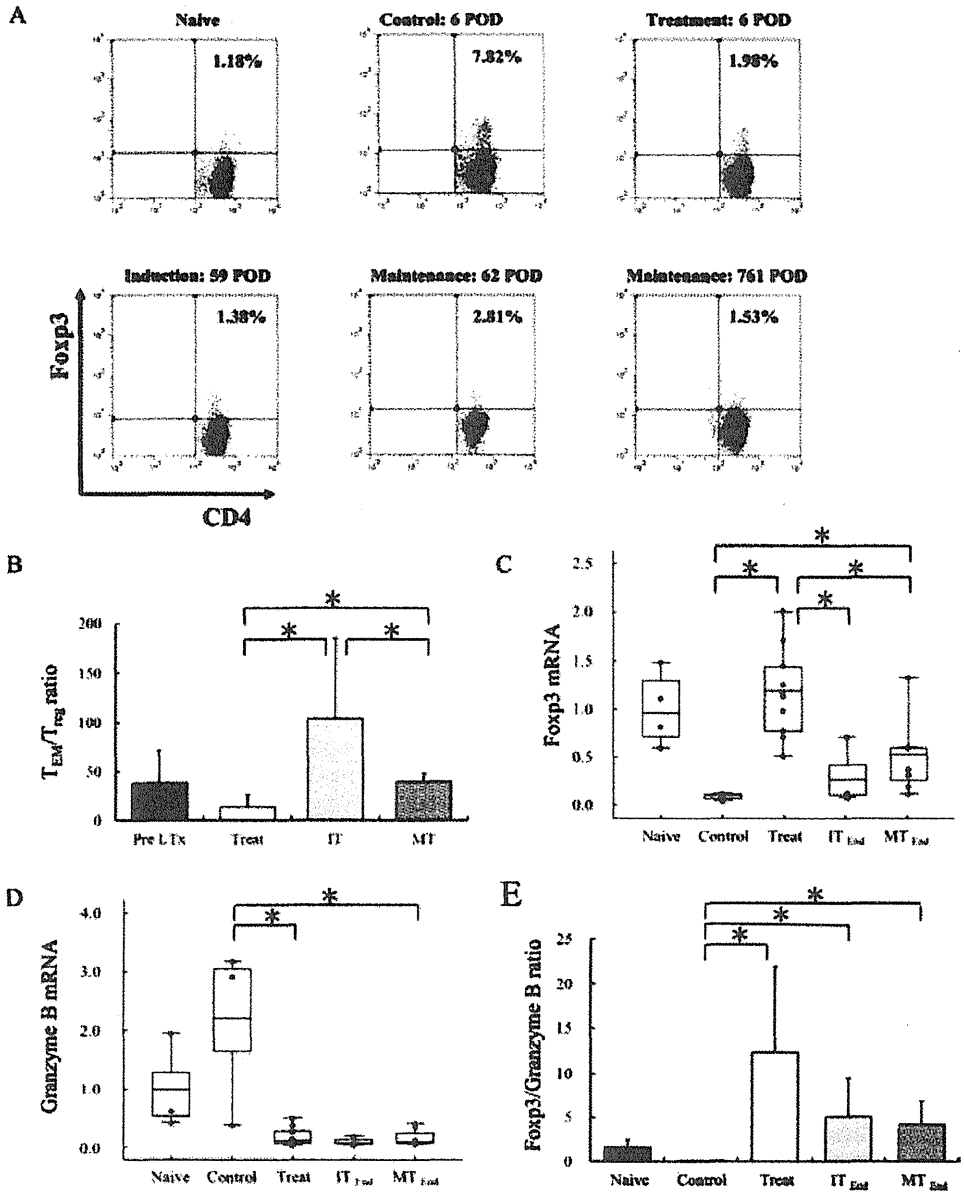

Figure 6: T_{reg} population in the periphery and graft and expression of intragraft gene transcripts. (A) The $CD4^+Foxp3^+$ T-cell population in the periphery was assessed by flow cytometry, and representative dot-plot analyses data are shown. The $CD4^+Foxp3^+$ T cells did not increase at all, except in animals that did not receive ASKP1240 treatment and acutely rejected the liver allograft. (B) The T_{EM}/T_{reg} ratio was examined before and after ASKP1240 treatment. Results at before LTx (Pre-LTx, n = 10), at POD 10 (Treat, n = 10), at 1 month after LTx with induction treatment (IT, n = 3) and at 8 months after LTx with maintenance treatment (MT, n = 3) are shown. After drug cessation, the T_{EM}/T_{reg} ratio was significantly increased. A significant difference was observed between the induction and maintenance treatments. The expression levels of Foxp3 (C) and Granzyme B (D) were assessed by real-time polymerase chain reaction (RT-PCR) in the liver specimens. Tissue samples were obtained from naive monkeys (Naïve, n = 4), untreated control allografts at the time of necropsy (Control, n = 3), all of the ASKP1240-treated animals at POD 10 (Treat, n = 10), and animals given the induction (IT_{End}, n = 3) and maintenance (MT_{End}, n = 7) ASKP1240 treatments at the end time point. The end point is defined as the time of necropsy. Each transcript was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results are given as fold-change differences with respect to that of naive monkeys, and presented as whisker plots for the 25th and 75th percentiles, mean and maximum and minimum values with individual data. No upregulation of Foxp3 transcript was observed, in even the long-term surviving animals compared with the animals during ASKP1240 administration (C). The intragraft expression level of Granzyme B increased in the control group, whereas the Granzyme B level was suppressed by ASKP1240 treatment (D). The Foxp3/Granzyme B ratio was significantly increased by ASPK1240 treatment (E) (*$p < 0.05$, Mann–Whitney *U*-test).

during treated and maintained, whereas the levels of Foxp3 (Figure 6C), TGF-β, IL-10, Perforin, IL-15 and IFN-γ (data not shown) transcripts did not correspond to freedom from graft rejection. ASKP1240 treatment significantly increased the Foxp3/Granzyme B ratio as compared with that of the nontreated control (Figure 6E).

Discussion

