

Expression of c-jun/c-fos mRNA Indicates Persistent Myocardial Stretch During Asphyxia-Induced Cardiac Arrest

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Summary

Right ventricular dysfunction is a key clinical issue for the viability of donation-after-circulatory-death (DCD) heart transplantation. DCD hearts with volume overload have the potential to exhibit aggravated right ventricular dysfunction following heart transplantation. The c-jun/c-fos mRNAs are genes that immediately respond to myocardial cell stretch. We assessed myocardial cell stretch during asphyxia-induced cardiac arrest by measuring c-jun/c-fos mRNA expression levels. The trachea was dissected and ligated to initiate asphyxiation in anesthetized Wistar rats under paralyzed ventilation. The hearts were harvested at 4 time points: 0, 15, 30, and 45 minutes after the termination of ventilation. Free walls of the right and left ventricles and the interventricular septum were sectioned. Total RNA was extracted from these tissues, and cDNA was synthesized using reverse transcription. The c-jun/c-fos mRNA expression levels were quantified using the droplet digital polymerase chain reaction method. In the left ventricle, c-jun/c-fos expression levels rapidly increased at 15 minutes, but the expression levels returned to the baseline level at 30 minutes after tracheal ligation. In contrast, in the right ventricle, c-jun/c-fos expression levels gradually increased and peaked 30 minutes after tracheal ligation. Myocardial cell stretching in the right ventricle is prolonged after asphyxia-induced cardiac arrest compared to that in the left ventricle, which may lead to right ventricular dysfunction after DCD heart transplantation.

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Key words: Donation after circulatory death, Heart transplantation, Animal model, Early response genes, Right ventricle

Transplantation of donation-after-circulatory-death (DCD) hearts remains challenging. The use of DCD hearts sometimes leads to primary graft dysfunction, requiring extracorporeal membrane oxygenation (ECMO) support. In fact, Chew, *et al.* reported that 35% of DCD heart recipients required temporal ECMO support to recover from cardiopulmonary bypass.¹⁾ During the agonal period after the respiratory arrest, the DCD heart experiences warm ischemia, a rapid surge of catecholamines, and volume and/or pressure overload, which may aggravate ventricular function, resulting in early graft dysfunction.

Although volume overload is a critical cause of early graft dysfunction,²⁻⁴⁾ the pathophysiological impact of volume overload on both the left and right ventricles during the DCD process is not fully understood. White, *et al.* reported that an $18 \pm 7\%$ increase in the right ventricular (RV) volume and a $12 \pm 9\%$ decrease in left ventricular (LV) volume occurred at the time of circulatory arrest in

the porcine DCD model.⁵⁾ Because of the thin muscle wall, the RV seems to be more anatomically tolerant to volume overload than the LV. To evaluate the effect of volume overload during the DCD process on both the LV and RV, we focused on early response genes.

The c-fos and c-jun messenger RNAs (mRNA) have been reported to be immediate early response genes for cardiac mechanical stress. Komuro, *et al.* demonstrated that c-fos mRNA expression was enhanced within 15 minutes by cardiomyocyte stretching.⁶⁾ Schunkert, *et al.* showed that c-fos and c-jun mRNAs began to accumulate at 15 minutes after systolic wall stress.⁷⁾ Sakai, *et al.* also showed that pressure stress greater than 80 mmHg enhanced c-fos mRNA expression after 30 minutes.⁸⁾ Therefore, c-fos and c-jun mRNA level measurements are beneficial for evaluating the pathophysiological impact of volume and/or pressure overload during the DCD process. Northern blotting and real-time polymerase chain reaction (PCR) have been used in previous studies for measuring

