

## Cardioprotective Effects of Tetrahydrobiopterin in Cold Heart Preservation after Cardiac Arrest

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**Background:** It has recently been shown that tetrahydrobiopterin (BH<sub>4</sub>), an essential cofactor of nitric oxide synthase (NOS), reduces ischemia-reperfusion myocardial injury. The aim of this study was to determine if supplementation with BH<sub>4</sub> after cardiac arrest followed by cold heart preservation would exert a cardioprotective effect against ischemia-reperfusion injury.

**Materials and Methods:** Isolated perfused rat hearts were subjected to 4°C cold ischemia and reperfusion. Hearts were treated with cold cardioplegic solution with or without BH<sub>4</sub> just before ischemia and during the first 5 min of reperfusion period. Effects of BH<sub>4</sub> on left ventricular function, myocardial contents of high-energy phosphates, and nitrite plus nitrate were measured in the perfusate, before ischemia and after reperfusion. Moreover, the effect of BH<sub>4</sub> on the cold-heart preservation followed by normothermic (37°C) ischemia was determined.

**Results:** BH<sub>4</sub> improved the contractile and metabolic abnormalities in reperfused cold preserved hearts that were subjected to normothermic ischemia. Furthermore, BH<sub>4</sub> significantly alleviated ischemic contracture during ischemia, and restored the diminished perfusate levels of nitrite plus nitrate after reperfusion.

**Conclusion:** These results demonstrated that BH<sub>4</sub> reduces ischemia-reperfusion injury in cold heart preservation. The cardioprotective effect of BH<sub>4</sub> implies that BH<sub>4</sub> could be a novel and effective therapeutic option in the preservation treatment of donor heart after cardiac arrest. (*Ann Thorac Cardiovasc Surg* 2006; 12: 95–104)

**Key words:** myocardial ischemia-reperfusion injury, oxygen free radicals, tetrahydrobiopterin, nitric oxide synthase, heart preservation

### Introduction

Recently, heart transplantations have been performed in Japan. However, at present it is difficult to obtain donor hearts. Consequently, if ischemic-reperfusion injury can be reduced by preservation of the donor heart by some means after cardiac arrest, this may help the advancement of heart transplantation in Japan. The availability of tetrahydrobiopterin (BH<sub>4</sub>) is essential for the catalytic

activity of nitric oxide synthases (NOS).<sup>1)</sup> A close link between cellular availability of BH<sub>4</sub> and nitric oxide (NO) synthesis has recently been demonstrated in a number of different cell types.<sup>2,3)</sup> Biochemical evidence revealed that activation of purified constitutive NOS in the presence of suboptimal levels of BH<sub>4</sub> results in uncoupling of oxygen reduction and arginine oxidation.<sup>4)</sup> In agreement with these results, Cosentino et al.<sup>5)</sup> recently proposed that in isolated canine coronary arteries depleted of BH<sub>4</sub>, endothelial NOS may serve as a source of oxygen free radicals (OFRs). Furthermore, in human aortic endothelial cells exposed to prolonged stretching, inhibition of BH<sub>4</sub> synthesis has been shown to increase markedly the production of superoxide anions.<sup>6)</sup> These results indicate that NOS itself can be a potential source for endothelial production of OFRs, and that decreased availability of BH<sub>4</sub>

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may cause a shift in balance between production of protective NO and toxic OFRs.<sup>4)</sup> Such an imbalance, in turn, could result in endothelial dysfunction and oxidative vascular injury as described in a number of vascular diseases.<sup>7,8)</sup>

Accordingly, these findings led to the hypothesis that when there is an insufficiency of BH<sub>4</sub>, such dysfunctional NOS participate in oxidative injury, especially under pathological conditions including ischemia-reperfusion. In fact, administration of exogenous BH<sub>4</sub> has been shown to reduce post-ischemic endothelial dysfunction,<sup>9)</sup> post-transplantation lung edema, OFRs injury in grafts,<sup>10)</sup> and ischemic renal injury.<sup>11)</sup> Moreover, a recent experimental study suggested that intracellular BH<sub>4</sub> levels are reduced after ischemia-reperfusion.<sup>9)</sup>

Previously, we investigated the effectiveness of BH<sub>4</sub> on ischemia-reperfusion myocardial injury in the isolated perfused rat heart using Langendorff apparatus and discovered that BH<sub>4</sub> reduces ischemia-reperfusion injury.<sup>12)</sup> Furthermore, it was confirmed that a decrease in BH<sub>4</sub> accelerates ischemia-reperfusion injury.<sup>13)</sup> Although further studies are necessary concerning the cardioprotective effect of BH<sub>4</sub>, it is likely that studies addressing the role of BH<sub>4</sub> on the heart may have a significant impact on clarification of the pathogenesis of ischemic heart disease and the preservation of donor hearts.

## Materials and Methods

The animals used in this study were used in accordance with the Guidelines for Animal Experimentation of the University of the Ryukyus, and the experimental protocol was approved by the Animal Care Committee of this institution. No mention of when the rats were humanely sacrificed.

### Experimental preparation

Male Sprague-Dawley rats (Japan SLC, Inc., Shizuoka, Japan) weighing 260 to 310 g were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg) and were given 1,000 IU/kg body weight sodium heparin intravenously. After thoracotomy, the heart was rapidly excised, the ascending aorta was cannulated, and retrograde perfusion of the heart was initiated on Langendorff apparatus at a constant pressure of 100 cm H<sub>2</sub>O. The isolated heart was perfused with Krebs-Henseleit solution (KHS) of the following composition (in mmol/L): NaCl 120, KCl 4.8, CaCl<sub>2</sub> 1.25, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0, and glucose 11.0, at 37°C. The perfusate was oxygenated

with 95% O<sub>2</sub>/5% CO<sub>2</sub> (partial pressure of O<sub>2</sub>>600 mmHg).

