Apoptosis amelioration through hypothermic reperfusion in heart transplant

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ABSTRACT

Objective: To investigate the effects of two different sets of graft temperature during perfusion on myocardial protection in the immediate post transplantation period in rats. **Materials and Methods:** Rats grouped into: Sham and two study groups, which include two set groups of heterotopic heart transplant perfused at two different temperature set. The studied groups underwent cuff method cervical heterotopic heart transplant. Myocardial cell injury and stress were assessed by measuring: Cardiac troponin-I, score of tissue injury, reactive oxygen species (ROS) and nitrogen, caspase 3 enzyme, and degree of myocardial apoptosis. The low set temperature (18°C) significantly reduced myocardial cell injury compared to 37°C reperfusion temperature. This cytoprotective effect of low temperature reperfusion phase was addressed by significant reduction in ROS and nitrogen and inflammatory cytokines, caspase 3, and myocardial apoptosis. **Conclusion**: Hypothermic reperfusion phase exerts cytoprotection in heart transplant through down regulation of oxygen, nitrogen reactive species, and inhibition of apoptosis.

Key words: Cytoprotection, hypothermic reperfusion phase, heart transplant

INTRODUCTION

Global myocardial ischemia is characterized by a series of conditions where the entire myocardium experiences a reduction or complete depletion in its supply of oxygen due to a lack of coronary arterial flow.[1] Global myocardial ischemia reperfusion injury (GMIRI) refers to cardiac tissue damage resulting from blood supply being returned to the tissue following a period of such ischemia.[2,3] Ischemia and reperfusion thus denote distinct phases of cardiac injury, with ischemia described as a situation where myocytes experience

adenosine triphosphate (ATP) depletion, acidosis, and lactate accumulation, and reperfusion involving the production of nitrogen and oxygen radicals.[1] The resulting damage incurred by cardiac tissue includes, but is not limited to reduced cell viability, increased evidence of cell necrosis, and apoptosis.[2,4] Overall, the structural and metabolic changes may be mild and thus be considered as reversible post-ischemic organ dysfunction, or they may be severe, leading to irreversible tissue damage.[4]

A focus of several recent research studies shows cardiac allografts to be naturally susceptible to ischemia and reperfusion injury during transplantation.[3] Furthermore, GMIRI is often the reason for inflammatory reactions following heart transplantation.[1] This remarkable inflammatory response is mediated by a number of cytokines, chemokines, adhesion molecules, transcription factors, and other proinflammatory compounds of the extracellular matrix.[5] This can have deleterious effects on the acute as well as long-term function and can result due to a plethora of cardiovascular complications.

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As a result, the control of such inflammatory reactions can have a profoundly positive influence in the outcome of cardiac transplantation.[5]

Research in the field of anti-inflammatory treatment strategies for GMIRI is in the early stages of development. Studies have shown that perfusing transplanted hearts at low temperatures is cardioprotective during the ischemic phase.[2] Though the exact mechanism by which inflammation is induced in GMIRI is a current topic of investigation, this paper will explore the cardioprotective effects of graded hypothermia against myocardial inflammation during the reperfusion phase in GMIRI.

Objective

This study is designed to assess the myocardial cytoprotective effects of hypothermic reperfusion (HR) phase after heart transplant.

MATERIALS AND METHODS

Study groups

Adult male Albino rats weighing 250-300 g with age from 4 to 5 months were housed in the animal shelter in a temperature-controlled (25°C) room with alternating 12-h light/12-h dark cycles. They were allowed free access to water and a chow diet until the start of the experiments. The Institutional Animal Care and Use Committee of Howard University reviewed and approved the experimental design of all animal experiments. After the 1st week of acclimatization, the rats were randomized into three groups. There were four animals in each group, the groups included:

- Sham group: A group in which the animals received anesthetic medications and cervical incisions.
- Normothermic reperfusion (NR) phase group: The animals underwent heterotopic cervical heart transplants with reperfusion phase temperature of 37°C.
- HR phase group: The animals underwent heterotopic cervical heart transplants with reperfusion phase temperature of 18°C.

Anesthesia and surgical procedure

Anesthesia

Intraperitoneal injections of a mixture of ketamine and xylazine were given to the rats in a dose of 100 and 10 mg/kg, respectively in order to anesthetize them and render them unconscious within 5-10 min.