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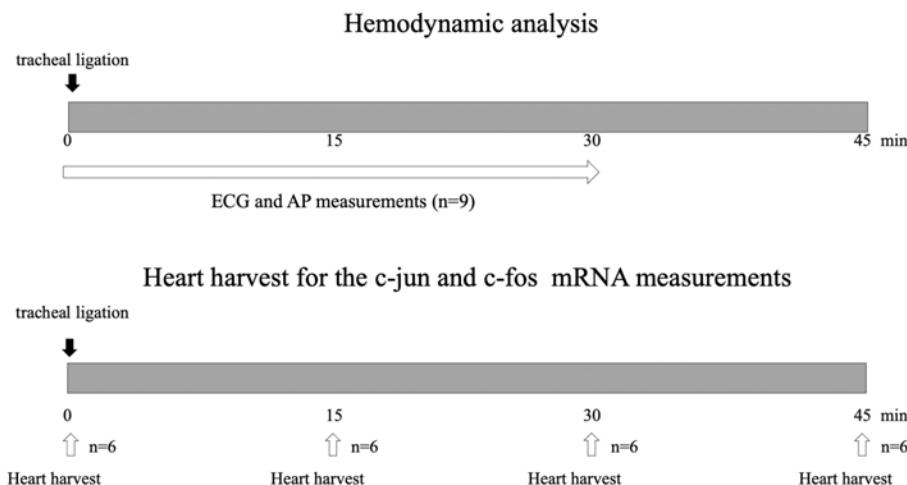


Figure 1. Experimental protocol. Upper panel shows the experimental timeline for electrocardiograms (ECG) and measuring arterial pressure (AP). Lower panel shows the experimental timeline for harvesting the heart for the c-jun and c-fos mRNA measurements. Nine rats were used for ECG and measuring AP. The heart was harvested at 4 time points: 0, 15, 30, and 45 minutes after tracheal ligation. Twenty-four rats were used for the c-jun and c-fos mRNA measurements.

c-fos and c-jun mRNA levels. Recent development of droplet digital PCR (ddPCR) technology allows us to precisely quantify mRNAs with low expression levels.⁹⁾ In this study, using ddPCR, we compared c-fos and c-jun mRNA expression levels between the LV and RV in a rat DCD model.

Methods

Ethical approval: All animals were cared for in accordance with the institutional guidelines and the 2011 “Guide for the Care and Use of Laboratory Animals” recommended by the U.S. National Institutes of Health. The experimental protocol was approved by the experimental animals committee of Okayama University (OKU-2021555).

Experimental protocol for circulatory death: Male Wistar rats (8 weeks old, 200 g) were intraperitoneally anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. The rats were placed on a heated surgical table to maintain body temperature around 38°C. Systolic arterial pressure (AP) was measured by inserting a catheter into the right carotid artery. Circulatory death was induced by asphyxiation, which was initiated by the ligation of the trachea without thoracotomy. Circulatory death was defined as the time at which the systolic arterial pressure decreased below 5 mmHg. Nine rats were used to measure hemodynamics, including electrocardiography (ECG) and AP, while 24 rats were used for the c-jun and c-fos mRNA measurements. The chest was opened by median sternotomy, and the heart was harvested at 0, 15, 30, or 45 minutes after tracheal ligation. Six rats were used for each time point (Figure 1). The LV and RV free wall and interventricular septum (IVS) tissues for each rat were resected and stored in the RNA Later Tissue Collection (Ambion, Carlsbad CA, USA) at -20°C.

RNA extraction and droplet digital PCR (ddPCR) as-

says: Total RNA was extracted from the LV and RV free walls and IVS tissues using RNA-Bee RNA isolation reagent, a buffer based on guanidine thiocyanate (Tel-Test, Friendswood, TX, USA). Briefly, tissue samples were homogenized in a buffer solution. The homogenate was added to chloroform and centrifuged ($20,400 \times g$ for 20 minutes) to remove the genomic DNA and proteins. The final precipitation was ethanol precipitation to obtain RNA and finally 1.2 µg of RNA was used in the next step. Total RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol.

The c-jun and c-fos mRNA levels from each sample were quantified by the ddPCR method as described previously.¹⁰⁾ Briefly, for ddPCR analysis, the ddPCR™ supermix for probes, droplet generation oil, gaskets and all other equipment were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). A QX200™ Droplet Digital™ PCR system (Bio-Rad) was used to generate and analyze the droplets. Probes were labelled with either FAM (for c-jun and c-fos) or HEX (for TATA-binding protein (TBP)). The primer and probe sets used were as follows: rat c-jun, Unique Assay ID: dRnoCPE5150088 (Bio-Rad), rat c-fos (fos), Assay ID: Rn02396760_g1 (Thermo Fisher Scientific, Inc. Waltham, MA, USA), and rat TBP, Unique Assay ID: dRnoCPE5188001 (Bio-Rad). The c-jun and c-fos mRNA expression levels were normalized to TBP mRNA levels.

