

A Study of the Myocardial Protective Effect of Rapid Cooling Based on Intracellular Ca, Intracellular pH, and HSP70

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Objective: It has been reported that rapid cooling of the heart during normothermic coronary circulation at reperfusion after ischemia promotes early recovery of cardiac function due to the positive inotropic effects on the myocardium produced by cooling. The aim of the present study was to investigate the myocardial protective effect of rapid cooling by measuring heat shock protein (HSP) levels and examining the relationship between cardiac function, intracellular Ca concentration, and intracellular pH after rapid cooling.

Methods: Isolated perfused rat hearts were subjected to ischemia for 60 minutes at a myocardial temperature of 37°C. One group of hearts (group R) was subjected to 3 minutes of rapid cooling ($\leq 4^\circ\text{C}$) 3 minutes after reperfusion was started. The other hearts (group C) were not cooled. Myocardial protection was then compared in the two groups by measuring heart rate, coronary flow, left ventricular contraction, intracellular Ca concentration (fura-2 fluorescence), and intracellular pH (BCECF-AM fluorescence) and examining HSP70 by western blot.

Results: (1) Left ventricular contraction was $105 \pm 0.05\%$ of the pre-ischemia value in group R after rapid cooling, whereas recovery was poor in group C ($63 \pm 2\%$). (2) The intracellular Ca concentration in group R rose to $141 \pm 8\%$ of the pre-ischemia value during ischemia, but decreased to $101 \pm 3\%$ at reperfusion after rapid cooling. In group C, the Ca concentration was $144 \pm 4\%$ of the pre-ischemia value during ischemia and rose further to $156 \pm 6\%$ at reperfusion. (3) In group R, the intracellular pH was 6.85 ± 0.05 during ischemia and rose after reperfusion was started, but after rapid cooling, decreased again to 7.43 ± 0.02 after 60 minutes reperfusion. In group C, the pH was 6.88 ± 0.06 during ischemia and rose to 7.90 ± 0.09 60 minutes after reperfusion was started. (4) HSP70 was detected in both groups, but the concentration (luminescence) was lower in group R than in group C.

Conclusion: Rapid cooling reduces myocardial injury by preventing intracellular Ca overload at reperfusion via inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and also decreases the appearance of HSPs, the so-called homeostasis proteins. (*Ann Thorac Cardiovasc Surg* 2003; 9: 301–6)

Key words: rapid cooling shock, $\text{Na}^+/\text{Ca}^{2+}$ exchange system, heat shock protein (HSP)

Introduction

Total body hypothermia and myocardial cooling are considered effective methods for protecting the myocardium during ischemia in heart surgery. However, the protec-

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tive effect of total body hypothermia and local myocardial cooling before cardioplegia-induced cardiac arrest is somewhat negated by rapid cooling contracture, which results in coronary artery spasm and the subsequent unequal supply of cardioplegic solutions. In the unarrested heart in which circulation is maintained, the positive inotropic effect of hypothermia on the myocardium causes rapid cooling contracture,¹⁻³⁾ but the mechanism is not well understood. We confirmed a change in myocardial contractile force when isolated rat hearts were cooled for 3 minutes during perfusion with a Langendorff apparatus, thus confirming that hypothermia during perfusion has a

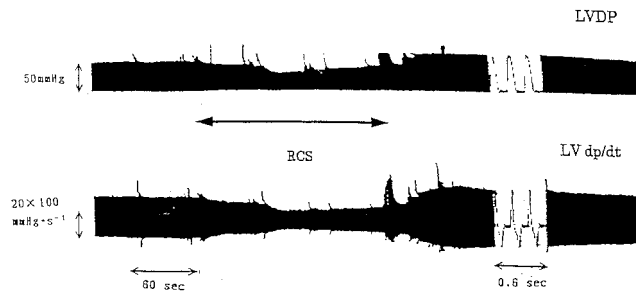


Fig. 1. Positive inotropism after myocardial hypothermia.

positive inotropic effect (Fig. 1). Heart rate decreased and contraction became weaker during rapid cooling. However, after rapid cooling, contractile force increased significantly ($p < 0.01$) to 32 ± 4 seconds and the left ventricular developed pressure was $120 \pm 10\%$ [means \pm standard error; (number of animals) $n=12$] higher than before rapid cooling. This experiment confirmed that rapid cooling has a positive inotropic effect. Since cardiac hypothermia during normothermic coronary circulation has a positive inotropic effect on the myocardium, we carried out rapid cooling at reperfusion after ischemia and found that this promotes early recovery of cardiac function.

