TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Successful transplantation of rat hearts subjected to extended cold preservation with a novel preservation solution

Kenji Wakayama,[1] Moto Fukai,[1] Kenichiro Yamashita,[1] Taichi Kimura,[2] Gentaro Hirokata,[1] Susumu Shibasaki,[1] Daisuke Fukumori,[1] Sanae Haga,[3] Mitsuru Sugawara,[4] Tomomi Suzuki,[1] Masahiko Taniguchi,[1] Tsuyoshi Shimamura,[1]Hiroyuki Furukawa,[1] Michitaka Ozaki,[3] Toshiya Kamiyama[1] and Satoru Todo[1]

1 Department of General Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan
2 Laboratory of Cancer Research, Department of Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Japan
3 Department of Molecular Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan
4 Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

Keywords
cold preservation, deuterium oxide, heart transplantation, preservation solution, rat.

Correspondence
Moto Fukai MD, PhD, Department of General Surgery, Hokkaido University Graduate School of Medicine, N-15, W-7, Kita-ku, Sapporo 060-8638, Japan.
Tel.: +81-11-706-5923;
fax: +81-11-706-7064;
e-mail: db7m-fki@hotmail.co.jp

Conflicts of Interest
The authors of this manuscript have no conflicts of interest to disclose.

Received: 19 December 2011
Revision requested: 15 January 2012
Accepted: 27 February 2012
Published online: 4 April 2012

doi:10.1111/j.1432-2277.2012.01469.x

Summary

Since prolonged cold preservation of the heart deteriorates the outcome of heart transplantation, a more protective preservation solution is required. We therefore developed a new solution, named Dsol, and examined whether Dsol, in comparison to UW, could better inhibit myocardial injury resulting from prolonged cold preservation. Syngeneic heterotopic heart transplantation in Lewis rats was performed after cold preservation with UW or Dsol for 24 or 36 h. In addition to graft survival, myocardial injury, ATP content, and Ca^{2+} -dependent proteases activity were assessed in the 24-h preservation group. The cytosolic Ca^{2+} concentration of H9c2 cardiomyocytes after 24-h cold preservation was assessed. Dsol significantly improved 7-day graft survival after 36-h preservation. After 24-h preservation, Dsol was associated with significantly faster recovery of ATP content and less activation of calpain and caspase-3 after reperfusion. Dsol diminished graft injury significantly, as revealed by the lower levels of infarction, apoptosis, serum LDH and AST release, and graft fibrosis at 7-day. Dsol significantly inhibited Ca^{2+} overload during cold preservation. Dsol inhibited myocardial injury and improved graft survival by suppressing Ca^{2+} overload during the preservation and the activation of Ca^{2+} -dependent proteases. Dsol is therefore considered a better alternative to UW to ameliorate the outcome of heart transplantation.

Introduction

Graft dysfunction in the early phase of reperfusion, due to ischemia and reperfusion injury (IRI), is still the critical problem to be conquered in clinical heart transplantation [1]. Since the heart is susceptible to cold IRI [2], the time limit for a safe heart graft is 4–6 h in clinical settings using University of Wisconsin solution (UW) [3]. Improvement of the graft quality after cold preservation is thus a very important issue, but the method of cardiac cold preservation has not been dramatically changed since the UW was introduced in 1988 [4]. For this reason, a better, alternative organ preservation solution is needed.

During cold preservation, harmful processes such as ATP depletion [5], Ca^{2+} overload [6], production of reactive oxygen species (ROS) [7], cellular acidosis [8], swelling [9], and cytoskeletal disruption [10] are initiated and progress. During subsequent re-warming ischemia and reperfusion, some of these harmful cascades, including ROS production, Ca^{2+} overload and downstream

activation of proteases [11], and delayed recovery of ATP production [12], are further enhanced. Prolonged cold preservation exacerbates these processes, and eventually causes cardiac graft injury.

We therefore developed Dsol, a novel organ preservation solution based on UW solution with a high sodium and low potassium component, modified impermeants, and deuterium oxide (D_2O) as solvent (Table 1). We expect the extracellular-type composition of this solution without hydroxyethyl starch (HES) to inhibit coronary endothelial injury and subsequent graft infarction after reperfusion [13,14]. In addition, the impermeants sucrose and mannitol, which cost less than raffinose, are expected to give the solution potent cellular protection and antioxidant effects [15,16]. Deuterium oxide (D_2O) has unique biological effects, including inhibition of cytosolic Ca^{2+} overload [17], and the stabilization of microtubules [18], actin cytoskeleton [19], plasma membrane [20], and membrane-bound proteins [21]. D_2O also accelerates ATP production by stimulation of glucose uptake, glycolysis [22], and mitochondrial respiration [23]. These properties could suppress Ca^{2+}-induced cellular damage, and maintain structural and functional homeostasis of cardiomyocytes. In previous studies, the efficacy of D_2O for liver and heart preservation [24], and D_2O-containing solutions for kidney [25], pancreas [26], and vascular tissue preservation [27] has been reported. However, the effects of D_2O-containing solution have not yet been explored in a heart preservation and transplantation model.

The aims of the present study were to test whether Dsol, in comparison to the widely accepted UW, could better inhibit myocardial injury in extended cold preservation and subsequent syngeneic transplantation of rat hearts.

Table 1. Composition of the preservation solutions.

	Dsol	UW
Additives (mM)		
NaOH	125	25
KOH	–	100
$MgSO_4$	5	5
KH_2PO_4	25	25
Lactobionate	100	100
Raffinose	–	30
Sucrose	20	–
Mannitol	10	–
Adenosine	5	5
Allopurinol	1	1
Glutathione	3	3
HES (g/l)	–	50
Solvent (%)		
H_2O	70	100
D_2O	30	–
Freezing point (°C)	0.3	−0.9

HES, Hydroxyethyl starch.

Materials and methods

Chemicals and reagents

All the chemicals and reagents were of the highest grade commercially available, and purchased from Wako Pure Chemical Co. (Osaka, Japan) unless otherwise noted.

Preparation of preservation solutions

UW solution (Viaspan®) was purchased from Bristol–Myers Squibb Co. (New York, NY, USA). Dsol was developed in our laboratory (Table 1). Deuterium oxide was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The freezing point of Dsol was 0.3 °C and we confirmed that Dsol would not freeze at 4 °C under the conditions employed herein. All solutions were filtered (0.45 μm) before use.

