

Effects of a Left Ventricular Assist Device on the Myocardium in Ischemia-reperfusion Injury

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Objective: It is suggested that apoptosis plays a role in heart diseases. The effect of left ventricular assist device (LVAD) on apoptosis at ischemia-reperfusion myocardial injury is unclear. We investigated the effect by assisting the ischemia-reperfusion myocardial injury models with LVAD.

Methods: Twelve swines were divided into two groups: the control group and LVAD group. The diagonal branch of the left coronary artery was occluded and released after 35 min. Reperfusion was performed, and observed for 3 hrs. The LVAD group was assisted by LVAD from 5 min before reperfusion to 3 hrs after it. Cardiac function, coronary flow, and cardiac tissue blood flow were measured. Pathologic assay was performed with terminal deoxynucleotidyl transferase-mediated dUTP in situ nick end labeling (TUNEL) and hematoxylin and eosin (HE) staining. mRNA of Bcl-xL and Bak were measured.

Results: Ejection fraction, cardiac output, and Emax in the LVAD group were improved ($p < 0.05$ vs. the control group). There were less necrotic cells in the LVAD group than in the control group. There were more TUNEL positive cells in the LVAD group than in the control group. mRNA of Bcl-xL and Bak in LVAD group were high.

Conclusion: The aggravation of cardiac dysfunction was limited to a minimum in the LVAD group. (*Ann Thorac Cardiovasc Surg* 2004; 10: 350–6)

Key words: cardiomyocyte apoptosis, left ventricular assist device, ischemia-reperfusion injury, Bcl-2 family

Introduction

Apoptosis is the mechanism of cell death that Kerr et al.¹⁾ researched and advocated by investigating the morphological changes occurring in ischemic hepatic cells. It was thought that there are few apoptotic cell deaths in the myocardial cells, which are the terminally differentiating cells considered to not proliferate generally. As regards apoptosis of myocardial cells in heart diseases, ever since Gottlieb et al.²⁾ reported the fragmentation of the DNA in the infarcted myocardium of rabbits that had 30 min ischemia and 4 hrs reperfusion, several studies have been

described on animal models of myocardial infarction and human autopsies.^{3,4)} Furthermore, the reports, which suggest that apoptosis plays a role in the causes or the progress of heart diseases such as ischemic cardiac muscle injury and heart failures, have been published consistently.^{5,6)}

In the field of cardiac surgery, Bartling et al. have reported that apoptosis was decreased by long-period assistance with a left ventricular assist device (LVAD) in end-stage heart failures such as dilated cardiomyopathies etc.⁷⁾ Moreover, it has also been reported that apoptosis is caused by cardioplegic arrest.⁸⁾ LVAD is the most effective method of assisted circulation, and its clinical effectiveness has been recognized. The effect of LVAD on ischemia-reperfusion myocardial injury has already been demonstrated,⁹⁾ however, the details are still unclear. In addition, no study investigating the effect of LVAD on apoptosis in acute ischemia-reperfusion myocardial injury has been conducted.

In this study, we investigated the effect on apoptosis in

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Received May 19, 2004; accepted for publication September 2, 2004.

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cardiac muscle by assisting the ischemia-reperfusion myocardial injury models with LVAD.