Objectives

The aim of the present study was to investigate the myocardial protective effects of rapid cooling by examining the appearance of heat shock proteins (HSP) after rapid cooling and studying the correlation between HSP and cardiac function, intracellular Ca concentration, or intracellular pH.

Methods

The study was conducted in accordance with animal welfare standards based on Guidelines for Laboratory Animals (the Zoological Society of Japan).

Protocol (Table 1)

All processes were carried out at 37°C .

An isolated perfused rat heart model was set up using the method described by Inoue.⁴⁾

Male Wistar rats (body weight: 240-250 g) were systemically heparinized by injecting 3.5 mg (3,500 IU) of heparin into the peritoneal cavity under inhalation anesthesia with ether mixed with oxygen. A laparotomy was performed, and the thoracic cavity was then opened by

Table 1. Protocol (Langendorff perfusion)

Perfusion	Ischemia	Reperfusion
15 min 37°C	60 min 37°C	60 min 37°C

■RCS (+/-) 3 min after reperfusion

making an incision in the diaphragm. The heart was immediately excised together with the lungs and surrounding tissue and immersed in ice-cold Krebs-Henseleit bicarbonate (KHB) buffer to induce arrest. The ascending aorta was rapidly cannulated in the Langendorff mode, and the heart was restarted by perfusion with KHB solution. The KHB solution was gassed with 95% O_2 + 5% CO_2 to obtain a pH of 7.40 ± 0.05 , a PO_2 of 600 ± 100 mmHg, and a PCO_2 of 40 ± 5 mmHg.

The hearts were subjected to ischemia for 60 minutes, after which reperfusion was started. Three minutes after initiation of reperfusion, one group of hearts was rapidly cooled ($\leq 4^\circ\text{C}$) for 3 minutes: R group ($n=18$). The other group of hearts was not subjected to rapid cooling: C group ($n=19$). Myocardial temperature, heart rate, coronary flow, left ventricular contraction, intracellular Ca concentration (fura-2 fluorescence), and intracellular pH (BCECF-AM fluorescence) were then measured. Finally, the left ventricular myocardium was collected and homogenated, and HSP70 was measured by western blot.

Experimental apparatus (Fig. 2)

Using a glass perfusion apparatus with a double-tube structure, the perfusion fluid was maintained at a temperature of $37 \pm 0.5^\circ\text{C}$ by circulating warm water through the outer tube. A glass aortic cannula with an outer diameter of 2.0 mm and an inner diameter of 1.8 mm was inserted into the ascending aorta of the isolated heart, and the heart was then secured inside the heart chamber. Langendorff perfusion was carried out at 90 cm of water column pressure. A transverse incision was made in the pulmonary artery, a temperature probe was inserted into the apex of the heart, and left ventricular contraction was measured with an FD-pick-up. Heart rate was measured by placing electrocardiographic (ECG) electrodes on the anterior wall of the right ventricular outflow tract and the right atrial wall, taking care not to damage the coronary arteries. Coronary flow measured by collecting the coronary effluent that dripped into the heart chamber for 1 minute every 5 minutes.