Statistical analysis: Heart rate and systolic arterial pressure data are presented as the mean $\pm$ standard error of the mean (SEM). Expression data of c-jun and c-fos mRNA are represented as the mean $\pm$ SEM. Expression data were analyzed statistically using one-way analysis of variance (ANOVA), followed by Tukey’s test, using SPSS statistical software (SPSS for Windows, version 27; SPSS Inc., Chicago, IL, USA). Statistical significance was set at

Table. Heart Rate and Systolic Arterial Pressure Data

Time after tracheal ligation (minutes)	0	2.5	5	15	30
Heart rate (bpm)	238 ± 7	95 ± 4	56 ± 2	20 ± 4	20 ± 3
Systolic AP (mmHg)	97 ± 5	97 ± 7	23 ± 7	4.7 ± 0.4	5.3 ± 0.7

Bpm indicates beat per minute; and AP, arterial pressure. Values are expressed as the mean ± standard error.

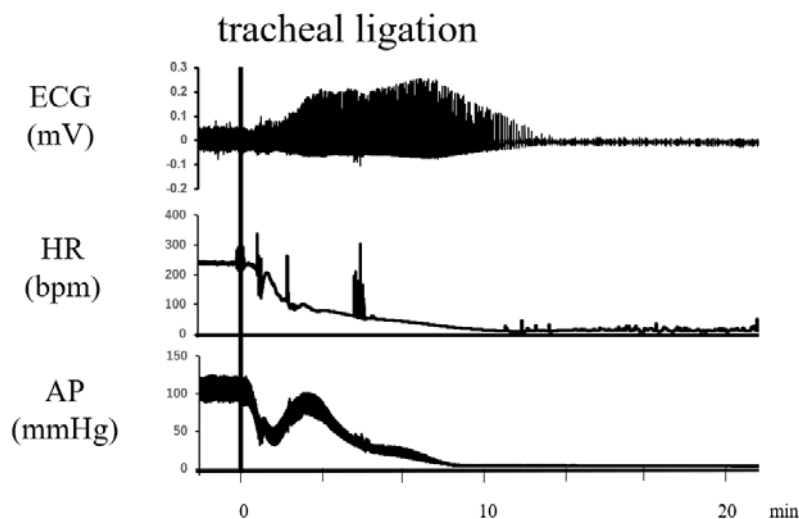


Figure 2. Typical changes in electrocardiogram (ECG) voltage, heart rate (HR), and arterial pressure (AP) before and after tracheal ligation were recorded.

$P < 0.05$.

Results

Changes in electrocardiogram and arterial pressure:

The Table shows the changes in heart rate and systolic arterial pressures after tracheal ligation. Figure 2 shows the typical changes in electrocardiogram voltage, heart rate, and arterial pressure before and after tracheal ligation. The heart rate progressively decreased, while the arterial pressure (AP) transiently increased approximately 2-3 minutes after tracheal ligation. Median time of circulatory death was 6.1 minutes (interquartile range (IQR): 5.8-7.5 minutes).

Expression of c-jun mRNA in RV, LV, and IVS: The c-jun/TBP mRNA expression level ratios in the RV, LV, and IVS tissues are shown in Figure 3. In the RV tissue, the c-jun mRNA expression level rapidly increased at 15 minutes (4.40 ± 0.68) compared to that at 0 minutes after the trachea ligation (1.41 ± 0.09 , $P < 0.001$). At 30 minutes after the ligation, a further increase in the c-jun mRNA expression level (6.27 ± 0.31) was observed compared to that at 15 minutes ($P = 0.027$). This increase remained until 45 minutes after the ligation (6.59 ± 0.19 , $P = 0.008$ versus 15 minutes).

In the LV tissue, the expression level of c-jun mRNA rapidly increased at 15 minutes (7.42 ± 0.12) compared to that at 0 minutes after the trachea ligation (5.26 ± 0.27 , $P < 0.001$). At 30 minutes after the ligation, the c-jun mRNA expression level (2.36 ± 0.39) significantly decreased below the baseline level ($P < 0.001$ versus 0 min-

utes). This decrease was maintained until 45 minutes after the ligation (3.24 ± 0.32 , $P = 0.0013$ versus 0 minutes).