Animals

The experiments were approved by the institutional Animal Care Committee, and were conducted under the guidelines for animal care and use of Hokkaido University. Inbred male Lewis (LEW) rats weighing 250–350 g were purchased from Kyudo Co., Ltd. (Saga, Japan), and were used as both donors and recipients. They were maintained in a specific pathogen-free facility, and were used for the experiments without fasting.

Cell culture and reagents

H9c2 cells (passage 18–25; CRL-1446™; American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat inactivated bovine serum (Gibco–Invitrogen, Carlsbad, CA, USA), and penicillin–streptomycin (Gibco), under 95% air/5% CO_2 at 37 °C. To assess the cytosolic Ca^{2+} concentration, a FRET-based Ca^{2+} indicator, the Premo Cameleon Calcium Sensor™ (Molecular Probes Inc. Eugene, OR, USA), was transduced into the H9c2 cells according to the manufacturer's instructions. Briefly, the cells were incubated in a growth medium containing an appropriate amount of vector at room temperature for 4 h, then incubated for another 16 h in a fresh growth medium containing expression-enhancer solution. Cells (4×10^4 cells/well) were plated on a 96-well culture plate for fluorescent measurement overnight under the normal growth conditions.

Heterotopic cardiac transplantation

Heterotopic heart transplantation was performed as previously described [28]. Briefly, after anesthetization with isoflurane inhalation, sodium heparin (1000 U/kg) was intravenously administered to the donor. Then, the heart was perfused in situ with 4 °C UW or Dsol from the aorta. The heart was rapidly excised and preserved in each solution at 4 °C. Recipients underwent a mid-line abdominal incision after anesthesia. The ascending aorta and pulmonary artery of the donors were anastomosed to the recipient's infra-renal abdominal aorta and inferior vena cava, respectively. The warm ischemic time was strictly adjusted to 25 min.

Experimental protocol *in vivo*

The grafts were transplanted after 24-h cold preservation in UW or Dsol solution (UW24 or Dsol24 group, respectively), 36-h preservation in UW or Dsol solution (UW36 or Dsol36 group, respectively), or no preservation (non-preserved control: NPC group). Graft survival was followed for 7 days. In the 24-h preservation groups, rats were sacrificed at 1 and 24 h after reperfusion (R1h and R24h, respectively). Grafts at the end of 24-h cold preservation (CP24h) in UW and Dsol solution, and normal heart controls (NHC) were also sampled. Graft infarction, apoptosis, serum biochemistry, inflammatory cells infiltration, high energy phosphates content, calpain and caspase 3 activities were assessed. At 7 days after reperfusion, rats were sacrificed to examine the level of graft fibrosis.

Graft survival

Graft survival was examined by palpation through the abdominal wall by two independent examiners in a blinded manner. Graft loss was defined as total stasis or the absence of any wall movement by direct inspection.

Infarction

Cardiac infarct size was assessed at R1h and R24h by triphenyltetrazolium chloride (TTC) staining as previously described [29]. Briefly, the excised hearts were incubated for 12 min in 1.5% TTC (w/v) in PBS at 37 °C, and fixed in 10% formalin-PBS thereafter. After taking microscopic images, the infarct area was calculated using computerized planimetry.

Apoptosis

Graft apoptosis was assessed at R24h by terminal dUTP nick end-labeling (TUNEL) staining as previously described [30]. Nuclei were counterstained with hematoxylin. TUNEL-positive cells were counted in five randomly selected HPFs (magnification ×400) adjacent to the necrotic area, the so-called area at risk, in a blinded manner. Mononuclear cells, cells without myofiber, or cells located at the interstitium were excluded as inflammatory cells. Results were expressed as the average number of TUNEL-positive cells per single HPF.

Infiltration of polymorphonuclear neutrophils (PMNs) and monocytes

The numbers of infiltrating inflammatory cells were assessed by counting the number of PMNs and monocytes at R24h. The grafts were fixed in 10% formalin-PBS, embedded in paraffin, and stained with hematoxylin-eosin (HE) for the PMNs count. Graft samples were also embedded and frozen in an OCT compound. Immunohistochemical (IHC) staining for monocytes/macrophages was performed with a mouse anti-rat CD68 antibody (AbD Serotec, Oxford, UK). Then the samples were incubated with a biotinylated goat anti-mouse IgG secondary antibody (DAKO, Cambridge, UK) and streptavidin-biotin-peroxidase (DAKO) in sequence. Detection of antibody binding was performed with 3,3'-diaminobenzidine (DAKO). Cells were counterstained with hematoxylin.

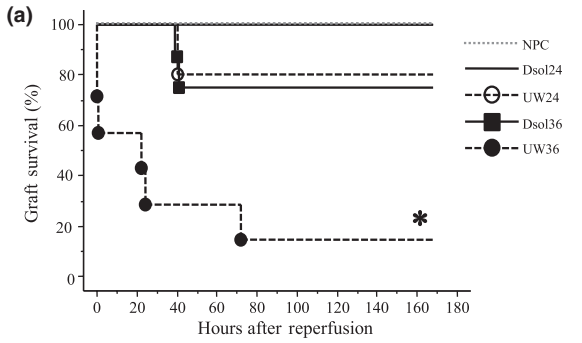
The numbers of PMNs and monocytes/macrophages were counted in 10 randomly selected HPFs for each section.

High energy phosphates

The levels of tissue adenine nucleotides (ATP, ADP, AMP) before preservation, at the end of 24-h cold preservation, and at R1h were measured as previously described [5]. Grafts were snap-frozen and homogenized in 20 μl/mg of ice cold 0.3 M perchloric acid with 0.01% (w/v) EDTA using a Polytron homogenizer (Kinematica Inc., Bohemia, NY, USA). After centrifugation (2200 *g*, 10 min, 4 °C), the supernatant was neutralized by 5 N KOH. An aliquot (20 μl) was analyzed by HPLC (Eicom, Kyoto, Japan). The dry-to-wet weight ratio of the tissue was separately measured by lyophilization. Myocardial adenine nucleotides were expressed as micromole per gram dry weight (μmol/g dw). Total adenine nucleotide (TAN) was calculated as the sum of ATP, ADP, and AMP.