A thin-wall latex balloon was inserted into the left ventricle (LV) through the left atrium to monitor LV pressure (LVP). The balloon was filled with bubble-free saline. The ventricle was loaded with 5-10 mmHg of the initial LV end-diastolic pressure (LVEDP), and this balloon volume was maintained throughout the experiments. LVP was measured with a pressure transducer (TP-400T, Nihon Kohden Corp., Tokyo, Japan) and the first derivative (dp/dt) of LVP was derived from differentiating the signal of LVP using an electronic differentiator (ED-601G, Nihon Kohden Corp.). LV developed pressure (LVDP) was estimated from LV systolic pressure (LVsP) and LVEDP. The mean coronary flow (CF) was measured with an electromagnetic flow probe (FF-030T, Nihon Kohden Corp.) attached to the aortic cannula, which was connected to an electromagnetic flowmeter (MFV-3200, Nihon Kohden Corp.). Heart rate (HR) was counted by a cardiometer (AT-600G, Nihon Kohden Corp.) triggered by the pressure pulse. After 10 min of equilibration, the hearts were atrially paced throughout the experiments by an electronic stimulator (SEN-3301, Nihon Kohden Corp.). Pacing rate was set at 110% of the natural beat during the stabilizing period of Langendorff perfusion as described previously.<sup>12,13)</sup>

### Experimental protocol

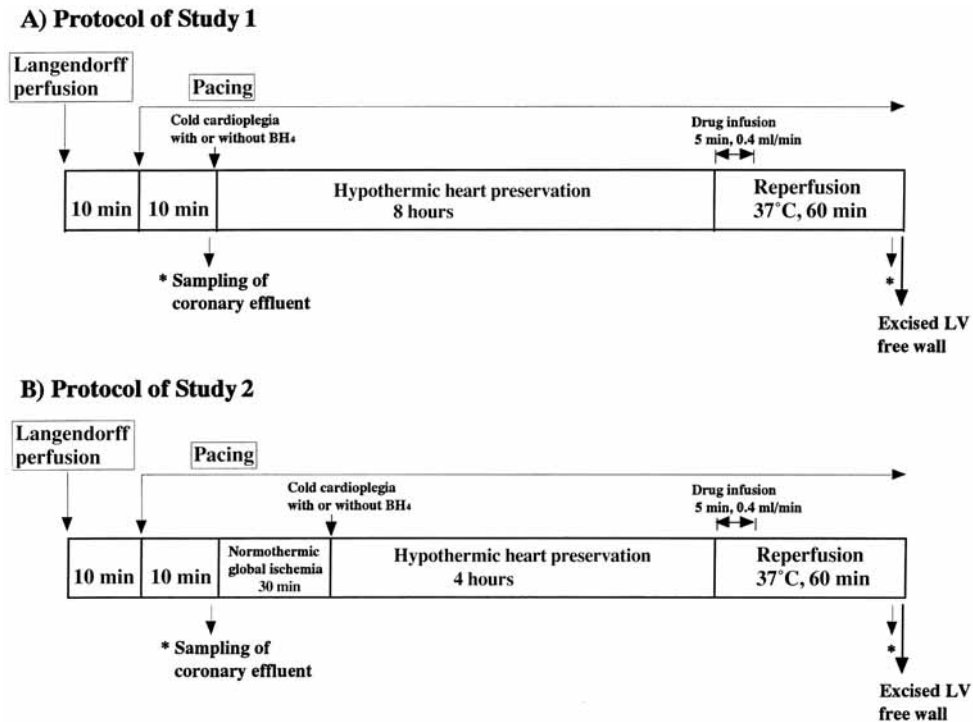
The schema of study is illustrated in Fig. 1.

#### Study 1 (Fig. 1A)

All hearts were perfused for 10 min to stabilize hemodynamics before the experiment was started. Five min after the beginning of atrial pacing, baseline values of cardiovascular parameters were measured.

The hearts were divided into control and BH<sub>4</sub> groups, and were infused with Miotecter<sup>®</sup> (so called St. Thomas No. 2 cardioplegic solution) of the following composition (in mg/L): NaCl 6,428.4, KCl 1,192.8, CaCl<sub>2</sub>-2H<sub>2</sub>O 176.4, MgCl<sub>2</sub>-6H<sub>2</sub>O 3,252.8, and NaHCO<sub>3</sub> 840.0 (control group, n=8), or BH<sub>4</sub> (Miotecter<sup>®</sup> with BH<sub>4</sub>, BH<sub>4</sub> group, n=8) at 4°C for cardioplegic arrest. Cold cardioplegic solution (Miotecter<sup>®</sup>, 20 ml/kg) with or without BH<sub>4</sub> (2.5 mg) was infused at an infusion rate of 10 ml/min by using an infusion pump (Model 11 or 22, Harvard Apparatus Co., Holliston, Mass., USA).

After cardioplegic arrest was obtained, the hearts were subjected to cold ischemia (hearts preservation) at 4°C for 8 hours, followed by 60 min reperfusion. The ischemic hearts were submerged in a chamber filled with the same cardioplegic solution with or without BH<sub>4</sub>. This solution



**Fig. 1.** Schema of study protocol.

**A:** Protocol of study 1. Cold cardioplegic solution (Miotecter®, 20 ml/kg) with or without BH<sub>4</sub> (2.5 mg) was used. After cardioplegic arrest was obtained, the hearts were subjected to cold ischemia (hearts preservation) at 4°C for 8 hours, followed by 60 min reperfusion.