In the IVS tissue, the expression level of c-jun mRNA did not change significantly at 15 (5.73 ± 0.52) and 30 minutes (6.29 ± 0.13) compared to that at 0 minutes after the trachea ligation (4.97 ± 0.28). At 45 minutes after the ligation, the c-jun mRNA expression level (7.82 ± 0.33) was significantly increased compared to that at 0 ($P < 0.001$), 15 ($P = 0.004$), and 30 minutes ($P = 0.044$) after the ligation.

Expression of c-fos mRNA in RV, LV, and IVS: The c-fos/TBP mRNA expression level ratios in the RV, LV, and IVS are shown in Figure 4. In the RV tissue, the c-fos mRNA expression level significantly increased at 30 minutes (0.88 ± 0.20) compared to that at 0 minutes after the trachea ligation (0.22 ± 0.05 , $P = 0.028$). At 45 minutes after the ligation, the c-fos expression level returned to the baseline level (0.63 ± 0.15 , $P = 0.253$ versus 0 minutes).

In the LV tissue, the c-fos mRNA expression level rapidly increased at 15 minutes (0.81 ± 0.24) compared to that at 0 minutes after the ligation (0.21 ± 0.06 , $P = 0.035$). At 30 minutes after the ligation, the c-fos mRNA expression level (0.19 ± 0.03) returned to the baseline level ($P = 0.999$ versus 0 minutes).

In the IVS tissue, the c-fos mRNA expression level did not change significantly throughout the experiment.

Discussion

In this study, using a rat DCD model, early response gene (c-jun and c-fos mRNA) expression levels were

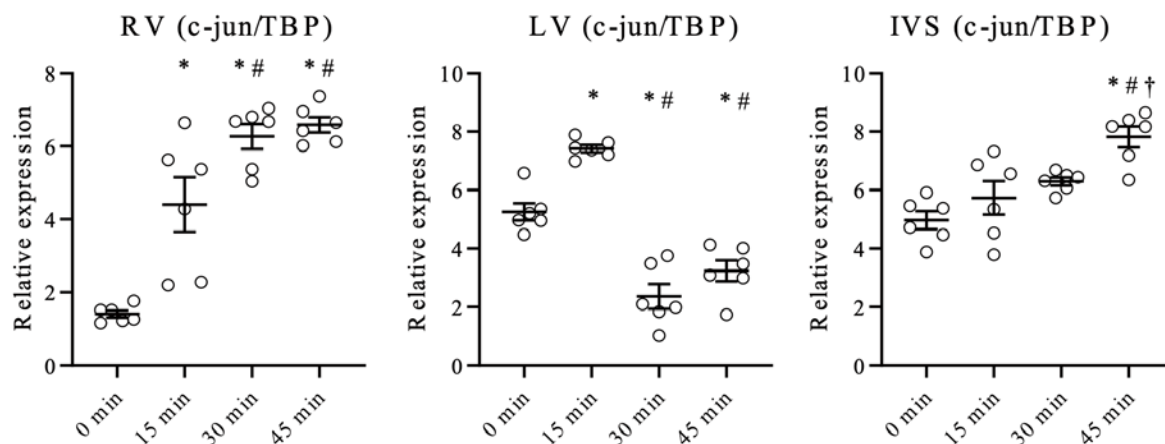


Figure 3. Expression dynamics of c-jun mRNA in DCD hearts. The expression of c-jun mRNA in the right ventricle (RV), left ventricle (LV) and interventricular septum (IVS) was measured up to 45 minutes after cardiac arrest. Each expression level was normalized by internal expression of TATA box binding protein gene (TBP). Values are expressed as the mean $\pm$ standard error of the mean (SEM). * P < 0.05 versus DCD 0 minutes group, # P < 0.05 versus DCD 15 minutes group, † P < 0.05 versus DCD 30 minutes by one-way ANOVA followed by Tukey's test.