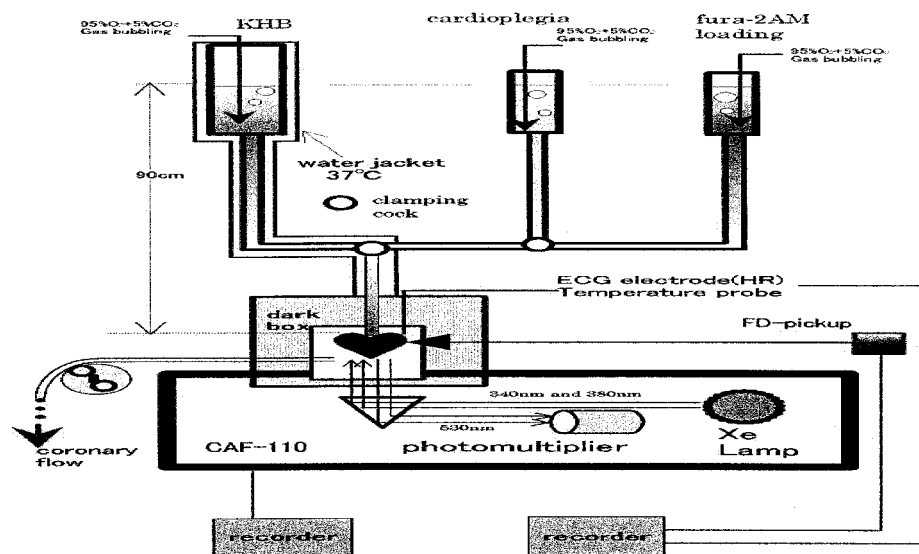


Fig. 2. Experimental apparatus.

Rapid cooling was achieved by removing the heart from the dark box of the apparatus used to measure the intracellular Ca concentration and intracellular pH and immersing the entire heart in a heart chamber filled with ice-cold saline (0–4°C) for 3 minutes. The heart was removed from the dark box only at the time of rapid cooling.

The intracellular Ca concentration was measured with a CAF-110 intracellular ion analyzer (JASCO Corp., Tokyo, Japan). The cells were loaded with fura-2 fluorescent dye for 15 minutes and then washed for 15 minutes, and the Ca concentration was calculated from the ratio of the fluorescence intensities emitted at 530 nm by excitation at 340 nm and 380 nm.

Fura-2AM in DMSO (Dojindo Laboratories, Kumamoto, Japan) esterified by an acetoxymethyl ester in 1 mMol dimethylsulfoxidepluronic acid solution was dissolved in oxygenated KHB solution together with the surfactant cremophor EL, and the heart muscle was perfused with this solution by the Langendorff method for 15 minutes to load the myocardial tissue and cells with fura-2AM. This method was used because all tissue and cells in a beating heart wave were to be loaded unlike in loading with fura-2 suspended cells and single myocardial cells.

Intracellular pH was measured with a Hitachi F-2000 spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) by loading the cells with BCECF-AM, washing the cells for 15 minutes, and calculating the pH from the ratio of the fluorescence intensities emitted at 530 nm by excitation at 450 nm and 490 nm.

Statistical analysis

Data were analyzed using ANOVA with the statistics program STATISTICA (StatSoft, Inc., Tulsa, OK, USA), and the experimental group were compared using Student's test. Results are expressed as mean±SE (standard error). Differences were considered significant at p value less than 0.05.

Results

Cardiac function and the intracellular Ca concentration are shown as a percentage of the pre-ischemia control value.

Heart rate (Fig. 3)

Heart rate was measured by placing ECG electrodes on the anterior wall of the right ventricular outflow tract and the right atrial wall.

In C group, the heart rate was 59±7% after 5 minutes reperfusion and 73±9% after 60 minutes of reperfusion.

In R group, the heart rate was 122±1% after 5 minutes of reperfusion, which was believed to be due to positive chronotropism. This later stabilized to 111.5±7% (p<0.05) after 15 minutes.

Coronary flow (Fig. 4)

Coronary flow measured by collecting the coronary effluent that dripped into the heart chamber for 1 minute every 5 minutes.