**B:** Protocol of study 2. The perfused hearts were subjected to normothermic global ischemia at 37°C for 30 min followed with 4 hours of cold ischemia and subsequently 60 min reperfusion.

was equilibrated in advance with a gas mixture of 95% O<sub>2</sub>/5% CO<sub>2</sub>, and maintained at 4°C. During ischemia, peak LVEDP and the time to onset of ischemic contracture for LVEDP to elevate more than 5 mmHg were measured. After completion of cold ischemia, the solution in the chamber was drained and the hearts were reperfused with an aerobic KHS for 60 min. BH<sub>4</sub> (1.25 mg/ml) and vehicle (KHS) were infused through a three-way valve placed just proximal to the aortic cannula by using an infusion pump during the first 5 min of reperfusion period. The dosages of BH<sub>4</sub> in coronary perfusate (nearly 100 μM) used in this study were chosen considering the previous in vitro findings that concentration-dependent NO production, which was estimated as L-citrulline formation by human endothelial NOS, was observed at doses of BH<sub>4</sub> ranging from 10 to 100 μM.<sup>14)</sup> The sampling of perfusate was performed before ischemia (baseline) and at 60 min after the initiation of reperfusion. Samples were stored at -20°C for measurement of NO<sub>x</sub> (nitrite plus nitrate) level.

At the end of experiments, sections of the LV free wall

were excised quickly and were frozen in liquid nitrogen. These frozen myocardial sections were used for energy metabolites in myocardial tissues.

#### Study 2 (Fig. 1B)

All hearts were perfused for 10 min to stabilize hemodynamics before the experiment was started. Five min after the beginning of atrial pacing, baseline values of cardiovascular parameters were measured.

The perfused hearts were subjected to 4 hours of cold ischemia followed with normothermic global ischemia at 37°C for 30 min, and subsequently 60 min of reperfusion. The ischemic hearts were submerged in a chamber filled with the modified KHS<sup>15)</sup> which contained 11.0 μM 2-amino-2-hydroxymethyl-1, 3-propanediol hydrochloride (Tris-HCl) instead of 11.0 μM glucose. This solution was equilibrated in advance with a gas mixture of 95% N<sub>2</sub>/5% CO<sub>2</sub> (partial pressure of O<sub>2</sub> <10 mmHg), and maintained at 37°C to prevent hypothermia-induced cardioprotection.

After normothermic global ischemia, the hearts were

divided into control and BH<sub>4</sub> groups, and were infused with vehicle (Miotecter<sup>®</sup>, ischemic-control group, n=8), or BH<sub>4</sub> (Miotecter<sup>®</sup> with BH<sub>4</sub>, ischemic-BH<sub>4</sub> group, n=8) at 4°C for cardioplegic arrest, in the same manner as study 1. Following this, the hearts were subjected to cold ischemia (heart preservation) at 4°C for 4 hours followed by 60 min reperfusion. BH<sub>4</sub> (1.25 mg/ml) and vehicle (KHS) were infused during the first 5 min of the reperfusion period as in study 1.

Sampling of perfusate and sections of the LV free wall were performed as described in study 1.

#### Determination of myocardial energy metabolites

The frozen myocardial tissue for measurement of energy metabolites was lyophilized for 6 hours. The dried tissue was homogenized with 0.6 M perchloric acid. The mixture was centrifuged at 12,000 rpm for 15 min at 2°C, and the supernatant was used for assay. Adenosine triphosphate (ATP) was determined by the firefly luminescence method, using an ATP monitoring agent (LL-100-2, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan) and a lumiphotometer (Minilumat LB9506, Berthold GmbH & Co. KG, Calmbacher, Germany).

#### Determination of myocardial tissue lipid peroxidation

The extent of lipid peroxidation in the frozen myocardial tissue was measured by the thiobarbituric acid (TBA) method<sup>15)</sup> with some modifications. The amount of TBA reactive substances was estimated as malondialdehyde (MDA) equivalents per gram wet myocardial weight. The developed color was read using a spectrophotometer (UV-2200A, Shimadzu Corp., Kyoto, Japan) at 532 nm. Commercially available 1,1,3,3-tetraethoxypropane (TEP) was used as a standard.

#### Determination of NO<sub>x</sub> level in the effluent

NO<sub>x</sub> levels in the effluent before ischemia and at 10 min after the initiation of reperfusion were analyzed using the Griess method with an automated NO detector-high-performance liquid chromatography system (ENO-20, Eicom Corp., Kyoto, Japan). In the non-ischemic groups, NO<sub>x</sub> level in the effluent was analyzed in the same time course as the ischemic groups. The absorbance of the color of the product dye was measured at 540 nm. Appropriate concentrations of NaNO<sub>2</sub> and NaNO<sub>3</sub> were used for constructing standard curves. The time/voltage change was traced by a data processor (EPC-300, Eicom Corp.). The concentration of NO<sub>x</sub> in the effluent was determined by comparing the peak area on the chromatogram with a stan-

dard. According to the manufacturer, the detection limit in this assay is 10 pmol/ml.

#### Drugs

The drugs employed in this study were (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH<sub>4</sub>) (Wako, Japan), Miotecter<sup>®</sup> (St. Thomas No. 2 solution, Kobayashi Pharmaceutical Co., Ltd., Tokyo, Japan), TBA (Sigma, Japan), 1,1,3,3-TEP (Sigma, Japan), NaNO<sub>2</sub> (Wako, Japan) and NaNO<sub>3</sub> (Wako, Japan).