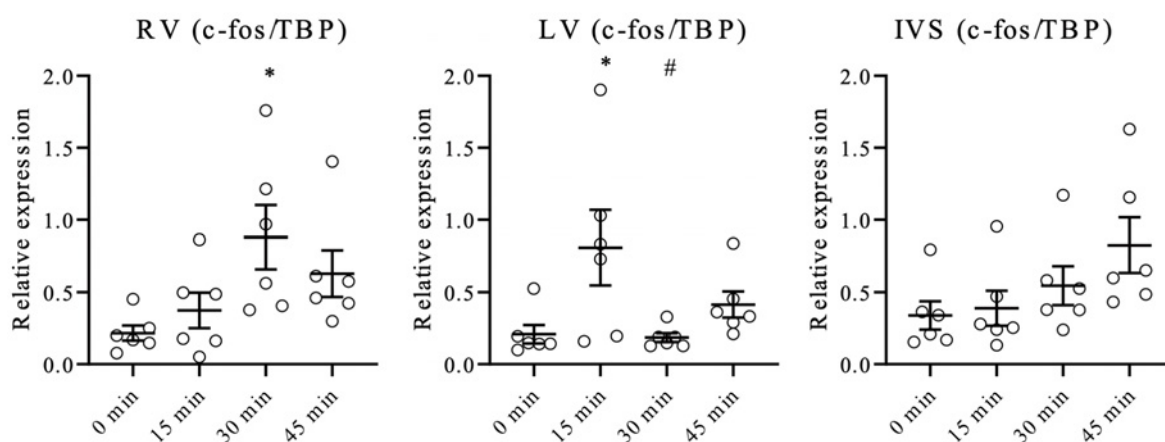


Figure 4. Expression dynamics of c-fos mRNA in DCD hearts. The expression of c-jun mRNA in the right ventricle (RV), left ventricle (LV) and interventricular septum (IVS) was measured up to 45 minutes after cardiac arrest by using the same samples that analyzed c-jun mRNA. Each expression level was normalized by internal expression of TATA box binding protein gene (TBP). Values are expressed as the mean $\pm$ SEM. * P < 0.05 versus DCD 0 minutes group, # P < 0.05 versus DCD 15 minutes group by one-way ANOVA followed by Tukey's test.

measured to examine the influences of volume overload and warm ischemia during the process of cardiac death from a time-point immediately after tracheal ligation, which represents the withdrawal of life support, to 45 minutes after tracheal ligation. In the RV free wall, an increase in c-jun mRNA expression level was observed at 15 minutes after tracheal ligation compared to that at 0 minutes after ligation, and the c-jun mRNA expression level continued to increase up to 45 minutes after ligation. In the IVS, unlike in the RV free wall, a slow increase in the c-jun mRNA expression level was observed. The c-jun mRNA expression level 45 minutes after ligation was higher than that at 0 minutes. In the LV free wall, the c-jun mRNA expression level 15 minutes after ligation was higher than that at 0 minutes; however, the expression level 30 minutes after ligation decreased to the baseline level. Interestingly, the expression dynamics of c-fos

mRNA in the RV, IVS, and LV showed a tendency similar to that of c-jun mRNA expression with elapsed time after tracheal ligation. These results may indicate that the molecular impact of myocardial cell stretch is different between the RV, IVS, and LV, even in the clinical settings of DCD heart transplantation.

c-jun and c-fos mRNA expression levels respond to mechanical stress and ischemia: Cardiac myocytes respond to pressure overload not only by increasing cell volume, but also by altering the expression levels of specific genes. The transcription of immediate early response genes, such as c-jun and c-fos mRNA, is activated rapidly and transiently within minutes of extracellular stimulation. The c-jun and c-fos mRNA have been reported to be the immediate response genes to mechanical stresses, such as myocardial cell stretch.¹¹⁾ A previous study in rats demonstrated that banding of the ascending aorta resulted in the

immediate induction of cardiac c-fos genes within 1 hour after the procedure.¹²⁾ Another time-course study revealed that c-jun and c-fos mRNAs were detectable as early as 15 minutes, peaked after 60 minutes, and returned to low levels 240 minutes after exposure to increased systolic wall stress.⁷⁾ However, these responses may be limited in a beating heart. Bauters, *et al.* demonstrated that an increase in coronary flow induces c-fos mRNA expression in isolated perfused rat beating hearts, but not in arrested hearts.¹³⁾

Ischemia-reperfusion injury has also been reported to cause a significant and rapid induction of c-jun and c-fos mRNA in LV myocardial tissues.¹⁴⁾ The c-jun and c-fos expression levels significantly increase after 30 minutes of coronary occlusion followed by 60 minutes of reperfusion. The induction of both genes was stronger in the ischemic myocardium after 120 minutes of coronary occlusion followed by 60 minutes of reperfusion. This finding may indicate that a longer ischemic duration results in higher expression levels of these genes. In the present study, c-jun and c-fos mRNA expression levels increased immediately after tracheal ligation. Although LV c-jun and c-fos mRNA expression levels peaked 15 minutes after tracheal ligation, levels of both genes gradually increased until 30-45 minutes in the RV. This may be associated with the difference in response to myocardial stretch and/or warm ischemia between RV and LV.