In C group, coronary flow was 60.5±6.5% after 5 minutes of reperfusion and 73±8.5% after 60 minutes of

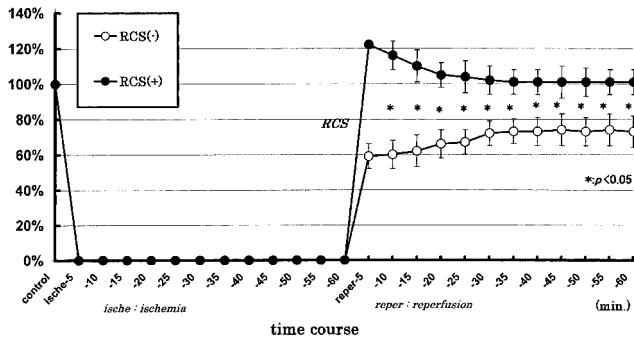


Fig. 3. Recovery of heart rate.

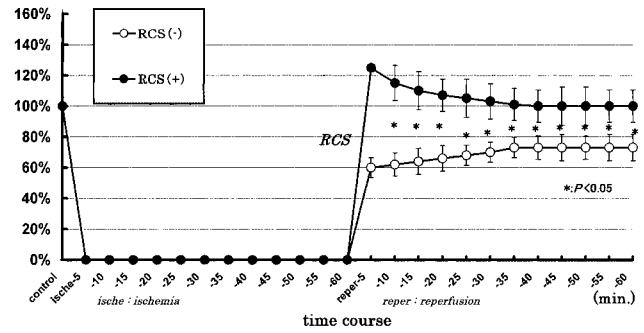


Fig. 4. Recovery of coronary flow.

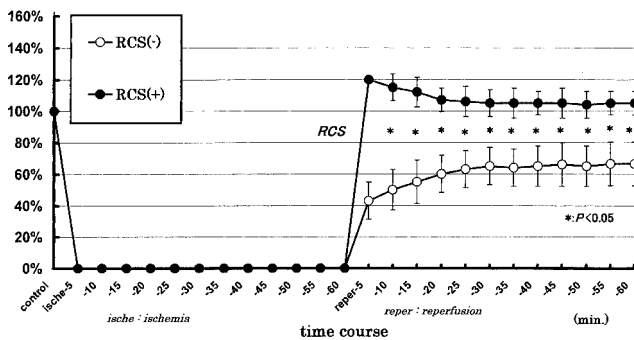


Fig. 5. Recovery of left ventricular contraction.

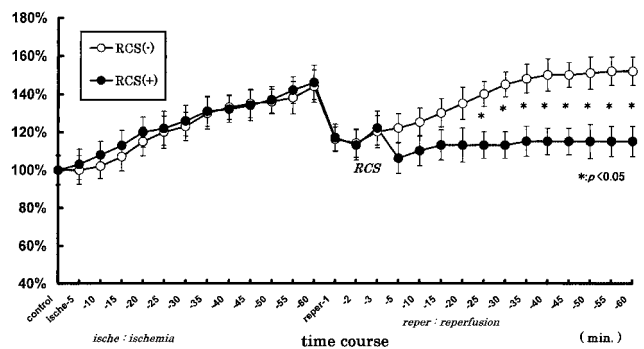


Fig. 6. Intracellular Ca concentration.

reperfusion.

In R group, coronary flow was $122 \pm 2\%$ after 5 minutes reperfusion and later stabilized to $112.5 \pm 10.5\%$ ($p < 0.05$) after 15 minutes.

Left ventricular contraction (Fig. 5)

The left ventricular contraction was measured with an FD-pick-up.

In C group, left ventricular contraction was $43 \pm 11\%$ after 5 minutes reperfusion and $63 \pm 2\%$ after 60 minutes reperfusion, indicating poor recovery.

In R group, left ventricular contraction was $122.5 \pm 7.5\%$ after rapid cooling and later stabilized to $105 \pm 0.05\%$ ($p < 0.05$).

Intracellular Ca concentration (Fig. 6)

The cells were loaded with fura-2 fluorescent dye for 15 minutes and then washed for 15 minutes and measured with a CAF-110.