Materials and Methods

This study was performed in 12 swines (39.6 ± 2.6 kg). All animals received humane care in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, as well as with the "Guide for the Care and Use of Laboratory Animals", prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH publication 86-23, revised, 1996). The animals were divided into two groups; 6 swines (the control group) were not supported by LVAD, while the other 6 swines (the LVAD group) were supported by LVAD. Each animal was anesthetized with an intravenous injection of 20 mg/kg sodium pentobarbital and ketamine chloride (1 mg/kg/h) and vecuronium bromide (0.02 mg/kg/h). After tracheal intubation, controlled mechanical ventilation was established at 20-25 beats/min with a tidal volume of 10-15 ml/kg by means of volume-controlled ventilation (Servo 900-E, Siemens-Elcoma Inc., Stockholm, Sweden). A venous line was inserted into the right carotid vein for injection. The left femoral artery was cannulated for monitoring the continuous aortic pressure. A conductance catheter (2012-6-27-P, Alpha Medical Instruments Inc., CA, USA) was inserted through the aortic valve into the left ventricle with the catheter tip placed at the apex. A catheter tip manometer (811-195S/ANP534, Sentron Inc., Roden, Netherlands) was also inserted into the left ventricle for monitoring the continuous left ventricular pressure. The left ventricular pressure-volume loop (P-V loop) was recorded by a Sigma-5 (Cardio Dynamics Inc., Zoetermeer, Netherlands), and LVAD was stopped temporarily because of undesirable noise. The left ventricular function was evaluated by the ejection fraction, cardiac output, Emax, and Tau. These data were calculated by analyzing the P-V loop with a Conduct PC (Cardio Dynamics Inc., Zoetermeer, Netherlands). A median sternotomy was performed. Subsequently, two ultrasonic flow transducers (T206, Transonic System Inc., Ithaca, NY, USA) were placed into the ascending aorta and the left coronary artery in front of the first diagonal branch. A tissue flow meter (ALF12RD, Advance Inc., Tokyo, Japan) was inserted 10 mm deep for measuring the segmental ischemic endocardial tissue blood flow.

Heparinization was performed systemically (1 mg/kg) in both groups. In the LVAD group, an inflow cannula

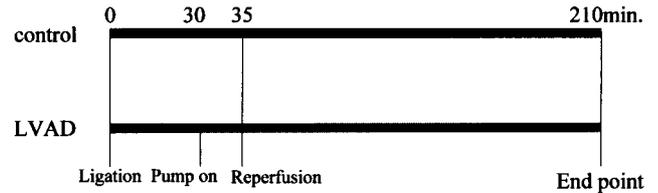


Fig. 1. Protocol.

(32 Fr, TF-2937-0, Edwards Lifesciences Inc., Irvine, CA, USA) was inserted into the left atrium, and an outflow cannula (5.2 mm, A211-52A, Stockert, Muenchen, Germany) was inserted into the ascending aorta. After recording the measurements, the first diagonal branch was occluded for 35 min. After the occluder of the first diagonal branch in the left coronary artery was released, reperfusion was performed for 3 hrs.¹⁰ The LVAD group swines were assisted by LVAD from 30 min after ischemia with a centrifugal pump (Gyro C1E3, Kyocera Inc., Kyoto, Japan). After having hemodynamic states stabilized, reperfusion was performed at 35 min after ischemia. They were assisted by LVAD to 3 hrs after reperfusion (Fig. 1). Subsequently, an ultrasonic flow transducer (T206, Transonic System Inc., Ithaca, NY, USA) was placed into the outflow cannula to measure the flow. Motor speed was increased until the assist rate (pump flow/total flow; total flow = native flow + pump flow) was approximately 75%.

Tissue samples were taken from the ischemic area after 3 hrs of reperfusion. Samples for real time polymerase chain reaction (RT-PCR) were rapidly excised, snap-frozen in liquid nitrogen, and then stored at -80°C . For standard pathologic study, tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and cut into 6- μm thick serial sections. Apoptosis was measured and evaluated by the measurements of transferase-mediated dUTP in situ nick end labeling (TUNEL) assay, Bcl-xL in the Bcl-2 family, and mRNA of the Bak. Bcl-xL and Bak were detected by PCR amplification and were measured. The following primers and probes were used.

Bcl-xL: Forward primer, 5'-CGT GGA GAG CGT AGA CAA GGA-3'; reverse primer, 5'-CCA AGG CTC TAG GTG GTC ATT C-3'; TaqMan probe, 5'-CAT CCA AGT TGC GAT CCG ACT CAC CT-3'.

Bak: Forward primer, 5'-GAT GAC ATC AAC CGG CGA TAC-3'; reverse primer, 5'-CCA GTT GAT GCC ACT CTC GAA-3'; TaqMan probe, 5'-TGA GTA CTT CAC CAA GAT CGC CTC CAG CT-3'.

The level of expression of Bcl-xL and Bak was normalized to 18SrRNA.