Mechanism of right ventricular mechanical stress in DCD hearts: In the first phase after asphyxiation, pulmonary vasoconstriction is observed, which is associated with an increased RV diastolic volume and pressure.⁵⁾ Elevated RV afterload results in a declined RV stroke volume and distension. A decrease in pulmonary blood flow causes a decrease in cardiac output and systemic blood pressure, which were initially observed following donor extubation in the clinical setting of DCD. After vasoconstriction, the hyperdynamic phase is initiated by a catecholamine surge, resulting in a transient increase in systemic blood pressure. In this study, a hemodynamic response was observed approximately 2-3 minutes after tracheal ligation. During the hyperdynamic phase, the RV diastolic volume and pressure continue to increase due to an increase in venous return to the RV. Because most of the total blood volume is contained within the venous capacitance system, sympathetic venoconstriction causes a decrease in systemic venous capacitance and an increase in RV preload.¹⁵⁾ Therefore, the RV becomes progressively more distended due to increased venous return and elevated pulmonary vascular resistance.⁵⁾ This mechanism may cause myocardial stretch to the RV, resulting in prolonged elevation of c-jun and c-fos mRNA expression levels.

Function of c-fos and c-jun mRNA: In dividing cells, when serum or growth factors are added to serum-starved cells, signals from growth factor-like ligands are rapidly transmitted, and transcription of c-fos and c-jun mRNA is induced via JNK. This phenomenon helps cells exit the G0 state and enter a cycle of active growth and division, inducing the expression of delayed early genes such as serum response factor (SRF) and MADS box transcription factors.¹⁶⁾ On the other hand, in cardiomyocytes, which are

non-dividing cells, the above-mentioned signals are known to be used as mechanostress signals or hypertrophic signals.^{11,12)} In cells under ischemic conditions such as global ischemia during DCD, the transcription of c-fos and c-jun mRNA induces delayed early genes such as SRF,¹⁷⁾ and these genes are responsible for maintaining of the cytoskeleton and cell survival (anti-apoptotic effect), which may contribute to the cardioprotection.

Clinical implications: Even under the controlled procurement in donation-after-brain-death (DBD) heart transplantation, RV dysfunction accounts for more than 50% of all early cardiac complications and 19% of early deaths post-operatively.¹⁸⁾ The mechanism of RV dysfunction in DCD heart caused by circulatory loads, warm ischemia, and reperfusion injury should be more complicated than that in DBD hearts. From an anatomical point of view, the thin walls and crescent shape make the RV a highly compliant chamber that may accommodate large increases in volume. However, the adaptive mechanism of the RV is not well suited to a sudden increase in pressure.¹⁹⁾ Cardiac arrest with a sudden pressure overload by asphyxiation in DCD hearts causes more myocardial damage than unloaded cardiac arrest in DBD hearts. Therefore, the mode of cardiac arrest should be considered when evaluating the cardiac function in DCD hearts for clinical applications. RV diastolic pressure progressively increases after respiratory arrest, reaches a peak in the agonal phase, and then gradually decreases after cardiac arrest.⁵⁾ However, an increase in RV volume remains after cardiac arrest. Reductions in RV pressure overload during the agonal period and RV volume overload in the process of cardiac arrest may prevent post-transplant right ventricular failure.²⁰⁾ This study demonstrated that c-jun and c-fos mRNAs were good biological markers of mechanical stress to the myocardium. Therefore, experiments focusing on these early response genes may be beneficial for evaluating effective cardioprotective methods during DCD heart transplantation.

Limitations: Because all tissue samples were stored in RNA Later solution at -20°C, histological examinations were not performed in this study. Also, further investigations are needed to evaluate the relationship between early response genes and histological changes during the process of DCD.

In conclusion, patterns of c-jun and c-fos mRNA expression revealed persistent myocardial stretching in the RV during asphyxia-induced cardiac arrest. The prevention of RV distension may be essential for successful DCD heart transplantation.

Disclosure

Conflicts of interest: The authors have declared no conflicts of interest.

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