Surgical operation

A cervical heterotopic heart transplantation (HHT) was carried out by using the cuff method.[6] Animals were intraperitoneally anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine.[7]

Donor operation

A transverse abdominal incision was performed. The viscera were slightly retracted to the left side, and 100 U of heparin was injected through the infrahepatic inferior vena cava (IVC). Approximately 3 min later, the thorax was opened through left and right dorsolateral incisions with a pair of rigid scissors, and the diaphragm was separated from the anterior chest wall at the same time. The right superior vena cava (SVC) was ligated and cut off at the distal side of the ligation with a segment of the suture left. The first branch of the aorta was ligated with a 6-0 silk suture, and the suture was retained to the first branch of the aorta. After cutting the aorta at the site distally close to its first branch, the heart was gently pulled upward by the silk sutures, which were attached to the right SVC and the first branch of the aorta. Retraction was maintained with forceps that were fixed to the corkboard. The left SVC, pulmonary arteries, pulmonary veins, and azygus vein were then ligated together with a 4-0 silk suture. Then, the heart was harvested from the donor and stored in a cold lactated Ringer's solution cooled to 4°C.

Recipient operation

A right anterolateral incision parallel to the neck was made. The jugular vein was isolated from its incoming branches. An 18-gauge cuff was sleeved, everted, and secured with a circumferential 6-0 silk suture using the conventional method that was previously reported. An 18-gauge cuff was always attached in this HHT series. The common carotid artery was ligated as far away from the subclavian artery as possible, and the manipulation was very gentle in order to prevent the injury of the endothelium and the formation of a thrombosis. The cuffing techniques of the artery were the same as those used with the jugular vein. After making a stay suture in the posterior wall of the right SVC of the graft heart, the graft was gently slipped over the cuff that had been fixed to the jugular vein of the recipient, and secured with a circumferential 6-0 silk suture. The anastomosis method between the donor aorta and the common carotid artery of recipient was the same as that used for the vein.

Controlling heart rate

Ventricular pacing was inserted to control rate of the transplanted heart (Medtronic model 5320 rapid atrial pulse generator) at a rate of 450 beats per min (bpm).

Controlling reperfusion temperature

The transplanted heart left open without skin closure and the animal maintained in sleep through frequent ketamine injection half of the initial dose. Controlled temperature normal saline (0.9%), 37 and 18°C for normothermic and hypothermic studied groups, respectively. Allowed to dripping frequently over the transplanted heart and its temperature is assessed by temperature probe beneath heart (CyQ 111 Precision Animal

Temperature Thermometer), a final step was removing all the bulldog clamps and making sure that the beating graft was working well with no congestion.

Determination of degree of myocardial injury

The cardiac troponin-I (cTnI)[7] and myocardial tumor necrosis factor alpha (TNFα)[7] in rat plasma were measured using the enzyme-linked immunosorbent assay (ELISA) kits.

Myocardial histopathology

After 3 h of HHT, the hearts were excised. The process of the slide preparation for the histopathological examination started with tissue fixation by 10% formalin, paraffin embedding, and staining with hematoxylin and eosin (H and E). Myocardial injury at the histopathological level was assessed based on the morphological criteria[6] and scored as follows: Score 0, zero damage; score 1 (mild), interstitial edema and localized necrosis; score 2 (moderate), widespread myocardial cell swelling and necrosis; score 3 (severe), necrosis with contraction bands, neutrophil infiltration and compressed capillaries; and score 4 (highly severe), diffuse necrosis with contraction bands, neutrophil infiltration, compressed capillaries, and hemorrhage.

1. Apoptosis detection using terminal transferase and biotin-16-dUTP (TUNEL fluorescent method). This protocol is used for detection and quantification of apoptosis (programmed cell death) at single cell level, based on labeling of deoxyribonucleic acid (DNA) strand breaks (TUNEL technology). Cleavage of genomic DNA during apoptosis may yield double stranded as well as single strand breaks ("nicks"), which can be identified by labeling free 3'-OH terminal with modified nucleotides in an enzymatic reaction. Positive controls: 1) Incubate sections with DNase I (3,000 U/ml in 50 mm Tris-HCl, pH 7.5, 1 mg/ml bovine serum albumin (BSA)) for 10 min at 15-25°C to induce DNA strand breaks, prior to labeling procedure. 2) Using known positive control is an alternative. Negative control: Incubate sections with label solution only (without terminal transferase) instead of TUNEL reaction mixture. Solutions and reagents: These solutions and all the above procedure were done according to the protocol of life science products and services company (IHC WORLD USA).