Fibrosis

Grafts excised at R7d were fixed in 10% formalin-PBS, embedded in paraffin, and stained with Masson's trichrome. After microscopic images were taken with a BIOREVO BZ9100 fluorescence microscope (KEYENCE, Osaka, Japan), they were processed using computerized



(b)

Graft survival								
NPC	>7d	>7d	>7d	>7d	>7d	>7d		
Dsol24	>7d	>7d	>7d	>7d	>7d			
UW24	40 h	>7d	>7d	>7d	>7d			
Dsol36	39 h	41 h	>7d	>7d	>7d	>7d	>7d	>7d
UW36	0 h	0 h	1 h	22 h	24 h	3d	>7d	

Figure 1 Seven-day cardiac isograft survival. Cardiac grafts were preserved for 24 h (UW24: $n = 5$; Dsol24: $n = 5$) or 36 h (UW36: $n = 7$; Dsol36: $n = 8$) following syngeneic heterotopic transplantation. Grafts without preservation were used as a non-preservation control (NPC; $n = 6$). (a) Survival curve after reperfusion. (b) Survival time of individual hearts in each group after reperfusion. Dsol significantly improved 7-day graft survival after 36-h cold preservation. $*P < 0.05$ by log-rank test, UW36 vs. Dsol36.

planimetry software (KEYENCE). The fibrotic area was expressed as the percentage of the total LV area.

Calpain and caspase 3 activation

To determine the levels of activation of calpain and caspase 3, calpain-specific cleavage of cytoskeleton-bound proteins (α-fodrin and talin) and cleavage of caspase 3 were assessed by a standard Western blot analysis [31,32]. The graft was homogenized with a glass-Teflon homogenizer in 4 ml/g of lysis buffer containing 25 mmol/l Tris–HCl, 150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/l EDTA, and 1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA) at pH 7.6. The homogenate was centrifuged for 15 min at 14000× *g* and 4 °C. The protein concentration of the resulting supernatant was determined with a bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA). Then, the proteins were separated with standard SDS–PAGE techniques. After transfer to a PVDF membrane, the proteins were probed with mouse anti-α-fodrin mAb (1:1000; Biomol, Plymouth Meeting, PA, USA), mouse anti-talin mAb (1:200; Sigma), and rabbit anti-caspase 3 Ab (1:1000; Cell Signaling, Danvers, MA, USA). Then, IgG-horseradish peroxide-conjugated anti-mouse or anti-rabbit secondary antibody (1:2500–1:10000; Amersham Bioscience, Buckinghamshire, UK) was applied for chemiluminescence detection (Amersham Bioscience). α-tubulin was detected with rabbit mAb to α-tubulin (1:1000; Cell Signaling) as an internal control. The cleaved bands of α-fodrin and talin were then normalized by the respective intact bands. Cleaved bands of caspase 3 were normalized by α-tubulin. The values were finally expressed as a percentage of the value in the normal heart controls.

Cytosolic Ca^{2+} concentration *in vitro*

Cells expressing a FRET based Ca^{2+} indicator, Premo Cameleon Calcium Sensor™, were subjected to 24-h cold preservation in UW or Dsol. Cameleon was excited at 370 nm to produce fluorescence from CFP detected at 480 nm in the Ca^{2+} -unbound form. In the Ca^{2+} -bound form, FRET occurred from CFP to YFP, resulting in the production of additional fluorescence at 535 nm. The mean fluorescent intensity at 535 nm (MFI_{535}) was expressed as a percentage of the MFI_{535} before preservation.

Statistical analysis

Data were expressed as the mean ± standard deviation or mean ± standard error of the mean as annotated. Graft survival was plotted by the Kaplan–Meier method, and was applied to a log-rank test for comparisons. One-factor ANOVA followed by *post hoc* test was applied as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

