0 hours	4.63±1.02	4.73±1.06	4.65±1.06
	after 3 hours	14.86±1.03	11.91±1.24*	9.65±1.05*.#
	after 6 hours	24.28±1.11	19.91±1.06*	15.98±1.14*.#
	after 9 hours	30.16±1.52	26.46±1.03*	23.56±1.17*.#
cTnI (ng/ml)	0 hours	0.35±0.07	0.36±0.11	0.36±0.11
	after 3 hours	0.63±0.06	0.56±0.07*	0.48±0.05*.#
	after 6 hours	4.68±0.39	3.82±0.37*	3.26±0.42*.#
	after 9 hours	6.88±0.51	5.90±0.52*	5.20±0.55*.#

*Compared with Group A, $P<0.05$; #Compared with Group B, $P<0.05$.

Table 2. ATP and MDA contents among three groups

Parameters	Group A	Group B	Group C
ATP (nmol/mg pro)	1.02±0.02	1.02±0.03*	1.01±0.02*.#
MDA (nmol/mg pro)	17.34±0.87	15.10±0.79*	11.92±0.83*.#

*Compared with Group A, $P<0.05$; #Compared with Group B, $P<0.05$.

Results

Comparison of serum creatine kinase-MB (CK-MB) and cardiac troponin I (cTnI) levels

Among three groups, it showed that the levels of CK-MB and cTnI displayed a remarkable increasing trend along with the preservation time (**Table 1**). It was found that the levels of serum CK-MB and cTnI between Group B and Group C were lower than those of Group A, and also the CK-MB and cTnI levels in Group C were lower than those of Group B ($P<0.05$). The above data indicate that when compared with standard hypothermic and static storage, completely beating status with normothermic blood perfusion could significantly reduce myocardial injury.

Comparison of MDA and ATP content

As **Table 2** showed after preserved 9 hours, ATP content of donor hearts of Group A and Group B were slightly lower than that of Group C; however, there was no statistical difference ($P>0.05$). However, when compared to Group A and Group B, MDA content of Group C was lower ($P<0.05$). These results might indicate that compared with standard hypothermic and static storage, the donor hearts perfused with normothermic blood solution and kept in a completely beating status exerts a slightly high energy requirement role. And the method of completely beating status with normothermic

blood perfusion in heart transplant could reduce oxidative stress reaction and oxygen free radical damage.

Myocardial apoptosis between three groups

Compared to Group A and Group B, a significant decrease in TUNEL-positive nuclear staining in left ventricular myocardium of rat heart was observed in Group C ($P<0.05$) (**Figure 1A, 1B**). The levels of apoptosis were dramatically higher in Group A and Group B than Group C, as assessed by detecting mRNA levels of Bax and Bcl-2 and protein expressions of Bax, Bcl-2, Caspase-3 and

Cleaved Caspase-3 ($P<0.05$) (**Figures 2, 3A and 3B**). These data indicate that completely beating status with normothermic blood perfusion inhibits myocardial apoptosis.

Discussion

With the development of scientific and technological factors, the methods of graft preservation are improved significantly and the availability of the donor hearts is also increased. Due to the duration of cold storage is still limited to 4 to 6 hours, during which period myocardial injury will occur in to more or less degree [4]. Thus, numerous methods, from modified preservation solution to preconditioning and perfusion preservation, have been studied, and then gradually focused on the approaches of continuous perfusion and keeping donor hearts in a beating status during the graft preservation to reduce the myocardial injury [11-13]. In this study, we found that compared with static cold storage and static continuous perfusion storage, the beating hearts preservation could effectively reduce myocardial injury and cardiac apoptosis in the long-term preservation.

On the biology markers of myocardial damage, serum Creatine Kinase-MB (CK-MB) and Cardiac Troponin I (cTnI) are most commonly detected, and these two kinds of biochemical markers of myocardial damage have significant specificity and sensitivity [14-16]. In our study,

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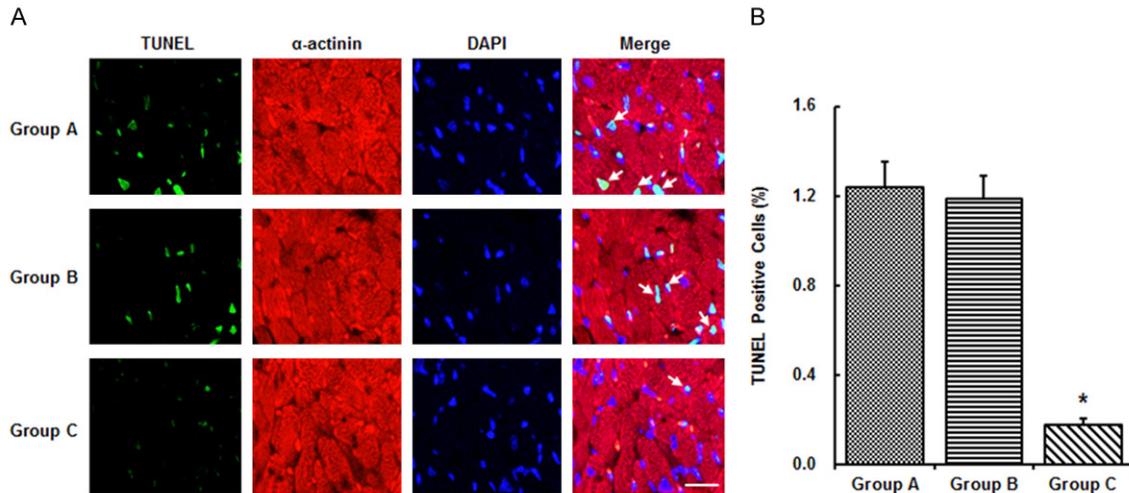


Figure 1. The TUNEL staining showed that the apoptosis rate is significantly decreased in beating status (Group C). A. TUNEL staining, green: TUNEL, red: α-actinin (cardiomyocytes), blue: DAPI (nucleus). B. The results of quantification for TUNEL staining. * $P < 0.05$ v.s. Group A and Group B.