Table 1. Hemodynamic data

		Baseline	Ischemia 30 min after	Reperfusion 180 min after
mAoP (mmHg)	Control	88.2±13	65.5±9.1	70.1±14
	LVAD	85.5±4.5	70.1±5.1	89.7±11
EF (%)	Control	59.6±6.9	44.5±6.8	35.6±5.2
	LVAD	61.5±3.5	41.1±9.0	57.5±8.7*
CO (L/min)	Control	2.50±0.56	2.07±0.28	1.70±0.16
	LVAD	2.52±0.49	1.93±0.66	2.11±0.34*
Emax (mmHg/ml)	Control	7.24±1.9	3.81±0.8	4.69±1.3
	LVAD	7.20±2.0	3.60±1.1	8.62±2.3*
Tau (ms)	Control	31.2±5.3	36.2±5.1	30.7±4.0
	LVAD	32.1±5.4	39.2±5.7	32.9±8.7

Values are expressed as the means ± SD

mAoP, mean aortic pressure; EF, ejection fraction; CO, cardiac output

* $p < 0.05$

The specimens obtained from the ischemic areas were fixed with 4% paraformaldehyde and stained with TUNEL staining and hematoxylin and eosin (HE) staining. These slides were observed under 100× magnification with a light microscope (Olympus BX50, Olympus, Tokyo, Japan). Further, the images were processed using an image scanner software (Viewfinder Lite Application 1.0.135, Pixera Corporation, CA, USA) and analyzed by using an image analysis software (Sigma Scan Pro, SPSS Inc., Chicago, IL, USA). In the TUNEL staining assay, the cells that became apoptotic were stained brown and were counted. In the HE staining assay, the cells that exhibited morphologic criteria such as swelling, cytoplasmic uniformity, and hyper eosinophilia were counted as necrotic cells. Necrotic and apoptotic cells were counted in five different fields of each slide, and the number of these cells was divided by the number of cells in the entire field of vision.

Statistical analysis was carried out by unpaired *t*-test. The values were expressed as mean ± standard deviation, and the difference was considered significant when the *p* value was 0.05 or less.

Results

Cardiac function

There were no significant differences in the heart rate between the two groups throughout the experiment. The mean aortic pressure in the LVAD group was higher than that in the control group during reperfusion. Ejection fraction, cardiac output, Emax, and Tau at 30 min after ischemia showed no significant differences between the LVAD and the control groups. Ejection fraction, cardiac

output, and Emax in the LVAD group were significantly better than those in the control group at 3 hrs after reperfusion as follows: ejection fraction (57.5±8.7% in the LVAD group vs. 35.6±5.2% in the control group, $p=0.0004$), cardiac output (2.11±0.34 L/min in the LVAD group vs. 1.70±0.16 L/min in the control group, $p=0.0238$), Emax (8.62±2.3 mmHg/ml in the LVAD group vs. 4.69±1.3 mmHg/ml in the control group, $p=0.0042$). Tau was not statistically significant at 3 hrs after reperfusion (Table 1).

Blood flow of coronary and cardiac muscle

The left coronary artery flow and the tissue blood flow decreased in both the groups 30 min after ischemia. Subsequently, they increased in the LVAD group and decreased slightly in the control group after a sudden rise at 10 min after reperfusion. There was a significant difference in the left coronary artery flow between the LVAD and control groups at 1, 2, and 3 hrs after reperfusion. Similarly, there was a significant difference in the tissue blood flow between the LVAD and control groups during reperfusion except at 10 min after reperfusion (Fig. 2-4).

Histopathology

In the HE staining assay, necrotic cells with swelling, cytoplasmic uniformity, and hyper eosinophilia were found in the ischemic area. The number (expressed as %) of necrotic cells identified by the HE staining was 4.37±1.23 in the control group and 0.90±0.53 ($p < 0.0001$) in the LVAD group. The low value in the LVAD group was statistically significant.

In the TUNEL staining assay, the apoptotic cells that were stained brown and revealed condensation of chro-

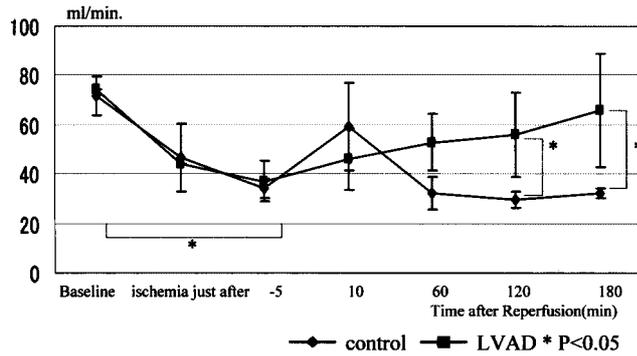


Fig. 2. Flow of left anterior descending coronary artery.

matin were found in the ischemic area. The number (expressed as %) of TUNEL positive cells was 0.51 ± 0.36 in the control group and 2.18 ± 1.63 ($p=0.03$) in the LVAD group (Fig. 5).