Procedure

A-Deparaffinize sections in two changes of xylene for 5 min each, and hydrate with two changes of 100% ethanol for 3 min each, and 95% ethanol for 1 min. B-Rinse in distilled water. C-Pretreatment: Use proteinase K digestion method. D-Note: For frozen sections or culture cells grown on slides, incubate with 0.2% Triton X-100 in phosphate buffered saline (PBS)-Tween for 30 min is required. Rinse sections in two changes of PBS-Tween 20, 2 min each. Preincubation: Incubate sections in TdT reaction buffer for 10 min. E-TdT

reaction: Incubate sections in TdT reaction mixture for 1-2 h at 37-40°C in humidified chamber. F-Stop reaction: Rinse sections in stop wash buffer for 10 min. G-Rinse in PBS-Tween 20 for 3 × 2 min. H-Detection: Incubate sections with fluorescein isothiocuanate (FITC)-avidin D in PBS for 30 min at room temperature. I-Rinse in PBS-Tween 20 for 3 × 2 min. J-Counterstain with propidium iodide (PI) or DAPI for 20 min. K-Rinse in PBS for 5 min. L-Mount sections with anti-fading mounting medium. M-Staining pattern: Nuclear fluorescent green.[8]

Determination of rat myocardial caspase 3 activity

Myocardial caspase 3 was assayed by using a caspase 3 colorimetric assay kit (Chemicon International) according to the manufacturer's protocol. The results were measured as amount of fold increment in this enzyme activity in the three studied groups.[9]

Reactive oxygen species assay

They are measured in the myocardial rats through oxidative stress measurement: Glutathione (GSH) myocardial level was measured using Quantichrom TM glutathione assay kit (from BioAssay Systems, USA). Malondialdehyde (MDA), the end product of lipid peroxidation, was analyzed according to the method of Buege and Aust[2] which based on the reaction of MDA with thiobarbituric acid (TBA) to form MDA-TBA complex, a red chromophore, which can be quantitated spectrophotometrically according to this method.

Reactive nitrogen species levels in rats' myocardial tissue

NOx (nitrite and nitrate, stable metabolites of NO) quantity in supernatants was determined by the Griess reaction and assayed utilizing a NOx concentration assay kit (R and D Systems). Nitrotyrosine is represented the *in vivo* ONOO⁻. The concentration of nitrotyrosine in rat cardiac tissue homogenate was determined via ELISA kit (Cell Sciences Inc., Canton, MA, USA), as previously described (Fan *et al*., 2005). In the homogenized myocardium and processed according to kit recommendations.

Statistical analysis

Statistical analysis was performed using ANOVA and Bonferroni correction for *post hoc* Student's tests, and a variable is considered significant if $P < 0.05$.

RESULTS

Rats with HR have decreased myocardial injury markers after 3 h of global IR injury:
1. The levels of cTnI (ng/ml) and TNFα were downregulated in the HR group compared with the normothermic group [Tables 1 and 2]. There are significant differences in cTnI (ng/ml) levels in rat plasma between the studied groups.

In rats with HR, the cTnI (ng/ml) in the rat plasma levels has an average of 4.26 ± 0.10, while the normothermic perfusion group has an average of 9.52 ± 0.24, and the sham group has an average of 1.36 ± 0.4.

TNFα (pg/ml) in myocardial rats with HR has an average of 4.79 ± 0.22, while the normothermic perfusion group has an average of 7.91 ± 0.28, and the sham group has an average of 1.01 ± 0.35 and it is significantly down regulated in HR group. As shown in Tables 1 and 2.

Tissue score of myocardial injury

The tissue score of myocardial injury was significantly decreased in the HR group compared with the normothermic group (6.38 ± 0.23 vs 6.38 ± 0.23, respectively) [Table 3].

HR decreased myocardial apoptosis after 3 h of global IR

In HR group, tissue sections stained with TUNEL stain shows significant reduction in apoptotic myocardial cells compared to NR. As in Figure 1a-d which shows average apoptic bodies seen in 10 fields for each tissue section in the three studied groups.

ROS in myocardium

After 3 h of reperfusion, the myocardium shows marked elevation in MDA and reduction in GSH compared to sham group (0.39 vs 0. 0.1 μmol/g, $P < 0.05$ and 1.07 vs 3.8 μmol/g, $P < 0.05$, respectively for MDA and GSH). In HR group, the myocardium shows significantly less increment in MDA, and significantly less decrement in GSH level compared to NR group (0.2 vs 0.39 μmol/g, $P < 0.05$ and 2.37 vs 1.07 μmol/g, $P < 0.05$, respectively for MDA and GSH). As shown in Figure 2a and b.