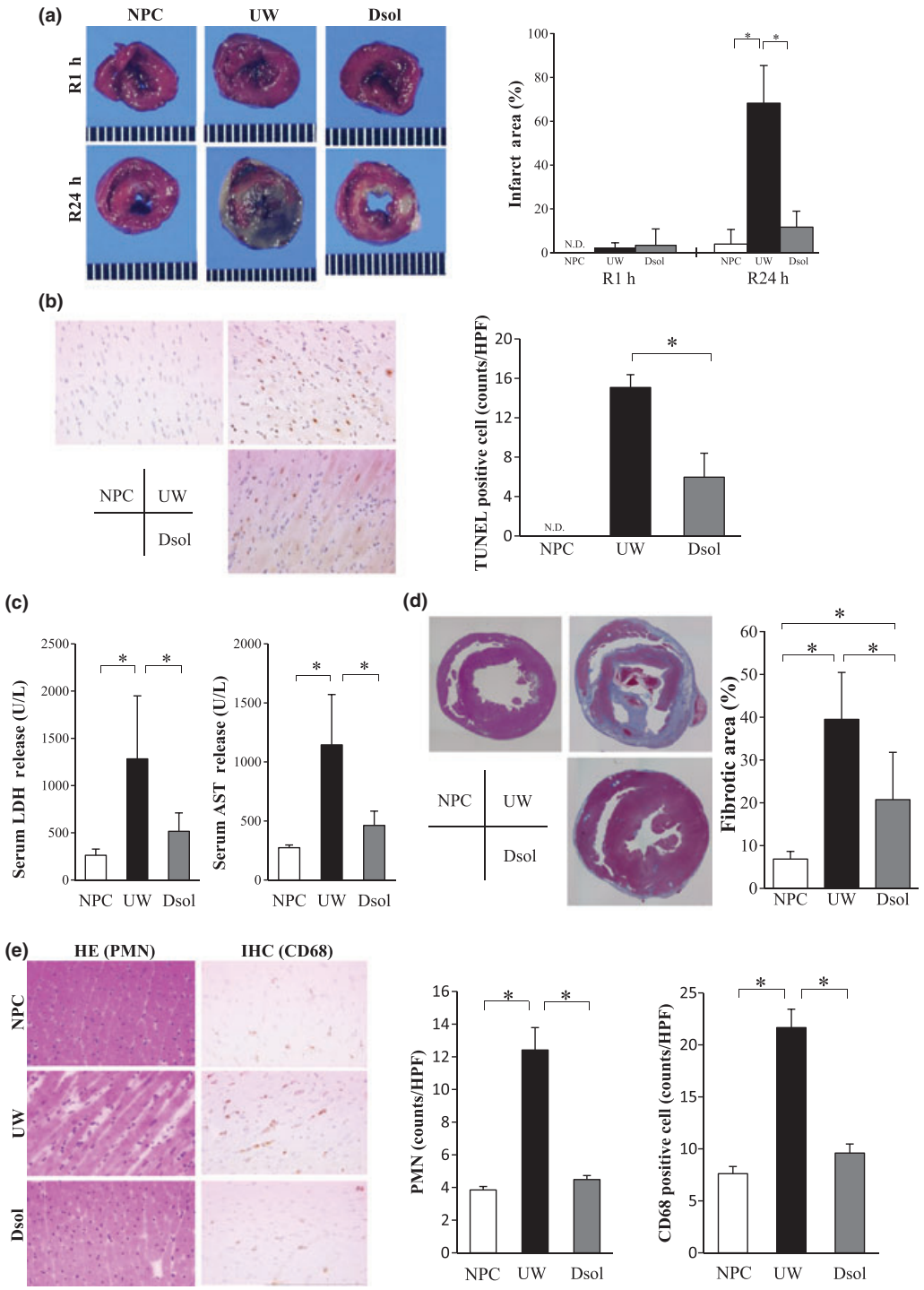
Dsol ameliorated graft survival

All hearts in the non-preservation control group (NPC) survived for 7 days (Fig. 1). In the 24-h cold preservation experiment, the rate of 7-day graft survival in the Dsol group was 100% (5/5), versus 80% (four of five) in the UW group. In the 36-h preservation experiment, the rate of 7-day graft survival was 75% (six of eight) in the Dsol group, whereas it was only 14% (one of seven) in the UW group ($P < 0.05$; Dsol36 vs. UW36).

Dsol decreased graft infarction, apoptosis, LDH and AST release

Graft infarction at 1 h after reperfusion (R1h) was not evident in all groups, and ranged from 0% to 3.4% of the total LV area. At R24h, the infarct area was 67.8% ± 16.5% of the total LV area in the UW group, whereas it was 11.7% ± 7.3% in the Dsol group ($P < 0.05$; Dsol vs. UW; Fig. 2a).

TUNEL-positive cells, i.e. apoptotic cardiomyocytes, were not found in the NPC group at R24h. The number of TUNEL-positive cardiomyocytes at R24h was signifi-



cantly smaller in the Dsol group (5.97 ± 2.44 counts/HPF) than in the UW group (15.1 ± 1.30 counts/HPF, Fig. 2b).

Serum LDH and AST levels in the UW group (1282 ± 667 and 1144 ± 427 IU/l, respectively) were significantly higher than those in the Dsol group (516 ± 195 and 463 ± 120 IU/l, respectively) at R24h (Fig. 2c).

Dsol reduced graft fibrosis

The fibrotic area at R7d was significantly larger in the UW group (39.5% ± 11.0%) than in the Dsol group (20.7% ± 11.1%) or NPC group (6.9% ± 1.8%, $P < 0.05$ for UW versus NPC and for UW versus Dsol, Fig. 2d).

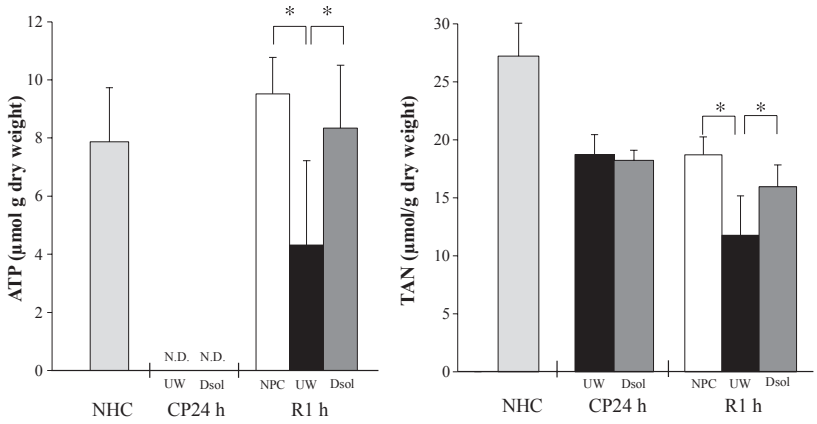


Figure 3 Graft ATP and total adenine nucleotide contents of the normal heart controls, after 24 h of cold preservation, and 1 h after reperfusion were measured by HPLC. Dsol was associated with significantly faster recovery of ATP and TAN content at 1 h after reperfusion. Values represent the mean ± SD, $n = 4$ each group, $^*P < 0.05$, Fischer's PLSD *post hoc* test. N.D., not detected; NHC, normal heart control; NPC, non-preservation control.

Dsol suppressed the infiltration of inflammatory cells

The number of polymorphonuclear neutrophils (PMNs) in the interstitium at R24h was significantly higher in the UW group (12.4 ± 1.37 counts/HPF) than in the Dsol group (4.5 ± 0.24 counts/HPF). The number of CD68-positive monocytes/macrophages at R24h was significantly higher in the UW group (21.7 ± 1.76 counts/HPF) than in the Dsol group (9.6 ± 0.87 counts/HPF, Fig. 2e).

Dsol improved the restoration of high energy phosphates after reperfusion

ATP content in the normal heart was 7.87 ± 1.86 (μmol/g dw), whereas ATP was not detected at the end of the 24-h cold preservation in either group. At R1h, it was significantly higher in the Dsol group (8.34 ± 2.16 μmol/g dw) than in the UW group (4.32 ± 2.90 μmol/g dw, Fig. 3). TAN was also significantly higher in the Dsol group (15.94 ± 1.89 μmol/g dw) than in the UW group (11.77 ± 3.39 μmol/g dw).

Dsol inhibited cold preservation-induced Ca^{2+} overload *in vitro*

After 24-h cold preservation, MFI_{535} increased to as much as 376% of the basal level in the UW group, whereas it increased to only 140% of the basal level in the Dsol group ($P < 0.0001$). Therefore, Dsol inhibited Ca^{2+} overload during cold preservation (Fig. 4a).