#### Data analysis

The data were analyzed by one-way analysis of variance, and paired and unpaired observations were analyzed with paired and unpaired t-tests, respectively. All values are presented as means ± standard error. Statistical significance was defined as p<0.05.

### Results

#### Study 1

##### *Effect of BH<sub>4</sub> on LV function*

Baseline values of cardiovascular parameters such as HR, LVsP, LVEDP, LVDP, max and min LV dp/dt, and CF were similar in all groups (Table 1).

During 8 hours of cold ischemia (period of heart preservation), LVDP and CF immediately decreased to almost zero in all groups after injection of cold cardioplegic solution (20 ml/kg) with or without BH<sub>4</sub>; this was continued for 8 hours of heart preservation at 4°C. During 60 min reperfusion these parameters showed partial recoveries (Fig. 2). Recoveries of LVDP and CF during reperfusion were comparable between the two groups (Figs. 2A and 2C). LVEDP increased during ischemia and increased again immediately after the onset of reperfusion in both groups. The increases in LVEDP were comparable between the two groups (Fig. 2B).

##### *Metabolic changes and NO<sub>x</sub> level in the coronary effluent*

Fig. 3A shows the high-energy phosphate ATP in the LV free wall at 60 min of reperfusion. In non-ischemic Langendorff perfusion hearts in this experimental system, myocardial contents of ATP was 30.3±3.6 μmol/g dry weight of myocardial tissue, which should reflect the state of myocardial oxidative metabolism (data not shown). There was a marked decrease of ATP in both cold ischemic hearts, although, the high-energy phosphate ATP content was comparable in the two groups (Fig. 3A).

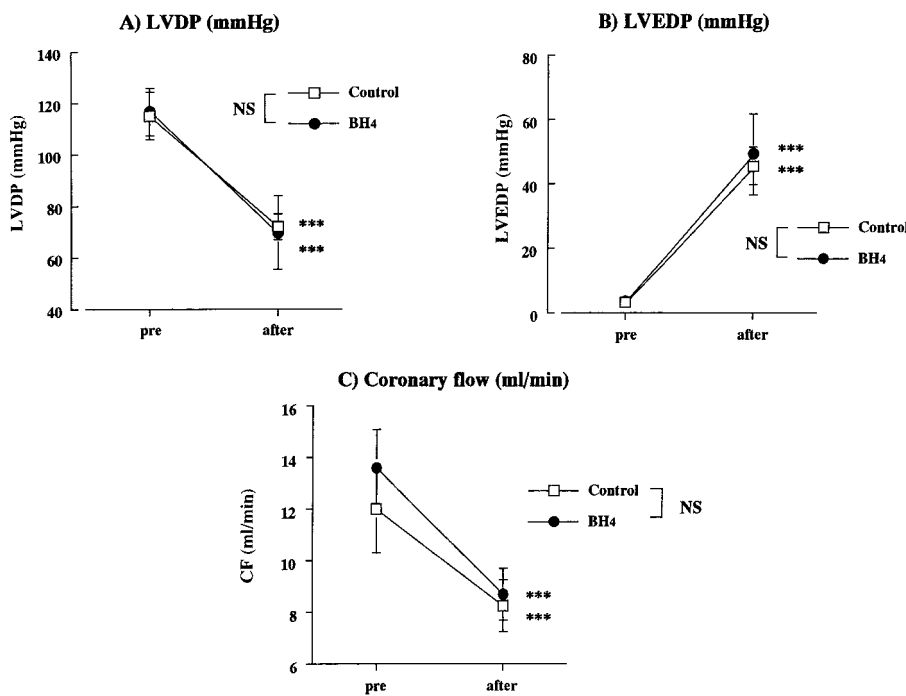
Baseline values (before ischemia) of NO<sub>x</sub> in the coro-

**Table 1. Baseline values of cardiovascular parameters**

Group	HR (beats/min)	LVsP (mmHg)	LVEDP (mmHg)	LVDP (mmHg)	Max LV dp/dt (mmHg/sec)	Min LV dp/dt (mmHg/sec)	CF (ml/min)
Control (n=8)	347.5±2.5	118.3±10.0	5.3±0.3	115.2±9.1	4,018.8±84.5	2,106.3±55.5	12.0±1.7
BH <sub>4</sub> (n=8)	347.5±2.5	120.3±10.2	5.4±0.4	116.8±9.2	3,987.5±89.5	2,081.3±54.2	13.6±1.5
Isc control (n=8)	347.5±2.5	111.0±9.5	6.3±0.4	108.8±7.6	4,118.8±121.7	2,212.5±66.6	13.0±1.2
Isc BH <sub>4</sub> (n=8)	347.5±2.5	112.5±8.9	5.6±0.4	109.9±8.9	4,031.3±54.2	2,206.3±44.8	13.8±1.4