Genetic analysis

Bcl-xL was found to be 2.48 ± 0.76 in the control group and 5.18 ± 2.99 in the LVAD group. The LVAD group demonstrated a higher value ($p=0.04$). Bak was found to be 10.0 ± 3.54 in the control group and 33.8 ± 46.2 ($p=0.24$) in the LVAD group. Bak/Bcl-xL was found to be 4.31 ± 1.79 in the control group and 7.11 ± 7.71 in the LVAD group. The LVAD group demonstrated a higher value ($p=0.41$) (Fig. 6).

Discussion

Cell death is classified into apoptosis and necrosis. Necrosis is a type of cell death that is a result of responses to an external injury. Cell membrane injury and disruption of intracellular metabolism lead to accumulation of water in the cell, which causes swelling because of a rise in the osmotic pressure.¹¹ In addition to this, the injury to the cell membrane also worsens. Finally, the cell membrane ruptures. Consequently, as the cellular contents are discharged to the exterior, inflammation is caused. On the other hand, apoptosis is a type of cell death that is regulated by a gene. Apoptosis generally occurs as a physiologic phenomenon; however, cellular shrinkage and a characteristic agglomeration of nuclei have been established as morphological characteristics of apoptosis.¹¹ As it advances, the cell is divided, and a few apoptotic corpuscles are formed. Subsequently, they are engorged by phagocytes such as macrophages. It has been demonstrated that no inflammation occurs during apoptosis as

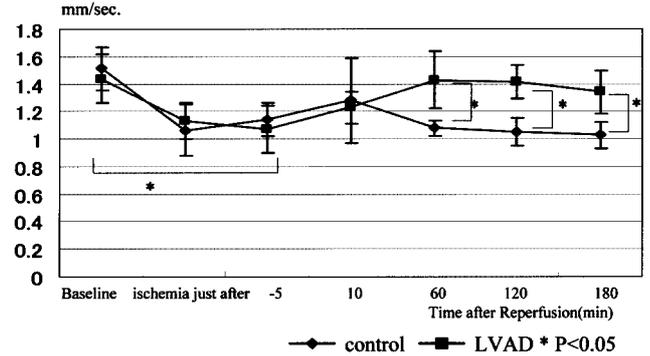


Fig. 3. Tissue blood flow velocity in cardiac muscle.

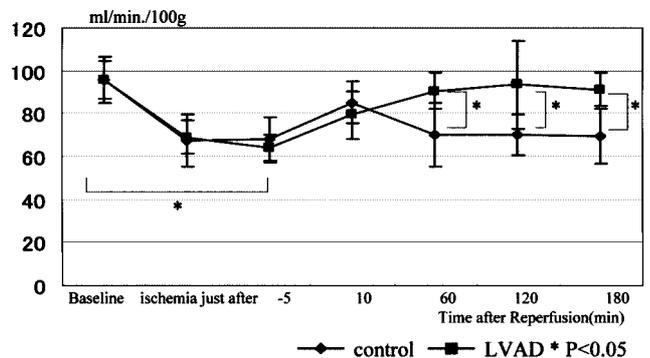


Fig. 4. Tissue blood flow in cardiac muscle.

the cell membrane is not ruptured and the cellular contents are not discharged.