Dsol inhibited calpain and caspase-3 activation

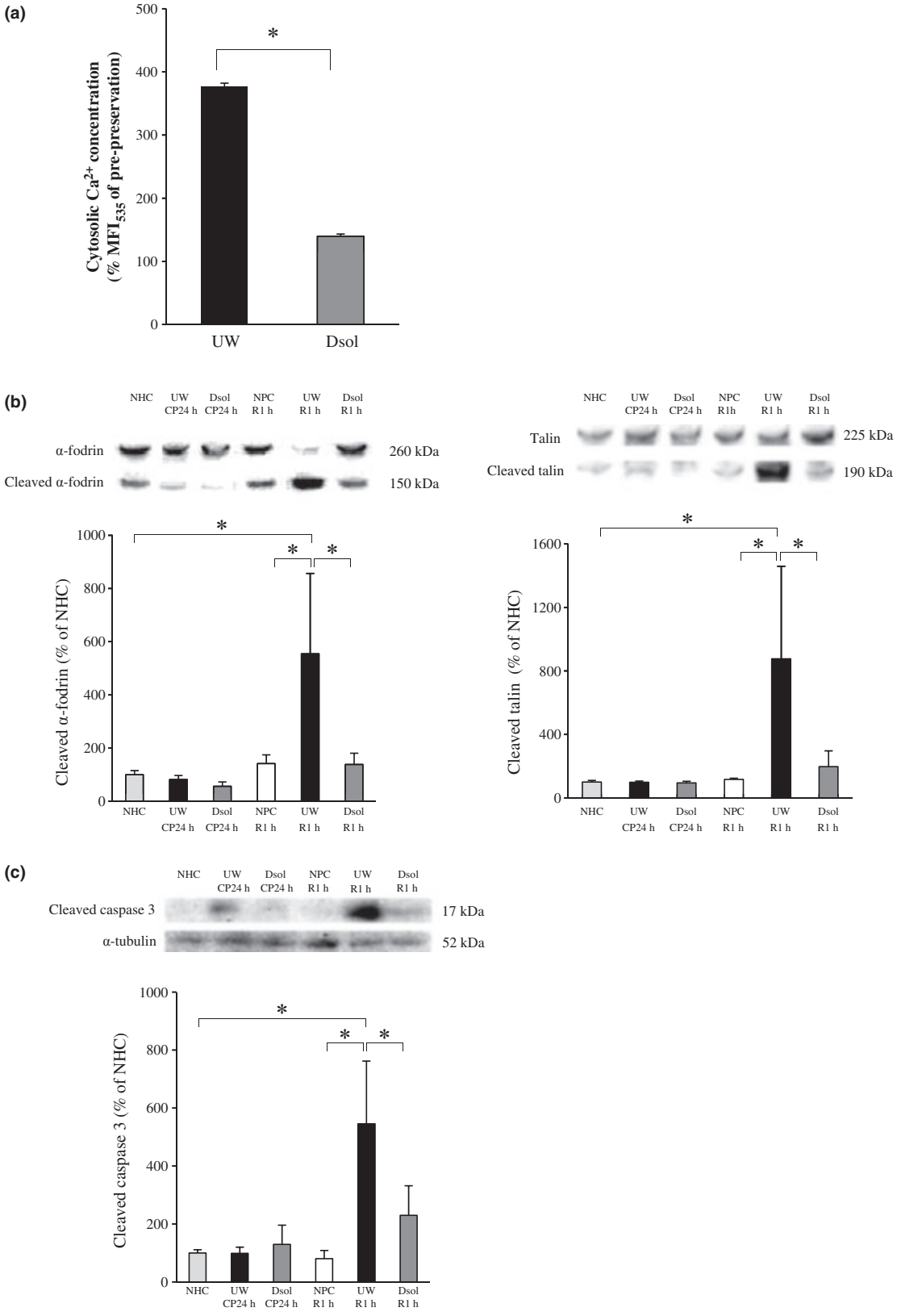
The calpain-specific substrates, talin and α-fodrin, were not cleaved at the end of the 24-h cold preservation period in either the UW or Dsol group (Fig. 4b). At R1h, they showed a significantly greater amount of cleavage in the UW group compared to the normal heart control group (NHC). Calpain-mediated cleavage was significantly suppressed in the Dsol group ($P < 0.05$ vs. UW, Fig. 4b).

The activations of caspase 3 by cleavage were assessed. The active cleaved fragments of caspase 3 (17 kDa) were significantly increased at R1h in the UW group compared to the NHC group ($P < 0.05$, vs. NHC), whereas they were significantly suppressed in the Dsol group ($P < 0.05$, vs. UW, Fig. 4c).

Discussion

In the current study, we demonstrated that the novel organ preservation solution Dsol improved cardiac graft survival after 36-h cold preservation. After 24-h preservation, Dsol markedly suppressed necrosis and apoptosis as

Figure 2 Graft injury after 24-h cold preservation and reperfusion. (a) Graft infarction at 1 h and 24 h after reperfusion as determined by TTC staining. Representative TTC-stained sections from grafts (Upper, R1hr; Lower, R24hr) and infarct size as measured by planimetry ($n = 6$ each group). Each point on the scale represents 1 mm. (b) Apoptosis of cardiomyocytes after 24 h of reperfusion as determined by TUNEL staining. Representative TUNEL-stained sections and TUNEL-positive myocardial cell counts in each section are shown ($n = 6$ each group). TUNEL-positive nuclei appear dark brown. Magnification ×400. (c) Serum LDH and AST release at 24 h after reperfusion (NPC: $n = 6$; UW: $n = 5$; Dsol: $n = 5$). (d) Graft fibrosis at 7 days after reperfusion as determined by Masson's trichrome staining. The fibrotic area stains blue, and the viable area stains red. Representative sections (original magnification: ×20) are shown, and the fibrotic area was calculated (NPC: $n = 6$; UW: $n = 4$; Dsol; $n = 5$). (e) Histological and immunohistochemical examination of graft-infiltrating PMNs and monocytes after 24 h of preservation and 24 h after reperfusion. Representative photographs of HE staining and immunohistochemical staining by anti-CD68 antibody (magnification ×400). CD68-positive cells appear brown. PMNs and CD68-positive cells were counted in HE and IHC, respectively ($n = 6$ each group). Dsol diminished graft injury significantly, as revealed by the lower levels of infarction, apoptosis, serum LDH and AST release, graft fibrosis and infiltration of inflammatory cells after reperfusion. Data are presented as the mean ± SD, $^*P < 0.05$ by the Tukey–Kramer *post hoc* test. NPC, non-preservation control; N.D., not detected.



compared to UW solution. Dsol also enabled rapid restoration of the high energy phosphate that had been exhausted from the grafts during the preservation period. Dsol was clearly shown to prevent the elevation of cytosolic Ca^{2+} concentration during cold preservation *in vitro*, and inhibited Ca^{2+} -dependent activation of calpain and subsequent activation of caspases-3, compared to UW solution *in vivo*. These data clearly demonstrated the advantage of Dsol over UW, with the former showing excellent inhibition of cardiac graft injury after prolonged simple cold static preservation and subsequent cardiac transplantation in rats.