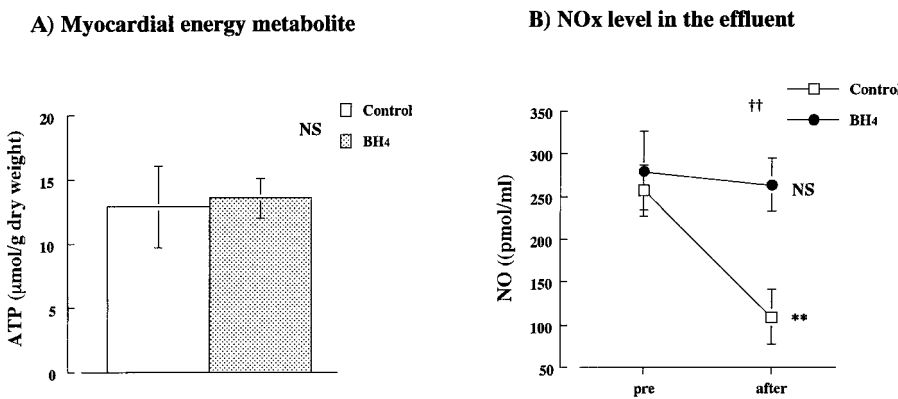
Control, 8 hours cold heart preservation without BH<sub>4</sub>; BH<sub>4</sub>, 8 hours cold heart preservation with BH<sub>4</sub>; isc control, 4 hours cold heart preservation after normothermic global ischemia without BH<sub>4</sub>; isc BH<sub>4</sub>, 4 hours cold heart preservation after normothermic global ischemia with BH<sub>4</sub>; HR, heart rate; LVsP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; max LV dp/dt, maximum left ventricular dp/dt; min LV dp/dt, minimum left ventricular dp/dt; CF, coronary flow.

All values are expressed as means±s.e. There were no significant differences among groups.



**Fig. 2.** Change in **A)** left ventricular developed pressure (LVDP), **B)** left ventricular end diastolic pressure (LVEDP), and **C)** mean coronary flow (CF) at 60 min of reperfusion followed by 8 hours cold heart preservation.

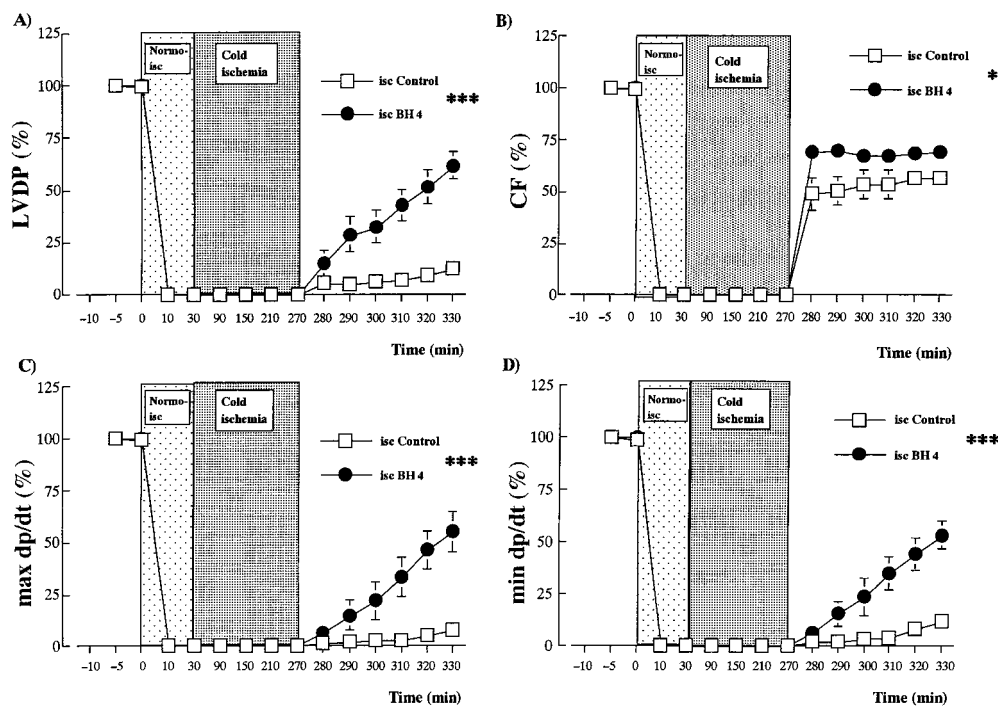
Each value represents mean±s.e. No significant differences were observed in the groups.



**Fig. 3.**

**A:** Metabolic changes.

**B:** NOx (nitrite plus nitrate) level in the coronary effluent. Each value represents mean±s.e. \*\*p<0.01, vs before ischemia, ††p<0.01, vs control group.



**Fig. 4.**  
**A:** Time-course of changes in LVDP.  
**B:** CF.  
**C:** Maximum LV dp/dt.  
**D:** Minimum LV dp/dt in ischemic-control group (n=8, open square) and ischemic BH<sub>4</sub> group (n=8, closed circle). Values are expressed as percentage of corresponding baseline values. Each value represents mean ± s.e. \*p<0.05, \*\*\*p<0.001.

nary effluent were not different in the two groups (Fig. 3B). In the BH<sub>4</sub> group, NO<sub>x</sub> levels after the reperfusion period was 263.5 ± 30.3 pmol/ml and this was similar to the baseline value of 280.0 ± 45.5 pmol/ml. By contrast, in the control group the level of NO<sub>x</sub> decreased markedly from 256.9 ± 30.5 pmol/ml before ischemia to 108.9 ± 32.5 pmol/ml after ischemia (p<0.01). The level of NO<sub>x</sub> in the BH<sub>4</sub> group after ischemia was significantly higher than that in the control group (p<0.01, Fig. 3B).