In this study, we have shown that a novel fully human anti-CD40 mAb, ASKP1240, markedly prolonged hepatic allograft survival in NHPs. According to the findings of our previous dose-dependent study on kidney transplantation (12), we adopted the induction and maintenance treatment protocols using ASKP1240 at a dose of 10 mg/kg. In contrast to renal allografts, the maintenance ASKP1240 treatment prevented rejection for the duration of therapy, and in one animal resulted in a long-term hepatic allograft acceptance. However, the induction ASKP1240 therapy resulted in graft chronic rejection after drug cessation. Prevention of allograft rejection with the maintenance-ASKP1240 treatment was associated with a potent suppression of both the direct and indirect cellular alloimmune responses, as assessed by the IFN-γ ELIspot assay. Furthermore, we found that the direct and indirect responses against third party or PHA antigens remained intact in the long-term survivors. These results were in contrast to the induction ASKP1240 treatment, which permitted recovery of both direct and indirect alloimmune responses after termination of ASKP1240 treatment. Hering et al. (26) demonstrated a marked suppression of IFN-γ–secreting donor-reactive T cells via the indirect pathway in recipient monkeys who had accepted porcine islet xenografts for over 100 days by using multiple immunosuppressants, including the anti-CD40L mAb.

The existence of memory T cells, which are resistant to various immunosuppressants (including costimulation blockers), has been regarded as a major barrier to the induction of transplantation tolerance (27). Weaver et al. (28) showed that elimination of memory T cells by combined LFA-3-Ig and CTLA4-Ig treatment contributed to kidney allograft acceptance in NHPs. Although the peripheral T_{EM} population increased shortly after termination of the induction ASKP1240 treatment, the T_{EM} cells were completely abolished by the maintenance ASKP1240 therapy, even after drug cessation. Along with suppression of cellular responses through the direct and indirect pathways, inhibition of T_{EM} cells seems to contribute to the ASKP1240 treatment's effect on the prevention of allograft rejection.

Previous studies have demonstrated that inhibition of the CD40–CD154 signaling pathway induces regulatory T cells (T_{reg}) (29) and that higher T_{reg} expression in the periphery is associated with an operational tolerance in a clinical LTx setting (30). We therefore assessed whether ASKP1240 treatment induced an immunoregulatory mechanism. In the results of our FACS and RT-PCR analyses, however, there was no clear evidence of T_{reg} induction by ASKP1240 administration. During the treatment period, the T_{EM}/T_{reg} ratio was suppressed; however, after drug cessation this ratio significantly increased, especially in the induction-treated animals. Although an increase in the T_{reg} population in the periphery or graft liver was not evident, a significant reduction of IFN-γ producing cells, antigen-specific $CD4^+CD154^+$ T cells, and T_{EM} by ASKP1240 administration suggests a possibility that suppression of allo-aggressive T cells by the maintenance ASKP1240 treatment consequently shifted the balance toward a tolerogenic state.