RNS in myocardium

HHT associated global IR induces significant elevation in NOx (nitrate/nitrite) and nitrotyrosine levels compared to sham group, (2.05 vs 0.13 mmol/g, $P < 0.05$ and 3.5 vs 0.06 nmol/g, $P < 0.05$, respectively for NOx and nitrotyrosine). In HR group the myocardium shows significant reduction in NOx and nitrotyrosine levels compared to NR group (1.07 vs 2.05 mmol/g, $P < 0.05$ and 3.5 vs 2.02 nmol/g, $P < 0.05$, respectively for NOx and nitrotyrosine). As shown in Figure 3a and b.

HR reduced myocardial expression of caspase 3 in HR group was significantly decreased compared with the normothermic group (2.4- vs 3.6-fold of elevation in regards to sham, respectively) [Figure 4].

DISCUSSION

Heart transplantation is considered as a process with global ischemia which is the time gap after donor heart removal and just before completion of transplantation to enter in reperfusion state. Reperfusion process is considered a trigger for many inflammatory processes that involved cells, chemokines, and cytokines; and these events are associated with different grades of myocardial injury that will end in some degree of myocardial dysfunction.[10]

The very early myocardial injury after reperfusion is usually considered within the first 3 h and it will be the determinant
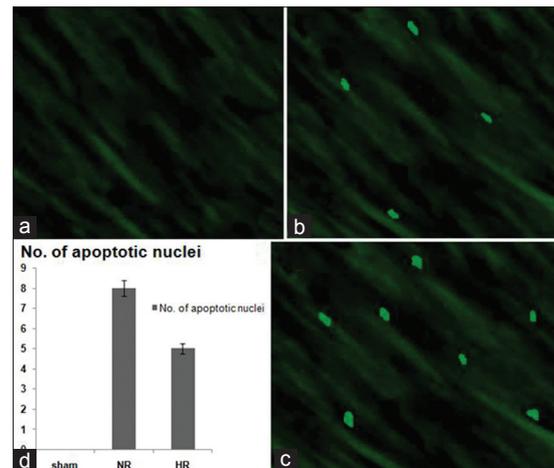
Table 1: Cardiac troponin-I in rat plasma cTnI (ng/ml)

Groups	cTnI (ng/ml)	P value
Sham	1.01±0.35	
Normothermic perfusion	7.91±0.28	<0.05*
Hypothermic perfusion	4.79±0.22	<0.05**

*Vs sham group, **vs normothermic perfusion

Table 2: TNFα in myocardial tissue in different groups

Groups	Cardiac TNF (pg/ml)	P value
Sham	175.93±8.34	
Normothermic perfusion	1200.56±29.41	<0.05*
Hypothermic perfusion	525±21.49	<0.05**

TNFα = Tumor necrosis factor alpha, *Vs. sham group, **vs control group

Table 3: Myocardial injury score

Groups	Total severity score of myocardial tissue injury	P value
Sham	1.00±0.14	
Normothermic reperfusion	6.38±0.23	<0.05*
Hypothermic reperfusion	6.38±0.23	<0.05**

*vs sham group, **vs control group

Figure 1: Apoptotic bodies were detected by fluorescent terminal transferase and biotin-16-dUTP (TUNEL) staining, only apoptic bodies visualized and stained green, (a) Sham group, (b) Hypothermic reperfusion (HR) group, (c) Normothermic repurfusion (NR) group, (d) Average no. of apoptic bodies in all groups. $P < 0.05$ HR group vs NR
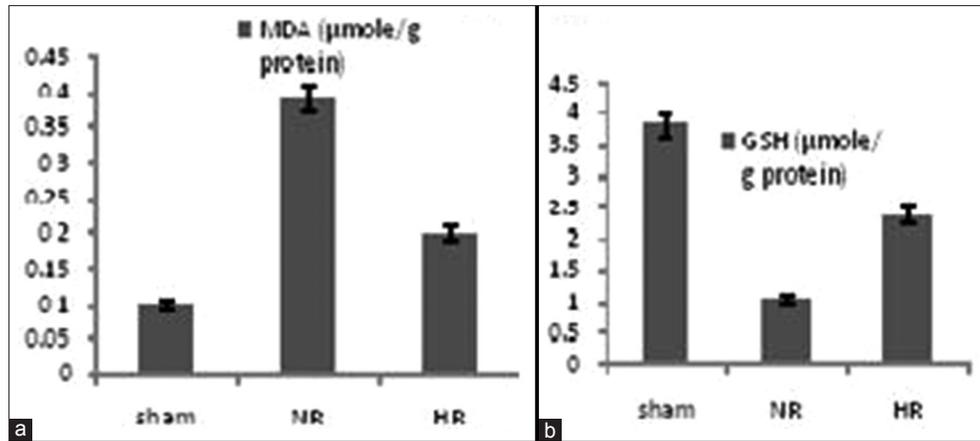
Figure 2: Myocardial levels of (a) malondialdehyde (MDA) and (b) glutathione (GSH) in all groups. *P* < 0.05 HR group vs NR
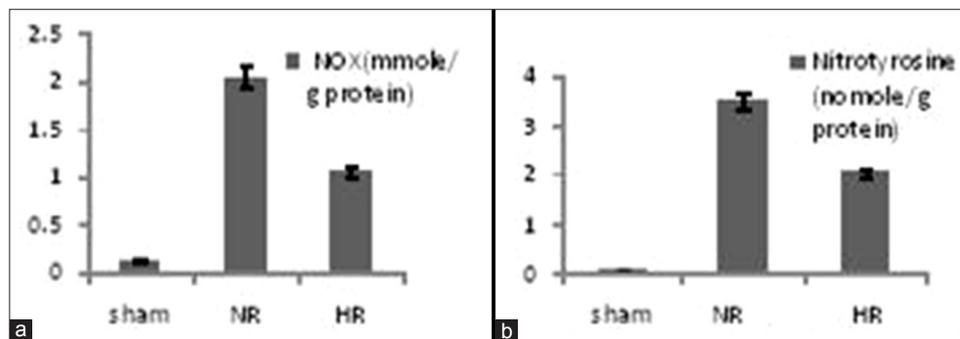