## Study 2

### Effect of BH<sub>4</sub> on LV function

During reperfusion of the ischemic BH<sub>4</sub> group significantly better recoveries of LVDP, max and min LV dp/dt, and CF were observed than in the ischemic-control group (Fig. 4). LVEDP increased during ischemia and increased again immediately after the onset of reperfusion (Fig. 5A). In the ischemic BH<sub>4</sub> group the increases in LVEDP both during ischemia and reperfusion were significantly smaller than that in the ischemic-control group (Fig. 5A). In addition, during ischemia, significantly delayed onset

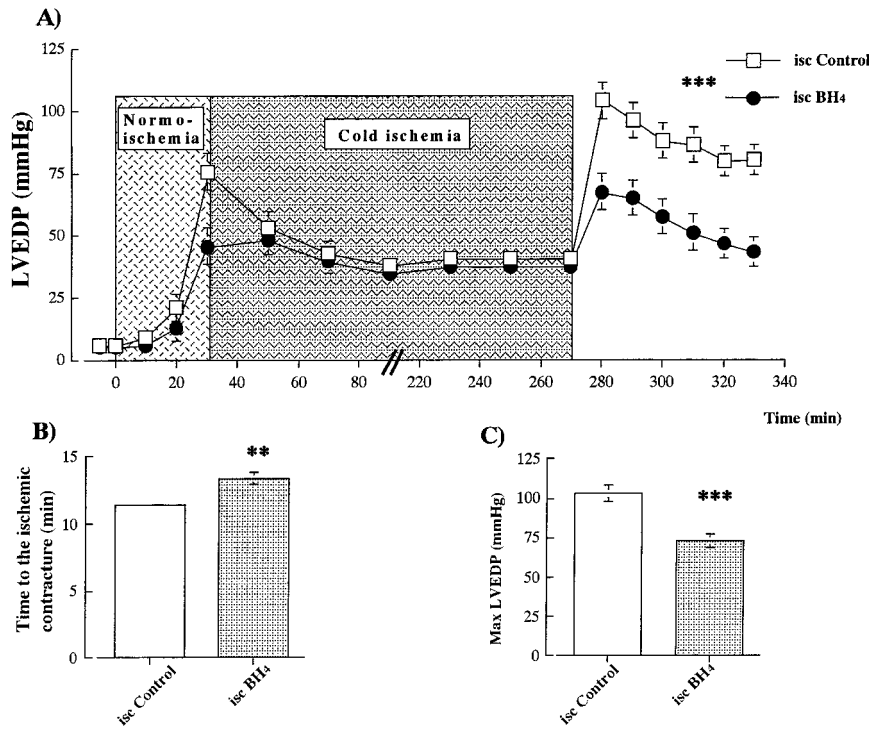
of ischemic contracture and lower maximal values of LVEDP were seen in the ischemic BH<sub>4</sub> group compared with the corresponding values in the ischemic-control group (Figs. 5B and 5C).

### Metabolic changes

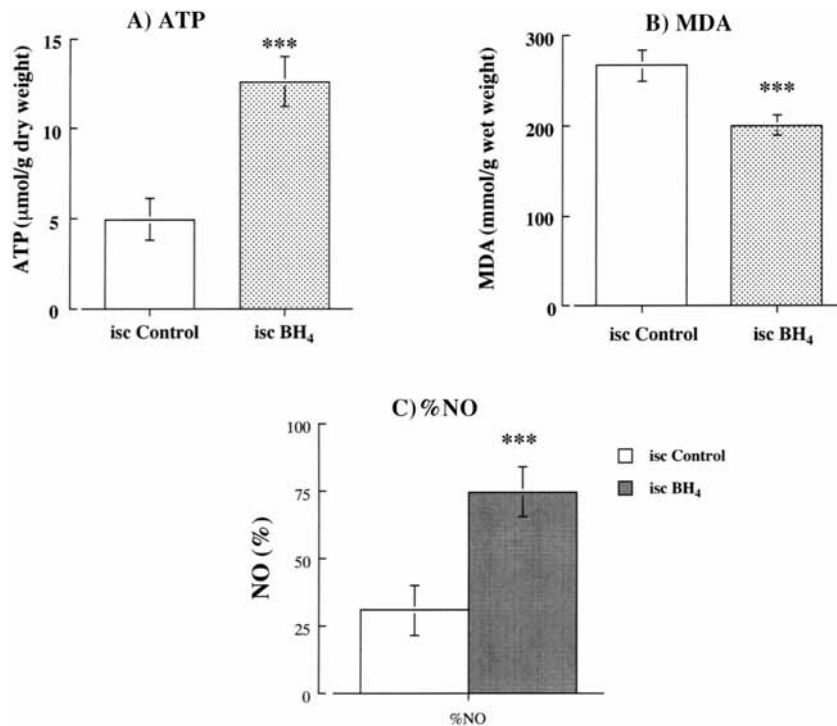
Fig. 6 shows the energy metabolism in the LV free wall at 60 min reperfusion performed after 30 min of normothermic ischemia with 4 hours cold heart preservation. The high-energy phosphate ATP levels in the ischemic-BH<sub>4</sub> group (12.6 ± 1.39 μmol/g dry weight) were significantly higher than that in the ischemic-control group (4.96 ± 1.16 μmol/g dry weight) (p<0.01, Fig. 6A). Myocardial content of MDA in the ischemic BH<sub>4</sub> group (200.65 ± 11.18 mmol/g wet weight) were significantly lower than that in the ischemic Control group (266.87 ± 17.25 mmol/g wet weight) (p<0.01, Fig. 6B).

### NO<sub>x</sub> level in the coronary effluent

There were no differences in the baseline values (before ischemia) of NO<sub>x</sub> level in the coronary effluent between



**Fig. 5.**  
**A:** Time-course of changes in left ventricular end-diastolic pressure (LVEDP).  
**B:** The time to onset of ischemic contracture.  
**C:** Peak LVEDP during ischemia.  
 Each value represents mean ± s.e.  
 \*\*p<0.01, \*\*\*p<0.001.