In addition inhibiting cellular immune responses, ASKP1240 completely suppressed both DSA and ADA formation during the treatment course. Of the three long-term–surviving liver transplant recipients, two developed DSA after cessation of ASKP1240; however, there was no sign of antibody-mediated rejection, which was confirmed by graft C4d and IgG staining. This result was in contrast to our previous ASKP1240 trial in kidney transplantation, in which the grafts became positive to C4d and IgG and all the renal allografts underwent chronic nephropathy (12). Indeed, humoral mechanisms of alloreactivity mediated by DSA appear to frequently operate along with cellular mechanisms (31). In this study, we did not directly assess a helper T-cell function. Therefore, we do not deny the possibility that incomplete suppression of helper T cells by ASKP1240 treatment permitted formation of DSA, although there was no histopathological evidence of humoral rejection in the liver grafts. Many studies have shown that development of DSA is a major risk for chronic renal allograft rejection (32) and positive C4d graft staining is a hallmark of chronic renal allograft rejection (33). In LTx, similar findings have been reported (34,35). However, it is well known that liver allografts are resistant to HLA incompatibility or positive cross-match (36), and this unique immunoprivilege of the liver grafts (37) may have caused a discrepancy in the efficacy of ASKP1240 on different organ transplants. Although we have demonstrated that donor specific effector function was completely suppressed but the responses against third-party antigens and PHA were maintained in the long-survived animal following treatment cessation, by considering forementioned results regarding the DSA, we could not identify whether a donor-specific tolerance was induced in the maintenance ASKP1240-treated transplant recipients.

Fibrotic change of the portal tract was indicated by protocol biopsies in all grafts at 1 month after LTx. The degree of fibrosis varied and did not correspond to the type of ASKP1240 treatment protocol. Insufficient immunosuppression can be a risk factor of development of graft fibrosis and chronic rejection (38); however, this explanation seems unlikely because the fibrosis was already noted after LTx when both the cellular and humoral alloimmune

responses were abolished by ASKP1240 administration, as shown by several assays. Rather, this rapid peri-portal fibrotic change seems to be a hallmark of IRI, which has been shown previously to play a major role in graft fibrosis in the liver (39) and other organs (40). In fact, the cynomolgus monkey liver was very susceptible to ischemia: in our preliminary study, most of recipient monkeys died within 24 hours after LTx because the veno-veno bypass was not applicable in these small monkeys. Instead, we adopted a technique of clamping the SMA during an anhepatic phase (manuscript in preparation). Even using this unique technique, a severe IRI to the hepatic allograft was not completely abolished, as indicated by a considerable elevation of serum AST and ALT levels following LTx.

In the graft liver of the animals treated with a short course induction of ASKP1240, cellular infiltrates increased and the fibrotic area expanded after cessation of ASKP1240; finally, the bile ducts were microscopically lost, which indicated progression of chronic allograft rejection. In contrast, histopathological changes—including signs of neither acute nor chronic rejection—were present in the liver grafts when ASKP1240 treatment was maintained. In addition, these animals experienced repeated cholangitis that required fasting and antibiotic treatment. Two animals were eventually lost due to abscess formation. The episodes of cholangitis provoked biliary cirrhotic changes to the graft livers; persistence of fibrosis and bile duct proliferation (but not disappearance) were confirmed by histology. Indeed, similar microscopic features were reported by Gotoh et al. (41) in long-term–surviving liver grafts under FK506 treatment in cynomolgus monkeys. We used the ImmuKnow® for the purpose of assessing an immunological state, because it has been shown as an useful tool in clinical transplantation (42). In our study, however, ImmuKnow® values did not decline even when the animals suffered from cholangitis or liver abscess. We do not deny the possibility that ASKP1240 treated animals were over-immunosuppressed, especially when ASKP1240 treatment was maintained. However, the only infectious complication apparent in the treated animals was cholangitis/biloma, and we consider that this complication occurred because of the biliary reconstruction method, cholecystoduodenostomy, in the current LTx model.