In case of ischemia-reperfusion myocardial cells, events such as the discharge of Ca from sarcoplasmic reticula, the activation of Na-Ca exchanger, the rise in the permeability of the cell membrane, etc. occur. Therefore, the intracellular Ca concentration is increased. Subsequently, the hydrolysis of Adenosine Triphosphate (ATP) is advanced in the mitochondria. On the other hand, the contraction of the cardiac muscle is temporarily reinitiated due to oxygen supply. The remaining ATP is consumed more rapidly, and the high energy phosphate gets exhausted.^{12,13} Subsequently, the cells die in a similar manner as in necrosis. The sudden increase in the intracellular Ca due to reperfusion causes a temporary increase in the contraction of the cardiac muscle. In this study, the results indicated that the flow of the left anterior descending coronary artery (LAD) in the control group was increased temporarily during the 10 min after reperfusion. However, in the LVAD group, the flow of LAD after reperfusion did not increase rapidly, although the flow of

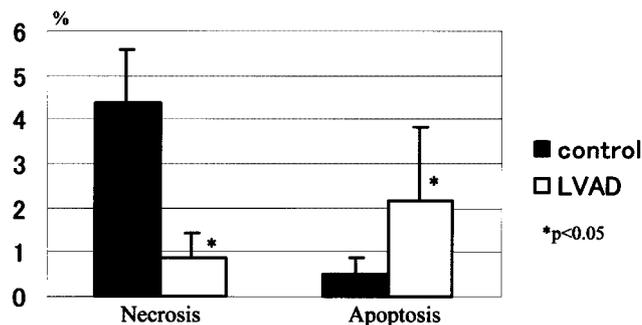


Fig. 5. Percentage of necrotic and apoptotic cells.

LAD had increased. It is thought that these phenomena arose because left ventricular unloading due to the LVAD effect and the improvement of the coronary flow contributed to the improvement of the hypoxia, which helped to keep both, the increase of the intracellular Ca concentration and the contraction that had occurred at reperfusion under control. The increase in the intracellular Ca concentration due to reperfusion acts as a factor inducing apoptosis. It has been showed that apart from the intracellular Ca concentration, physical stress, super oxide, nitric oxide, hypoxia, tumor necrosis factor α (TNF α), intracellular surplus Ca, etc. are associated with the occurrence of apoptosis in the myocardial muscle.¹⁴⁻¹⁹⁾ These extracellular stimuli are transmitted to the mitochondria, and subsequently, cytochrome c is released from the mitochondria through Voltage Dependent Anion Channel (VDAC).^{20,21)} The protein group of the Bcl-2 family plays a role in this release. The Bcl-2 family comprises Bcl-2, Bcl-xL, Mcl-1, etc., which have anti-apoptotic effects and Bax, Bak, Bad, etc., which have pro-apoptotic effects. It is thought that a process of cell death such as apoptosis is caused because the activation of caspase-3 is led by cytochrome c, and the DNA fragmentation and the

agglomeration of chromatin occurs.

In this study, in the Bcl-2 family, Bcl-xL was measured as an anti-apoptotic factor, and Bak was measured as a pro-apoptotic factor. Bcl-xL was directly combined with VDAC, and the release of cytochrome c was blocked. Bak was also combined with VDAC, leading to the release of cytochrome c. There were no significant differences in Bak; however, both Bcl-xL and Bak in the LVAD group were higher than those in the control group. Although there were no significant differences in Bak/Bcl-xL in the control group, Bak/Bcl-xL was high in the LVAD group. It was inferred that the Bcl-2 family had an influence in promoting apoptosis. Furthermore, from the results of TUNEL staining, it was evident that apoptosis had occurred to a significantly greater extent in the LVAD group than in the control group. However, there were no significant differences in the Bcl-2 family. Nevertheless, there were significant differences in the TUNEL staining examinations. Therefore, it seemed that a cascade, which did not mediate the measured Bcl-2 family in this study, was related to this phenomenon. This is the cascade through which intracellular stimuli such as TNF α , FasL, etc. activate caspase-8 and caspase-3, leading to apoptosis. It was thought that it was necessary to examine these stimuli after this study.