**Fig. 6.** Energy metabolites and NOx (nitrite plus nitrate) level in the coronary effluent.  
 ATP, adenosine triphosphate; MDA, malondialdehyde.  
 %NO are expressed as a percentage of the values before ischemia.  
 Each value represents mean ± s.e.  
 \*\*\*p<0.001

the two groups. In the ischemic-control group (n=8), the levels of NOx decreased markedly from 340.6 ± 68.5 pmol/ml before ischemia to 76.9 ± 16.7 pmol/ml after ischemia (p<0.001). By contrast, in the ischemic-BH<sub>4</sub> group (n=8), there was a decrease of NOx levels from

380.0 ± 79.1 pmol/ml before ischemia to 247.5 ± 32.7 pmol/ml after ischemia. In the ischemic-BH<sub>4</sub> group the NOx level after reperfusion was significantly higher than that in the ischemic-control group (p<0.001). As shown in Fig. 6C the NOx level in the ischemic-BH<sub>4</sub>

group after ischemia ( $75.0 \pm 9.4\%$ ), which is expressed as a percentage of the value before ischemia, was significantly higher than that in the ischemic-control group ( $31.1 \pm 9.3\%$ ) ( $p < 0.001$ ).

## Comments

Recently, heart transplantation has been performed in Japan. However, at the present it is difficult to find donor hearts. In Japan, generally brain death is not accepted. A person's death is considered to be cardiac arrest. Therefore it is difficult to deal with cooling and drug treatment for example before cardiac arrest. It will be important from now on to study whether donor heart protection by treatment after the normothermic cardiac arrest is possible. If the heart came to a standstill due to anoxia and there was no protection against ischemia, tissue damage would occur to the heart. To date, no studies have been reported with hearts in which function has been interrupted by controlled arrest through cardioplegia, and subsequently resuscitated for use in heart transplantation. However, if ischemia-reperfusion injury can be mitigated through the preservation of the donor heart by some means after cardiac arrest, this is likely to advance the development of heart transplantation.

It has been reported that the suppression of energy consumption of the heart by lowering of the temperature to  $4-7.5^\circ\text{C}$ <sup>16)</sup> is important in heart preservation when using St. Thomas fluid for soaking. The physiological basis for the protection of cells by low temperature during ischemia is the suppression of metabolic demands in organs.<sup>17)</sup> The result of study 1 reported here suggests that there is a significant protective effect by low temperature when the heart is subjected to 8 hours of cold preservation. The preservation via ATP and the effect of BH<sub>4</sub> for the attenuation of myocardial injury after reperfusion were not clearly demonstrated. However, BH<sub>4</sub> was effective in increasing the NO<sub>x</sub> level.

Previously we reported that relatively low concentrations of BH<sub>4</sub> attenuates myocardial ischemia-reperfusion injury in isolated perfused rat hearts,<sup>12)</sup> and that inhibition of BH<sub>4</sub> synthesis impairs the myocardial production of NO and aggravates myocardial ischemia-reperfusion injury.<sup>13)</sup> Inhibition of BH<sub>4</sub> synthesis also impairs baseline values of cardiovascular parameters such as LVsP, LVDP, max and min LV dp/dt, and CF. Deficiency of BH<sub>4</sub> seems to accelerate endothelial dysfunction.<sup>13)</sup> It is thought that the increased oxidative injuries in hearts subjected to ischemia-reperfusion are, at least in part, due to insuffi-

ciency of BH<sub>4</sub>, resulting in reduced activity of NOS. This may lead to a favorable shift in the balance between increases in levels of protective NO and decreases in levels of deleterious OFRs.<sup>2)</sup> These findings may suggest that the beneficial effects of BH<sub>4</sub> are derived from decreasing OFRs and increasing NO production.<sup>2,13)</sup> However, an increase in NO did not affect an increase in CF and did not lead to an obvious improvement of cardiac function.<sup>12,13)</sup>

It is thought that an increase in endogenous NO itself is important for the improvement of cardiac function. When vasodilating metabolites were washed from the myocardium, the vascular tone increased.<sup>18)</sup> Recently, Shen et al.<sup>19)</sup> suggested that endogenous NO increases myocardial O<sub>2</sub> consumption. Thus, a part of the cardioprotective effects of BH<sub>4</sub> might be explained by the increased generation of NO. In addition, Vásquez-Vivar et al. reported that BH<sub>4</sub> inhibits the generation of superoxide.<sup>20)</sup> They suggested that BH<sub>4</sub> has a protective effect against cerebral ischemia-reperfusion injury due to decreased OFRs and increased levels of NO produced by NOS. NO is synthesized from L-arginine by NOS and is a highly reactive free radical and a potent vasodilator.<sup>10,21)</sup> Some investigators have reported that treatment with BH<sub>4</sub> reduces ischemia-reperfusion induced tissue injury.<sup>9,10)</sup> They suggested that the increase in BH<sub>4</sub> content may protect against OFR-induced ischemia-reperfusion tissue injury, and that decreased endothelium-derived NO activity may worsen the ischemic tissue damage and moreover that this may involve BH<sub>4</sub> depletion.