In previous reports using the same model, the graft function of the UW-preserved rat hearts after transplantation was recovered in the 12-h preservation group [33], whereas it was impaired in the 18-h preservation group [34]. Further, 24-h preservation in UW raised the possibility of graft loss due to the critical ischemia/reperfusion injury [5]. Infarction of grafts after prolonged cold preservation presents a risk of graft loss. To avoid graft loss in such cases, previous reports have suggested the importance of suppressing graft infarction to below 15% of the total area of individual grafts after reperfusion [35,36]. In the present study, Dsol suppressed the graft infarction in just 11% of total area of grafts, and prevented graft loss completely. On the other hand, UW induced 68% graft infarction and resulted in graft loss in 20% of grafts after 24-h cold preservation. In addition, the surviving grafts in the UW-preserved group tended to beat more weakly than the Dsol-preserved grafts. However, we could not evaluate graft function in this study because we employed a non-functional model. Functional assessment using a functional model remains a challenge for future studies. However, the present results do indicate that Dsol has a more powerful protective effect than UW solution, although this protective effect appeared more evident after 36-h preservation.

Necrosis at the center of the infarction and apoptosis around the necrotic area, the so-called area at risk (AAR), are closely related to graft survival and contractile function [37]. After prolonged cold preservation and reperfusion, cardiomyocytes fell into necrosis for various reasons, including hypercontracture, insufficient blood flow due to vascular failure, and activation of necrosis-inducing proteases [14,38,39]. In the present study, UW could not prevent necrotic cell death, as demonstrated by TTC staining, AST and LDH release, and eventual graft fibrosis, which was consistent with a previous report [35], whereas Dsol achieved nearly complete inhibition. Necrotic cardiomyocytes induced infiltration of inflammatory cells in the UW group but not in the Dsol group. These cells, in turn, damage viable cardiomyocytes by secreting inflammatory mediators [40]. Therefore, the prevention of necrosis also has important implications in terms of stopping this harmful cycle. Cardiomyocytes that manage to just avoid necrosis often fall into apoptotic cell death within the AAR [37]. We demonstrated that abundant TUNEL-positive apoptotic myocardia were found at the AAR in UW-preserved hearts, whereas they were significantly suppressed in the Dsol group. Dsol prevented cell death not only by preventing necrosis but also by preventing apoptosis.

Cytosolic Ca^{2+} overload during prolonged cold preservation and Ca^{2+} -dependent activation of calpain and caspases after reperfusion play a central role in cellular necrosis and/or apoptosis. Calpain is activated by cytosolic Ca^{2+} overload, and activated calpain, in turn, induces necrosis by cleavage of cytoskeletal proteins such as α-fodrin and talin [39]. Calpain also triggers apoptosis by caspase-12 activation [41], and Bid [42] and Bax cleavage [43], followed by caspase 3 activation. Among the many unique properties of D_2O, such as stabilization of the microtubules [18], actin cytoskeleton [19], plasma membrane [20], and membrane-bound proteins [21], we focused on the ability of D_2O to suppress the elevation of cytosolic Ca^{2+} concentration [17]. D_2O is reported to inhibit calcium influx via the plasma membrane L-type Ca^{2+} channel [44] as well as calcium efflux from the sarcoplasmic reticulum (SR) to the cytosol [45]. Our present *in vitro* study demonstrated that cytosolic Ca^{2+} concentration was elevated up to 3.8-fold after 24-h cold preservation in the UW group. Elevated cytosolic Ca^{2+} at the end of the cold preservation period in turn leads to the activation of Ca^{2+} -dependent proteases, and thereby protease-induced necrosis and apoptosis of cardiomyocytes after reperfusion. In this study, the major source of aug-

Figure 4 (a) The cytosolic Ca^{2+} concentration of H9c2 cardiomyocytes after 24-h cold preservation was assessed by using a Premo Cameleon Calcium Sensor™. After 24-h cold preservation, MFI_{535} increased to as much as 376% of the baseline level in the UW group, versus 140% of the baseline level in the Dsol group. Values represent the mean ± SD, $n = 6$ each group. $^*P < 0.0001$ by Fischer's PLSD *post hoc* test. (b and c) Western blotting analyses of calpain and caspase-3 activity in the cardiac grafts after 24 h of cold preservation and 1 h of reperfusion. (b) Activated calpain mediated the cleavage of α-fodrin and talin. Representative Western blots of cleavage of intact α-fodrin (260 kDa) to a cleaved fragment (150 kDa), and intact talin (225 kDa) to a cleaved fragment (190 kDa) are shown. Semi-quantitative analyses are shown below. (c) Representative Western blots of cleavage of caspase-3 to the active fragments of caspase-3 (17 kDa). The results of the semi-quantitative analyses are shown below. Dsol significantly inhibited calpain and caspase-3 activation after reperfusion. All values are expressed as the mean ± SD, $n = 3$, $^*P < 0.05$, Turkey–Kramer *post hoc* test. NHC, normal heart control; NPC, non-preservation control.

mented cytosolic Ca^{2+} during preservation should be the efflux from SR, because both UW and Dsol are Ca^{2+} -free solutions. The D_2O present in the Dsol could inhibit Ca^{2+} release from SR and suppressed the elevation of cytosolic Ca^{2+} concentration during cold preservation. Accordingly, Dsol dramatically suppressed the activation of these degradative Ca^{2+} -dependent proteases thereafter. This property of D_2O should be a key mechanism of the graft protection with Dsol.

In addition to cellular death, the energy state, which is established mainly by mitochondrial oxidative ATP production, is closely related to the cardiac kinetics after transplantation. Flameng *et al.* reported that the impairment of ATP restoration after reperfusion, even if the ATP content was maintained at the end of 24-h cold static preservation, causes cardiac contractile dysfunction after transplantation [12]. Although Dsol failed to preserve ATP content during cold preservation in the present study, rapid recovery of ATP content was clearly shown at 1 h after reperfusion. Meanwhile, UW failed to recover ATP synthesis, even though graft infarction was not evident.