As in our previous studies (11,12), ASKP1240 did not cause obvious adverse events in the treated monkey liver recipients. Of all the animals, two experienced transient anemia. In their peripheral blood smear, achantrocytes were observed, and this phenomenon was diagnosed as spur cell anemia due to liver dysfunction (43). This anemia emerged at 2–3 months after LTx, and the animals recovered gradually but spontaneously from anemia during the ASKP1240 treatment course, corresponding to the improvement of liver function. The administration of ASKP1240 reduced peripheral non-T lymphocytes and suppressed germinal center formation in the spleen and lymph nodes at the time of autopsy (data not shown). These phenomena were also noted in our earlier studies (11,12); this effect seems to be mediated via the inhibition of CD40 signaling pathway by ASKP1240, as the CD40–CD154 axis induces B-cell proliferation, antibody production, immunoglobulin isotype switching and germinal center formation (44–47).

Ligation of CD40 to CD154 on T cells plays a crucial role in cellular immunity. Signals via CD40 on dendritic cells (DCs) and monocytes engender these cells into a fully competent antigen-presenting cells (APCs), that can reciprocally trigger the cognate T cells to clonally expand and differentiate (45). Also, CD40 activation leads to cell proliferation, antibody production and immunoglobulin isotype switching in B cells that puts forward humoral immunity (44). On the basis of the role of CD40 signaling in the immunity, interruption of CD40/CD154 interaction can result in inadequate antigen presentation and a deficiency in both cellular and humoral immune responses. Indeed, in this study, we have demonstrated that inhibition of CD40 signal by ASKP1240 completely suppressed antidonor cellular responses and inhibited generation of DSA and ADA during the treatment course in the LTx recipients. Because we did not directly evaluate the effects at a cellular level in the current study, we cannot explain the exact mechanisms of immunomodulatory effect achieved by treatment with ASKP1240. Further studies are warranted to elucidate this subject.

In conclusion, a fully human anti-CD40 mAb, ASKP1240, induced potent immunosuppressive effects for hepatic allografts in NHPs without causing serious side effects. CD40 blockade by ASKP1240 ameliorates cellular and humoral alloimmune responses and prevented rejection for the duration of therapy, and in one animal resulted in a long-term acceptance in cynomolgus monkeys, especially when treatment is sustained. The present results indicate that ASKP1240 is a promising agent for immunosuppression in LTx.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found the online version of this article.

Figure S1: Changes in peripheral T_{EM}/T_{reg} ratio of a long-survived animal (#8).

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ORIGINAL ARTICLE

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

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

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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.

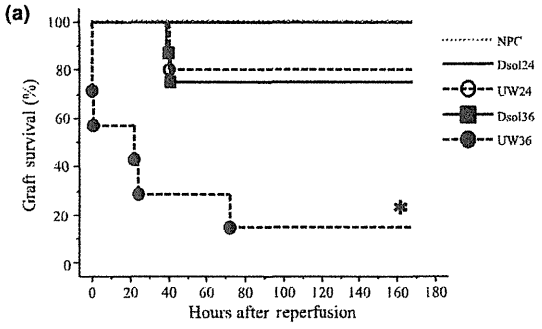
The numbers of PMNs and monocytes/macrophages were counted in 10 randomly selected HPFs for each section.