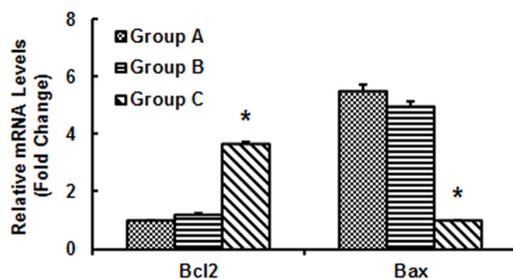


Figure 2. The mRNA expression levels of Bcl2 and Bax. The results of Real-Time PCR demonstrated that the mRNA levels of Bcl2 were increased, but Bax were reduced significantly in beating status (Group C) compared with static cold storage (Group A) and static normothermic blood perfusion (Group B). * $P < 0.05$ v.s. Group A and Group B.

it showed that not only stopped but also beating perfusion preservation methods all led to the increase of CK-MB and cTnI levels along with the preservation time. However, in the normothermic perfusion beating group, the serum CK-MB and cTnI were slightest. These data might reflect that compared to static and cold storage methods, the donor hearts preserved in the beating status perfused with normothermic blood solution could effectively reduce myocardial injury. In addition, ATP contents of Group A and Group B were slightly lower than that of Group C, but, there was no statistical difference. However, MDA content of Group A and Group B were obviously higher than that of Group C. These indicated that compared with

standard hypothermic and static storage, completely beating status with normothermic blood perfusion did not obviously increase energy requirement. MDA is the end product of lipid peroxidation metabolism, and its content could directly reflect the speed and strength of lipid peroxidation [17-19]. Therefore, our data indicated that the method of completely beating status perfused with normothermic blood solution could reduce oxidative stress reaction and oxygen free radical damage. The mechanism of protective role of this method might be related to providing the metabolic substrate, eliminating the metabolic wasters, reducing the oxygen free radical production, avoiding the influence of hypothermia and rewarming from preservation to implantation and improving the microvascular blood flow and so on [20].

Completely beating status with normothermic blood perfusion, a marked decrease in TUNEL-positive nuclear staining in the sectioned left ventricular myocardium was observed when compared with the methods of standard hypothermic and static storage. Furthermore, the levels of apoptosis, as assessed by detecting mRNA levels of Bax and Bcl-2 and protein expressions of Bax, Bcl-2, Caspase-3 and Cleaved Caspase-3 were significantly inhibited in donor hearts which kept in a completely beating status by perfused with normothermic blood solution. Hence, this method could effectively inhibit myocardial apoptosis and then reduce myocardial injury. The mechanism of

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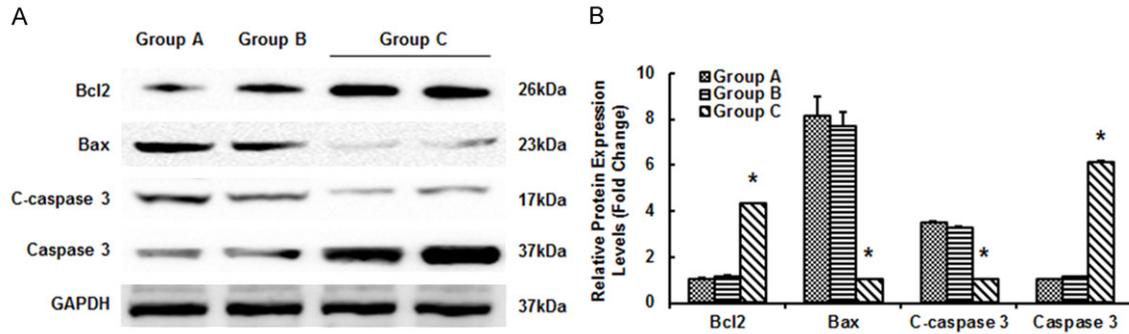


Figure 3. The protein expression levels of apoptosis molecules. The results of Western blotting demonstrated that the protein levels of Bcl2 and Caspase 3 were increased, but Bax and cleaved-caspase 3 were reduced significantly in beating status (Group C) compared with static cold storage (Group A) and static normothermic blood perfusion (Group B). A. The represented western blot. B. The quantification results for western blot bands in (A). * $P < 0.05$ v.s. Group A and Group B.

inhibiting myocardial apoptosis might be related to improving myocardial tissue perfusion and avoiding the undesirable influence of myocardial ischemic changes. With normothermic blood perfusion, the beating donor hearts were preserved for 9 hours. Compared with static cold storage, these results indicate that this approach may be more suitable for long-term donor heart preservation. From the myocardial injury to the cardiac apoptosis, all supported the advantage of the method of keeping the donor hearts in a completely beating status by perfused with normothermic blood solution in the improvement of heart preservation in the process of hearts transplantation in the future.

However, this study still has some limitation, for example, the sample size is small and the data should be confirmed by a larger sample and larger animal experiment. In present study, we only studied myocardial injury and cardiac apoptosis, and we have not done heart transplantation by using these donor hearts, therefore, other parameters such as cardiac function, the efficacy of heart implantation such as early performance of cardiac function, toxic metabolite removal and immune rejection with the host or other clinical events also need to be tested. In addition, the long-term efficacy of completely beating status with normothermic blood perfusion should be confirmed in the future.

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Disclosure of conflict of interest

None.

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