Although the intracellular-type component and HES adopted by UW can potently prevent cellular swelling during cold preservation, they tend to induce graft infarction as a result of coronary endothelial injury [13,14]. Therefore, we adopted the extracellular-type component without HES for Dsol. In this respect, the concept of Dsol is similar to that of Celsior [46], which showed better preservation than UW within a relatively short period [47], but not after extended cold preservation [48,49]. The reasons for the potent protection by Dsol even after a prolonged period could be the modified impermeants and D_2O, which could compensate for the demerits of the extracellular-type composition. Modified impermeants such as mannitol and sucrose, which *per se* have cytoprotective [15] and anti-oxidative effects [16], could reduce organ swelling. Other properties of D_2O, in addition to the inhibition of Ca^{2+} -overload, such as stabilization of the microtubules [18], actin cytoskeleton [19], plasma membrane [20], and membrane-bound proteins [21], could help Dsol to inhibit graft injury.

In conclusion, Ca^{2+} overload initiated during cold preservation induces the activation of harmful proteases, and subsequent apoptosis and necrosis of cardiomyocytes after reperfusion, finally leading to graft loss. A novel organ preservation solution, Dsol, was shown to be superior to UW solution at inhibiting myocardial injury during extended cold preservation and subsequent syngeneic transplantation of rat hearts by inhibiting Ca^{2+} overload during cold preservation and subsequent activation of proteases. This solution could reduce the mortality of heart transplantation. Moreover, the protective effect of this solution could prolong the safe preservation time of cardiac grafts and increase the opportunities for organ distribution.

Authorship

KW, MF, KY and ST: designed the experiments. KW and MF: wrote the article. KW, MF, TK, GH, SS and DF: contributed to the acquisition of data and analysis. SH, TS, MT, TS and HF: provided expertise. MF and MS: provided new reagents. KW, MF, KY, TK and ST: interpreted the data.

Funding

This study was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (No. 2224904800), and by grants-in-aid for Young Scientists (2006-103) and for Scientific Research (2007-87, 2008-33) from the Regional R&D Proposal-Based Program of the Northern Advancement Center for Science & Technology of Hokkaido, Japan.

Acknowledgement

We thank Keyence Co. (Osaka, Japan) for their excellent technical advice and support in obtaining the pathological images.

References

1. Taylor DO, Edwards LB, Aurora P, *et al.* Registry of the International Society for Heart and Lung Transplantation: twenty-fifth official adult heart transplant report–2008. *J Heart Lung Transplant* 2008; **27**: 943.
2. Keck BM, White R, Breen TJ, Daily OP, Hosenpud JD. Thoracic organ transplants in the United States: a report from the UNOS/ISHLT Scientific Registry for Organ Transplants. United Network for Organ Sharing. International Society for Heart and Lung Transplantation. *Clin Transpl* 1994; 37.
3. Jahania MS, Sanchez JA, Narayan P, Lasley RD, Mentzer Jr RM. Heart preservation for transplantation: principles and strategies. *Ann Thorac Surg* 1999; **68**: 1983.
4. Swanson DK, Pasaoglu I, Berkoff HA, Southard JA, Hegge JO. Improved heart preservation with UW preservation solution. *J Heart Transplant* 1988; **7**: 456.
5. Stringham JC, Southard JH, Hegge J, Triemstra L, Fields BL, Belzer FO. Limitations of heart preservation by cold storage. *Transplantation* 1992; **53**: 287.
6. Stowe DF, Varadarajan SG, An J, Smart SC. Reduced cytosolic Ca(2+) loading and improved cardiac function after cardioplegic cold storage of guinea pig isolated hearts. *Circulation* 2000; **102**: 1172.