In C group, the Ca concentration was $144 \pm 4\%$ during ischemia and rose further to $156 \pm 6\%$ at reperfusion.

In R group, the Ca concentration rose to $141 \pm 8\%$ during ischemia but decreased to $101 \pm 3\%$ at reperfusion after rapid cooling ($p < 0.05$).

Intracellular pH (Fig. 7)

Intracellular pH was measured with a Hitachi F-2000 spectrofluorometer by loading the cells with BCECF-AM.

In C group, the pH was 6.88 ± 0.06 during ischemia and rose to 7.90 ± 0.09 60 minutes after reperfusion was started.

In R group, the pH was 6.85 ± 0.05 during ischemia and rose after reperfusion was started. However, the pH decreased again after rapid cooling and was 7.43 ± 0.02 after 60 minutes reperfusion ($p < 0.05$).

HSP70 (Fig. 8)

HSP70 was measured by the western blot method.

The black line that was particularly concentrated almost in the center of the figure was HSP70 kDa.

HSP70 was detected in both groups, but the concentration (luminescence) was lower in R group than in C group.

Discussion

The dramatic improvements in the outcome of heart surgery in recent years have been largely due to the result of advances in methods of myocardial protection. Such methods include widely used hypothermia and local myocar-

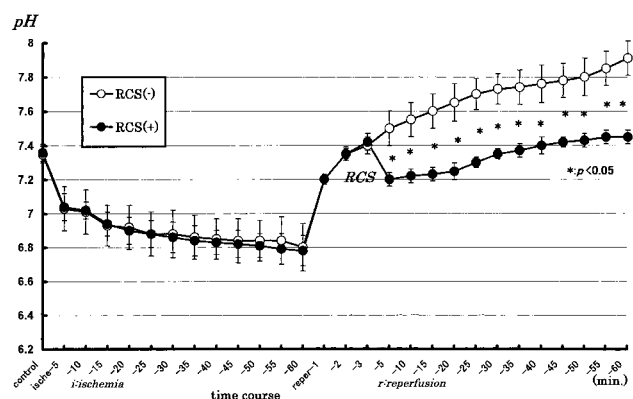


Fig. 7. Intracellular pH.

dial cooling, which protect the myocardium by lowering myocardial metabolism during ischemia, thus preserving high-energy phosphorylated compounds.

However, there is some debate among researchers as to the length of time and method of local myocardial cooling required to produce the best results. Sakai et al.^{5,6} and Horiuchi et al.⁷ of our department have reported that local cooling and infusion of cold cardioplegic solutions cause coronary artery spasm, resulting in the unequal supply of cardioplegic solution and a subsequent reduction in myocardial protection. Other studies have also found that the positive inotropic effect of cardiac hypothermia causes the myocardium to contract, resulting in injury.^{1-3,8} In a study using rabbits, Rebeyka et al.¹ observed that cardioplegic myocardial cooling in the unarrested state caused rapid myocardial contraction and subsequent myocardial damage that was apparent both in the biochemical findings and from left ventricular function. In the Rebeyka et al. study, the hearts were cooled before ischemia, and the rapid cooling referred to by Sakai et al. and Horiuchi et al. consisted of local cooling during ischemia induced by interruption of coronary perfusion. However, in the present study, the heart was cooled rapidly for a short period following resumption of normothermic perfusion (37°C) after ischemia. In a study of isolated rat hearts, Okada⁹ reported that rapid cooling during reperfusion after ischemia improved mitochondrial function and facilitated recovery of contraction.