Conversely, in study 2, cold heart preservation after normothermic ischemia clearly increased the myocardial injury, and BH<sub>4</sub> suppressed these injuries. Because ischemic injury is too strong, it is ineffective with cooling only after the normothermic cardiac arrest. Moreover, BH<sub>4</sub> also attenuated increases in lipid peroxidation estimated as the amount of MDA equivalents, decreases in ATP, an index of cardiac cell membrane damage, in the reperfused hearts. Increases in the tissue MDA level have been observed in such conditions that OFR-mediated damage is implicated.<sup>22)</sup> Furthermore, it is well known that both mechanical function of the heart and myocardial energy metabolism are markedly impaired after ischemia. In this study there was a significant difference in the CF after reperfusion. Therefore it is likely that the enhancement of NO-bioavailability by administration of BH<sub>4</sub> had a role in suppression of myocardial injury. In this regard it is of interest that the enhancement of NO-bioavailability by administration of BH<sub>4</sub> has been reported in the patients

with hypercholesterolemia.<sup>23)</sup>

There are no reports dealing with heart preservation after normothermic cardiac arrest, therefore, the result obtained with BH<sub>4</sub> in this study relative to its effect in heart preservation can not be confirmed by other such evidence. However, it was possible that BH<sub>4</sub>, which attenuates ischemia-reperfusion injury through modulation of NOS leading to a decrease in OFRs and increase in NO, decreases the injury induced by heart preservation after ischemia. This warrants further investigation as there is a limit to heart transplantation using donor hearts obtained from brain-dead subjects at present.

In this study, LVEDP increased during ischemia and increased again immediately after the onset of reperfusion (Fig. 5A). In the ischemic BH<sub>4</sub> group the increases in LVEDP both during ischemia and reperfusion were significantly smaller than that in the ischemic-control group (Fig. 5A). In addition, during ischemia, significantly delayed onset of ischemic contracture and lower maximal values of LVEDP were seen in the ischemic BH<sub>4</sub> group compared with the corresponding values in the ischemic-control group (Figs. 5B and 5C). Previously we reported that, BH<sub>4</sub> suppressed increases in LVEDP both during ischemia and reperfusion. Additionally, the time to onset of ischemic contracture in BH<sub>4</sub> treatment rat heart was significantly longer than that in BH<sub>4</sub> no treatment rat heart. Ischemic contracture has been suggested to be a sign of irreversible cell damage and its appearance is thought to coincide with the time when glycolytic ATP production ceases. Despite controversy concerning the validity of direct measurements of OFRs by electron paramagnetic resonance spectroscopy, some studies support the theory that OFRs are generated during myocardial ischemia in addition to reperfusion. In our previous study, administration of BH<sub>4</sub> before ischemia improved post-ischemic contractile dysfunction compared with administration of BH<sub>4</sub> after ischemia, but to a lesser extent than when BH<sub>4</sub> was given both before ischemia and just after reperfusion.<sup>12)</sup> Therefore, it is likely that the improvements of reperfusion injury by BH<sub>4</sub> were derived from the effects of BH<sub>4</sub> during ischemia in addition to reperfusion. Accordingly, it is possible that the effect of attenuation of injury during ischemia and at reperfusion is attainable even if BH<sub>4</sub> is administered just after normothermic cardiac arrest. These findings suggest that the beneficial effects of BH<sub>4</sub> are derived from scavenging of OFRs during not only reperfusion but also ischemia. Although the reperfusion time was short as may be the case if heart transplantation was being attempted, the recovery of car-

diac function in the early stage of reperfusion accurately indicated the suppression of injury of the preserved heart. The reason why we chose the 60-min reperfusion time was that in this model, mechanical functions in the non-ischemic hearts are known to be gradually attenuated over 60-min after reperfusion following the same time-course as the ischemic hearts in the preliminary experiments.<sup>12)</sup> Further studies will be required to confirm the effectiveness of BH<sub>4</sub> on the myocardial ischemia-reperfusion injury with a longer reperfusion time.

Result of study 1 demonstrated that, the influence of cooling (hypothermia) with or without BH<sub>4</sub> before cardiac arrest is significantly effective against the donor heart function. However, in study 2, because the ischemic injury was too severe, it was ineffective with cooling after the normothermic cardiac arrest. Moreover, because the ischemic injury was too severe in study 2, LV function was not recovered in the ischemic-control group after 8 hours of cold ischemia followed with normothermic global ischemia. Some studies support the theory that OFRs are generated during myocardial ischemia in addition to reperfusion. Therefore we chose 4 hours of cold ischemia followed with normothermic global ischemia in study 2.

At present, the mechanism of action of NO on myocardial ischemia-reperfusion injury is unclear in this model; however, Shen et al.<sup>19)</sup> have suggested that endogenous NO increases myocardial metabolic efficiency by reducing myocardial O<sub>2</sub> consumption. These beneficial effects of BH<sub>4</sub> are apparently caused by suppressing the myocardial cell damage via reduction of OFRs and increasing endogenous NO. However, we believe that the most likely mechanism of cardioprotection by BH<sub>4</sub> is the reduced generation of OFRs rather than increased generation of NO by NOS. Further studies, including combined treatment with BH<sub>4</sub> and SOD/catalase, may help to confirm the precise role of BH<sub>4</sub> on ischemia-reperfusion injury.

In conclusion, the present results may demonstrate that BH<sub>4</sub> lessens ischemia-reperfusion injury in donor hearts after cardiac arrest. Cold heart preservation after normothermic ischemia clearly increased the myocardial injury, and BH<sub>4</sub> suppressed these injuries. The cardioprotective effect of BH<sub>4</sub> seems independent of its intrinsic radical scavenging action, and is primarily attributable to improvement of dysfunctional NOS that may serve as a cause of oxidative injury. In this study, we did not measure oxygen-radical generation and BH<sub>4</sub> content in any heart. This would have been very useful to enhance our understanding of the mechanism behind BH<sub>4</sub> administration.

The current findings imply that supplementary administration of BH<sub>4</sub> may provide a novel strategy to prevent myocardial ischemia-reperfusion injury and associated complications. It is also possible that additional protection may be afforded by using L-arginine and/or free radical scavengers in combination with BH<sub>4</sub>. Further studies are required to confirm the effectiveness of such treatment modalities in the clinic.

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