7. Riess ML, Camara AK, Kevin LG, An J, Stowe DF. Reduced reactive O_2 species formation and preserved mitochondrial NADH and $[Ca^{2+}]$ levels during short-term 17 degrees C ischemia in intact hearts. *Cardiovasc Res* 2004; **61**: 580.
8. Thatte HS, Rhee JH, Zagarins SE, *et al.* Acidosis-induced apoptosis in human and porcine heart. *Ann Thorac Surg* 2004; **77**: 1376.
9. Batty PR, Hicks GL, DeWeese JA, Wang TC. Optimal osmolality for cold storage of the cardiac explant. *J Surg Res* 1990; **48**: 601.
10. Hall SM, Haworth SG. Effect of cold preservation on pulmonary arterial smooth muscle cells. *Am J Physiol* 1996; **270**: L435.
11. Kohli V, Gao W, Camargo Jr CA, Clavien PA. Calpain is a mediator of preservation-reperfusion injury in rat liver transplantation. *Proc Natl Acad Sci U S A* 1997; **94**: 9354.
12. Flameng W, Dyszkiewicz W, Minten J. Energy state of the myocardium during long-term cold storage and subsequent reperfusion. *Eur J Cardiothorac Surg* 1988; **2**: 244.
13. Drinkwater DC, Rudis E, Laks H, *et al.* University of Wisconsin solution versus Stanford cardioplegic solution and the development of cardiac allograft vasculopathy. *J Heart Lung Transplant* 1995; **14**: 891.
14. Cartier R, Hollmann C, Dagenais F, Buluran J, Pellerin M, Leclerc Y. Effects of University of Wisconsin solution on endothelium-dependent coronary artery relaxation in the rat. *Ann Thorac Surg* 1993; **55**: 50.
15. Lodge JP, Perry SL, Skinner C, Potts DJ, Giles GR. Improved porcine renal preservation with a simple extracellular solution–PBS140. *Transplantation* 1991; **51**: 574.
16. England MD, Cavarocchi NC, O'Brien JF, *et al.* Influence of antioxidants (mannitol and allopurinol) on oxygen free radical generation during and after cardiopulmonary bypass. *Circulation* 1986; **74**: III134.
17. Vasdev S, Gupta IP, Sampson CA, Longerich L, Parai S. Deuterium oxide normalizes blood pressure and elevated cytosolic calcium in rats with ethanol-induced hypertension. *Can J Cardiol* 1993; **9**: 802.
18. Marsland D, Tilney LG, Hirshfield M. Stabilizing effects of D_2O on the microtubular components and needle-like form of heliozoan axopods: a pressure-temperature analysis. *J Cell Physiol* 1971; **77**: 187.
19. Omori H, Kuroda M, Naora H, *et al.* Deuterium oxide (heavy water) accelerates actin assembly in vitro and changes microfilament distribution in cultured cells. *Eur J Cell Biol* 1997; **74**: 273.
20. Wenzel M, Fischer JH. [Organ preservation with heavy water–effect of D_2O concentration and temperature on organ swelling]. *J Clin Chem Clin Biochem* 1983; **21**: 83.
21. Ahlers J, Foret M, Lemm U. Does $2H_2O$ also protect membrane-bound enzymes? *Enzyme* 1983; **30**: 70.
22. Wals PA, Katz J. The effect of D_2O on glycolysis by rat hepatocytes. *Int J Biochem* 1993; **25**: 1561.
23. Urbauer JL, Dorgan LJ, Schuster SM. Effects of deuterium on the kinetics of beef heart mitochondrial ATPase. *Arch Biochem Biophys* 1984; **231**: 498.
24. Wenzel M, Holscher B, Gunther T, Merker HJ. [Organ preservation by heavy water (D_2O). Morphological and biochemical studies on heart and liver (author's transl)]. *J Clin Chem Clin Biochem* 1979; **17**: 123.
25. Fischer JH, Knupfer P, Beyer M. Flush solution 2, a new concept for one-to-three-day hypothermic renal storage preservation. Functional recovery after preservation in Euro-Collins, Collins' C2, hypertonic citrate, and F.2 solution. *Transplantation* 1985; **39**: 122.
26. Hesse UJ, Gores PF, Florack G, Sutherland DE. The use of D_2O (heavy water)-based solution for hypothermic preservation of the pancreas. *Transplant Proc* 1987; **19**: 4167.
27. Berwanger CS, Cleanthis TM, Hafez HM, Fuller BJ, Mansfield AO, Stansby G. Deuterium oxide-based University of Wisconsin solution improves viability of hypothermically stored vascular tissue. *Transplantation* 1998; **65**: 735.
28. Ono K, Lindsey ES. Improved technique of heart transplantation in rats. *J Thorac Cardiovasc Surg* 1969; **57**: 225.
29. Fishbein MC, Meerbaum S, Rit J, *et al.* Early phase acute myocardial infarct size quantification: validation of the triphenyl tetrazolium chloride tissue enzyme staining technique. *Am Heart J* 1981; **101**: 593.
30. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992; **119**: 493.
31. French JP, Quindry JC, Falk DJ, *et al.* Ischemia-reperfusion-induced calpain activation and SERCA2a degradation are attenuated by exercise training and calpain inhibition. *Am J Physiol Heart Circ Physiol* 2006; **290**: H128.
32. Marques CA, Keil U, Bonert A, *et al.* Neurotoxic mechanisms caused by the Alzheimer's disease-linked Swedish amyloid precursor protein mutation: oxidative stress, caspases, and the JNK pathway. *J Biol Chem* 2003; **278**: 28294.
33. Baxter K, Howden BO, Jin X, Jablonski P. Studies in a modified auxiliary abdominal rat heart transplantation model: preservation with colloid-free University of Wisconsin solution. *J Heart Lung Transplant* 1998; **17**: 532.
34. Masters TN, Fokin AA, Schaper J, Pool L, Gong G, Robicsek F. Changes in the preserved heart that limit the length of preservation. *J Heart Lung Transplant* 2002; **21**: 590.
35. Huang J, Nakamura K, Ito Y, *et al.* Bcl-xL gene transfer inhibits Bax translocation and prolongs cardiac cold preservation time in rats. *Circulation* 2005; **112**: 76.
36. Nakao A, Neto JS, Kanno S, *et al.* Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both. *Am J Transplant* 2005; **5**: 282.
37. Yaoita H, Ogawa K, Maehara K, Maruyama Y. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. *Circulation* 1998; **97**: 276.

38. Piper HM, Abdallah Y, Schafer C. The first minutes of reperfusion: a window of opportunity for cardioprotection. *Cardiovasc Res* 2004; **61**: 365.
39. Yoshida K, Inui M, Harada K, *et al.* Reperfusion of rat heart after brief ischemia induces proteolysis of calspectin (nonerythroid spectrin or fodrin) by calpain. *Circ Res* 1995; **77**: 603.
40. Kirklin JK, McGiffin DC. Control of the inflammatory response in extended myocardial preservation of the donor heart. *Ann Thorac Surg* 1999; **68**: 1978.
41. Nakagawa T, Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 2000; **150**: 887.
42. Chen M, Won DJ, Krajewski S, Gottlieb RA. Calpain and mitochondria in ischemia/reperfusion injury. *J Biol Chem* 2002; **277**: 29181.
43. Gao G, Dou QP. N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome *C* release and apoptotic cell death. *J Cell Biochem* 2000; **80**: 53.
44. Prod'hom B, Pietrobon D, Hess P. Direct measurement of proton transfer rates to a group controlling the dihydropyridine-sensitive Ca^{2+} channel. *Nature* 1987; **329**: 243.
45. Ikeda M, Suzuki S, Kishio M, *et al.* Hydrogen-deuterium exchange effects on beta-endorphin release from AtT20 murine pituitary tumor cells. *Biophys J* 2004; **86**: 565.
46. Menasche P, Termignon JL, Pradier F, *et al.* Experimental evaluation of Celsior, a new heart preservation solution. *Eur J Cardiothorac Surg* 1994; **8**: 207.
47. Michel P, Vial R, Rodriguez C, Ferrera R. A comparative study of the most widely used solutions for cardiac graft preservation during hypothermia. *J Heart Lung Transplant* 2002; **21**: 1030.
48. Mohara J, Takahashi T, Oshima K, *et al.* The effect of Celsior solution on 12-h cardiac preservation in comparison with University of Wisconsin solution. *J Cardiovasc Surg (Torino)* 2001; **42**: 187.
49. Kajihara N, Morita S, Tanoue Y, *et al.* The UW solution has greater potential for longer preservation periods than the Celsior solution: comparative study for ventricular and coronary endothelial function after 24-h heart preservation. *Eur J Cardiothorac Surg* 2006; **29**: 784.