After confirming that cooling has a positive inotropic effect, we carried out rapid cooling in the early stages of reperfusion after ischemia and investigated the effect on recovery of contractile force at reperfusion. The results showed that rapid cooling during perfusion increased the contractile force to 32 ± 4 seconds and the left ventricular

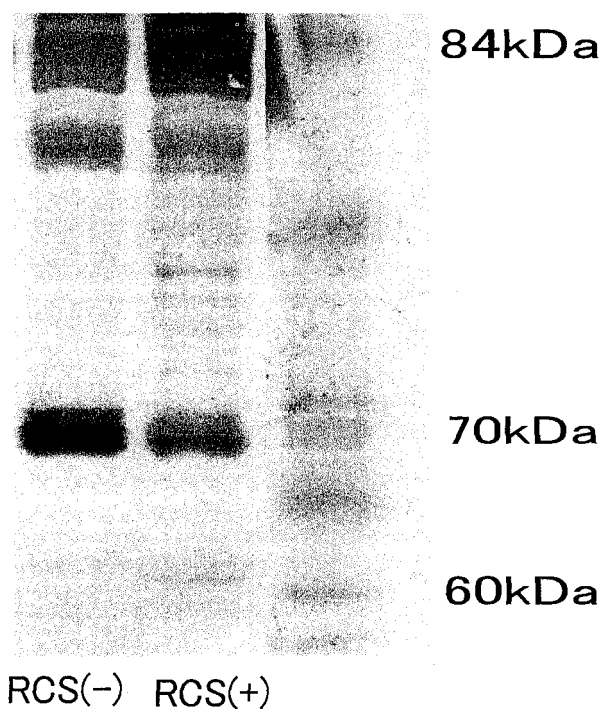


Fig. 8. Heat shock protein.

Western blot of protein obtained from the left ventricle of C group and R group.

developed pressure to $120 \pm 10\%$ of the value before cooling, confirming a positive inotropic effect. This was thought to be because, in the rapidly cooled heart, the sarcoplasmic reticulum releases sufficient amounts of Ca for contraction and because adenosine triphosphate (ATP) is preserved due to the effect of cooling on cyclic adenosine monophosphate production. This Ca and ATP is then used after rapid cooling, increasing the contractile force.

Ca, pH, and sodium dynamics within the cell play an important role in changes at the cellular level after ischemia-induced myocardial injury and reperfusion, and excessive concentrations of intracellular Ca eventually cause irreversible cell damage.¹⁰⁻¹³

However, even when ischemia is not severe enough to cause irreversible cell necrosis, the failure of cardiac function to recover after reperfusion results in transient contractile dysfunction, or myocardial stunning. The mechanism of myocardial stunning remains unknown, but a study by Kusaoka et al.¹⁴ suggests that intracellular Ca overload at reperfusion may be an important factor. Ca overload occurs via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. When reperfusion is started, the pH is rapidly corrected, acti-

vating the Na⁺/Ca²⁺ exchanger and causing Ca influx and overload. Stunning can therefore be reduced by slowing correction of acidosis at reperfusion, thus inhibiting the Na⁺/Ca²⁺ exchanger.¹⁵⁾

1) In the present study, a certain concentration of intracellular Ca was present after initiation of reperfusion because rapid cooling did not inhibit Ca-induced Ca release from the sarcoplasmic reticulum,^{16,17)} and that Ca was believed to be the trigger for early recovery of contractile force. Rapid cooling protected the myocardium at reperfusion after ischemia by slowing the sudden correction of acidosis that occurs at reperfusion, thus inhibiting the H⁺/Na²⁺ exchanger and, in turn, the Na⁺/Ca²⁺ exchanger, and preventing intracellular Ca overload.

2) Our findings suggest that as well as preventing intracellular Ca overload at reperfusion, rapid cooling inhibits the appearance of HSPs, the so-called homeostasis proteins, by reducing stress due to Ca overload-induced myocardial injury.

3) The exchange of information (unfolded protein response) between the endoplasmic reticulum chaperone and the nucleus induced by large amounts of ATP and NAD⁺ at reperfusion results in the movement of HSP70 into the nucleus, where it inhibits overactivation of poly (ADP-ribose) synthetase. However, since rapid cooling preserves ATP,¹⁸⁾ HSP70 was presumably proportionate.