Figure 3: Myocardial levels of (a) NOx and (b) nitrotyrosine, in all groups. *P* < 0.05 HR group vs NR
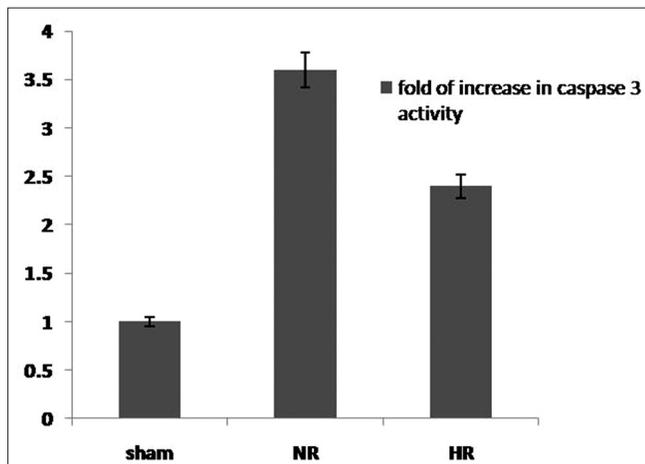

Figure 4: Quantitative analysis of caspase 3 activity in the three groups. *P* < 0.05 HR group vs NR

of subsequent extension and augmentation of further injury and dysfunction.[11] In this study, we addressed this early myocardial injury expressed by increased score of myocardial tissue injury and raised cardiac troponin and is associated with significant elevation of TNFα, and global myocardial reperfusion would induce oxidative stress and release of oxygen free radicals and RNS. These facts were already concluded in previous studies,[12] these reactive species were considered the early chemicals that induce myocardial cell injury after starting the reperfusion stage, and in this study this stage is also associated with significant increase in myocardial expression of caspase 3 enzyme which is the initial signal for apoptotic process. And in this research work in NR, apoptotic bodies were significantly formed, the cellular evidence of myocardial cell death after reperfusion. The levels of cTnI (ng/ml) and TNFα were downregulated in the HR group compared with the normothermic group. Additionally the tissue score of myocardial injury was significantly decreased in the HR group compared with the normothermic group. Myocardial reperfusion in low temperature would induce less expression of the injurious reactive oxygen and RNS and so less myocardial tissue stress. It had been widely recognized that, except for ROS, RNS plays an important role in mediating myocardial apoptosis during IR.[13] NO may react with superoxide, whose production is increased during post-ischemic reperfusion, to form the more toxic nitrating and oxidant agent ONOO⁻, an RNS. Peroxynitrite is a substantial trigger of cardiomyocyte apoptosis. ONOO⁻ has been shown to be highly reactive with a wide variety of molecules, including deoxyribose, cellular lipids, and protein sulfhydryl moieties; causing direct oxidative tissue damage. The deleterious effects of RNS are further exacerbated with increased ambient ROS. And HR state in this study would downregulate all the above processes and expressing less myocardial caspase 3 ends in less myocardial apoptosis the paced controlled heart rate

during HR should exclude the possible myocardial protection after global IR that could be resulted from hypothermia induced bradycardia.

In conclusion HR phase exerts cytoprotection in heart transplant through downregulation of oxygen, nitrogen reactive species, and inhibition of apoptosis.

ACKNOWLEDGMENTS

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