From these results, it is thought that the cardiac function could be preserved because there were few cells in the LVAD group which underwent necrosis, and a large portion of the cardiac muscle survived. Meanwhile, there were many cells that became apoptotic in the LVAD group. Consequently, it was inferred that many cells which could avoid being necrosed died due to apoptosis. It is considered that many cells became apoptotic; however, the spread of inflammation to the surroundings did not occur. This was advantageous for the heart. Consequently, the cardiac function could be preserved. As LVAD pro-

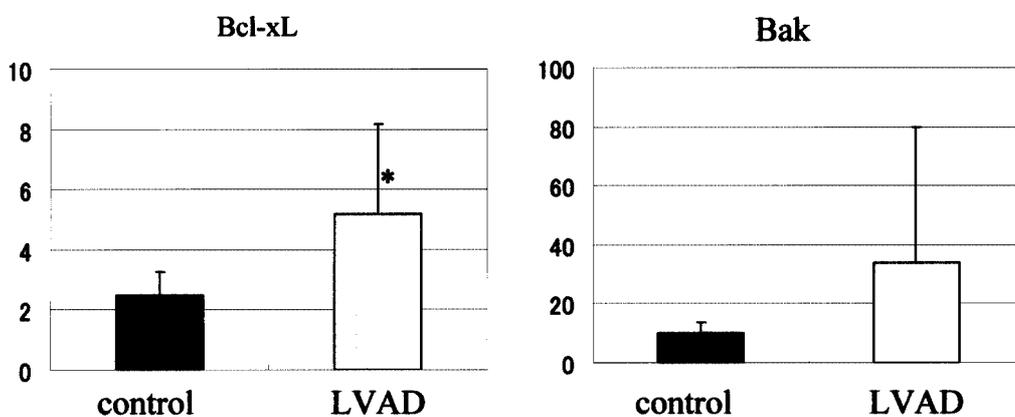


Fig. 6. Quantitation of the Bcl-2 family. The level of expression of Bcl-xL and Bak was normalized to 18SrRNA. *p<0.05

vides not only pressure assistance but also flow assistance, it is very effective in severe heart failure caused by ischemic heart disease as it leads to unloading and an increase in coronary flow.²²⁾ LVAD has been used clinically in actual treatments. It has been reported that improvement in the cardiac function by LVAD in cases of ischemia-reperfusion injury can be expected along with some improvements in the cardiac function.⁹⁾ It is considered that this is caused by the increase in the coronary flow and unloading achieved by LVAD. In this study, the hypoxia in the ischemic areas was improved by the increase in the coronary flow at the reperfusion. Moreover, the ATP consumption in the cardiac muscle was decreased by unloading, and the rise in the intracellular Ca concentration was also decreased. It is thought that necrosis was not caused as a result of these events. However, it was thought that the cells that necrosed in the control group could be led to be apoptotic by a rise in the intracellular Ca concentration and the extracellular stimuli by ischemia-reperfusion.

The influence on apoptosis was previously studied at our facilities by using assisted circulation by LVAD and percutaneous cardiopulmonary support on the 3 hrs ischemic heart model.²³⁾ The results indicated that apoptosis was decreased by mechanical assisted circulation. Since the cardiac muscle apoptosis was observed to a greater extent in reperfusion than in ischemia,²⁾ the ischemia reperfusion models were used in this study. As a consequence, results that were different from those of previous experiments were obtained since apoptosis was promoted by the assisted circulation. There are differences between the causes of cell injury in an ischemic state and an ischemia-reperfusion state. In the ischemic state, cytokines such as TNF α , released due to hypoxia, cause cell injury. On the other hand, in the ischemia-reperfusion state, the increase in the intracellular Ca concentration causes cell injury. It was thought that these stimuli acted as factors leading to apoptosis. Moreover, they were the cause of the varying results obtained when mechanical assisted circulation was performed. For explaining the pathological processes involved in the effect of LVAD on ischemia-reperfusion myocardial injury, it was thought that in the future, the estimations of the intracellular Ca concentration, TNF α , FasL, etc. will be required.

Conclusion

Cardiac muscle death due to ischemia-reperfusion injury was influenced by the mechanical assisted circulation with

LVAD, and necrosis was decreased. Therefore, the cardiac function was preserved. In addition, apoptosis was observed to a greater extent in the LVAD group. However, it was concluded that this type of cell death had little influence on the adjacent organelles, and the aggravation of the cardiac dysfunction was limited to a minimum.

Acknowledgements

I wish to thank Honorary Prof. Yukiyasu Sezai, Chief Prof. Nanao Negishi, Associate Prof. Motomi Shiono, and members of the study group at Nihon University School of Medicine, Department of Cardiovascular Surgery for correcting the manuscript. I also thank Mr. Yoshiki Taniguchi for his technical assistance.

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