Conclusion

Rapid cooling reduces stress caused by Ca overload-induced myocardial injury by inhibiting the sudden correction of acidosis at reperfusion after ischemia and also decreases the appearance of HSP70 by preserving ATP.

A summary of this paper was presented at the 24th Meeting of the Myocardial Metabolism Research Society.

References

1. Rebeyka IM, Hanan SA, Borges MR, et al. Rapid cooling contracture of the myocardium. The adverse effect of prearrest cardiac hypothermia. *J Thorac Cardiovasc Surg* 1990; **100**: 240–9.
2. Kusuoka H, Ikoma Y, Futaki S, et al. Positive inotropism in hypothermia partially depends on an increase in maximal Ca-activated force. *Am J Physiol* 1991; **261**:

- H1005-10.
3. Bridge JHB. Relationship between the sarcoplasmic reticulum and sarcolemmal calcium transport revealed by rapidly cooling rabbit ventricular muscle. *J Gen Physiol* 1986; **88**: 437–73.
4. Inoue K. Myocardial protective effect of various types of cardioplegic solutions in isolated rat hearts. *J Sho Med Assoc* 1979; **39**: 143–57.
5. Sakai T, Fujii K, Shimizu R. The “rapid cooling contraction” of the thymol-treated muscle fibers. *Jikeikai Med J* 1968; **15**: 187–201.
6. Sakai T, Fujii K, Takemoto N. Thymolcontracture and rapid cooling contraction of the thymol-treated muscle fibers. *Jikeikai Med J* 1964; **14**: 164–76.
7. Horiuchi M, Inoue K, Kashima T, et al. A study of cardioplegic solutions containing papaverine hydrochloride. *Nippon Kyobu Geka Gakkai Zasshi* 1988; **36**: 48–55. (in Japanese)
8. Monroe RG, Strang RH, LaFarge CG, Levey J. Ventricular performance, pressure-volume relationship and O₂ consumption during hypothermia. *Am J Physiol* 1964; **206**: 67–73.
9. Okada T. Facilitating effect of cold shock on recovery from anoxia-induced contractile depression in isolated rat heart and heart muscle. *Circ Res* 1988; **62**: 338–46.
10. Wier WG. Cytoplasmic [Ca²⁺] in mammalian ventricle: dynamic control by cellular processes. *Annu Rev Physiol* 1990; **52**: 467–85.
11. Guarnieri T. Intracellular sodium-calcium exchange dissociation in early contractile failure in hypoxic ferret papillary muscle. *J Physiol* 1987; **388**: 449–65.
12. MacLeod KT. Regulation and interaction of intracellular calcium, sodium and hydrogen ions in cardiac muscle. *Cardioscience* 1991; **2**: 71–85.
13. Mochizuki M. Molecular mechanism of ischemic myocardial injury. In: Yasaki Y ed.; *Molecular Basis of Heart Disease*. Yodosha Co., Ltd., Tokyo, 1994; pp 72–83.
14. Kusaoka H, Koretsune Y, Chacko VP. Excitation-contraction coupling in postischemic myocardium: does failure of activated Ca²⁺ transients underlie “stunning”? *Circ Res* 1990; **66**: 1268–76.
15. Hori M, Kitakaze M, Sato H, et al. Staged reperfusion attenuates myocardial stunning in dogs. *Circulation* 1991; **84**: 2135–45.
16. Endo M. Mechanism of myocardial cell contraction and relaxation and intracellular calcium handling. In: Yasaki Y ed.; *Molecular Basis of Heart Disease*. Yodosha Co., Ltd., Tokyo, 1994; pp 25–36.
17. Yamamoto H, Ichikawa H, Yamamoto F. Diltiazem abolishes the effect of ryanodine in St. Thomas’ Hospital cardioplegic solution on the post-ischemic functional recovery. *Nippon Kyobu Geka Gakkai Zasshi* 1995; **43**: 19–25. (in Japanese)
18. Hearse DJ, Stewart DA, Chain EB. Recovery from cardiac bypass and elective cardiac arrest. *Circ Res* 1974; **35**: 448.