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

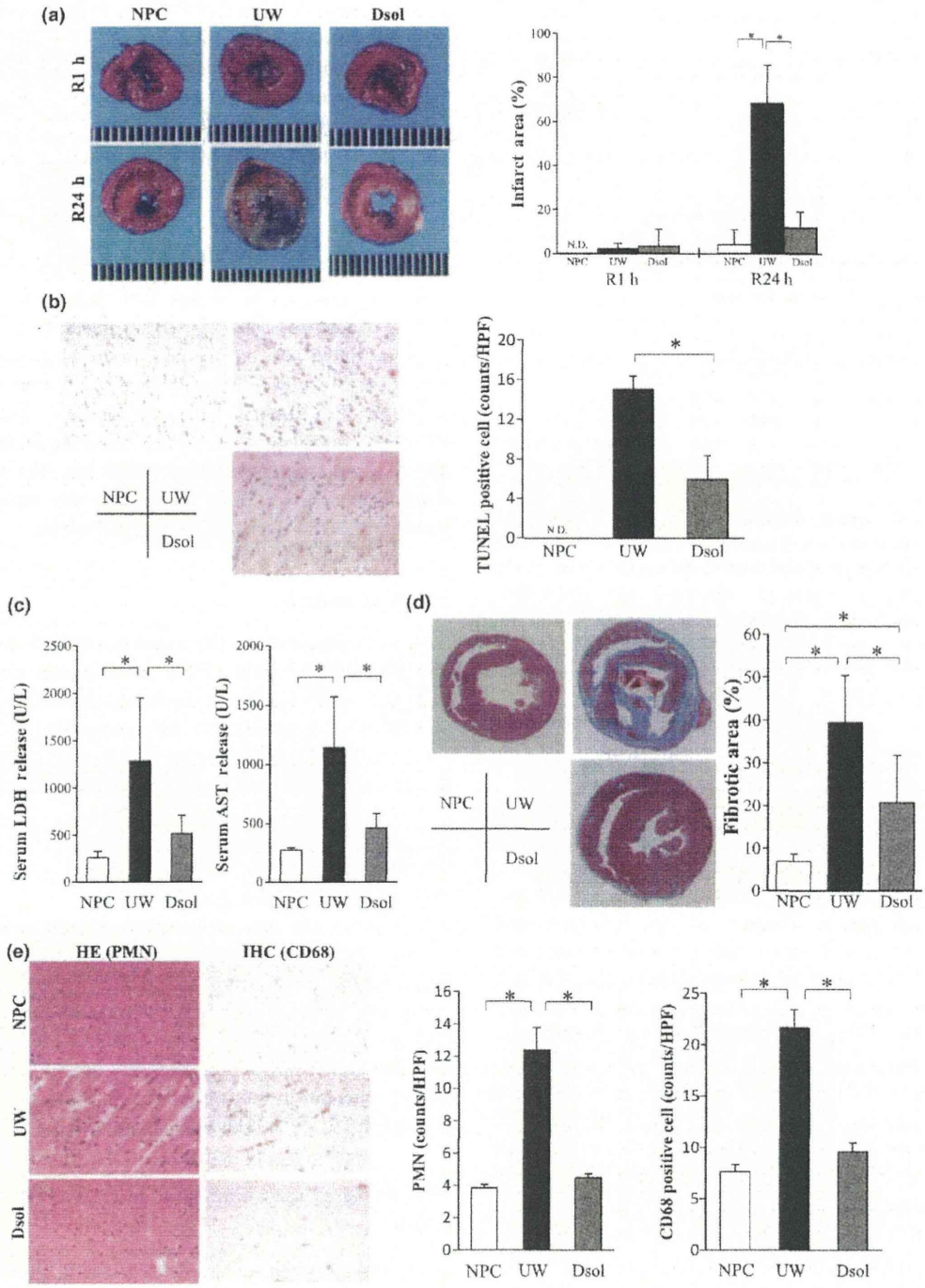
Dsol ameliorated graft survival

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).

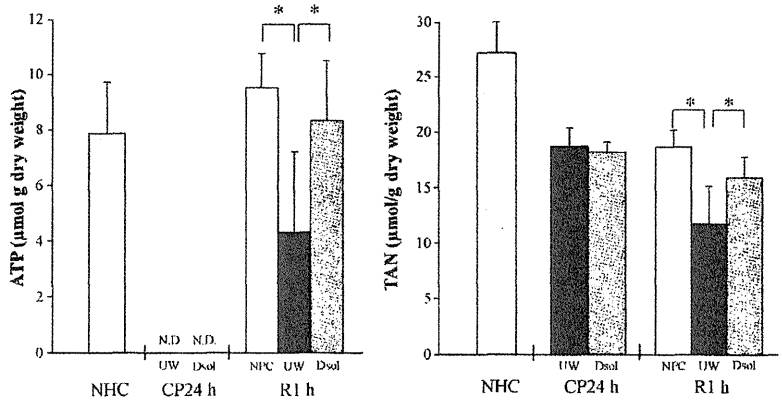


Figure 3 Graft ATP and total adenine nucleotide contents of the normal heart controls, after 24 h of cold preservation, and 1 h after reperfusion were measured by HPLC. Dsol was associated with significantly faster recovery of ATP and TAN content at 1 h after reperfusion. Values represent the mean ± 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

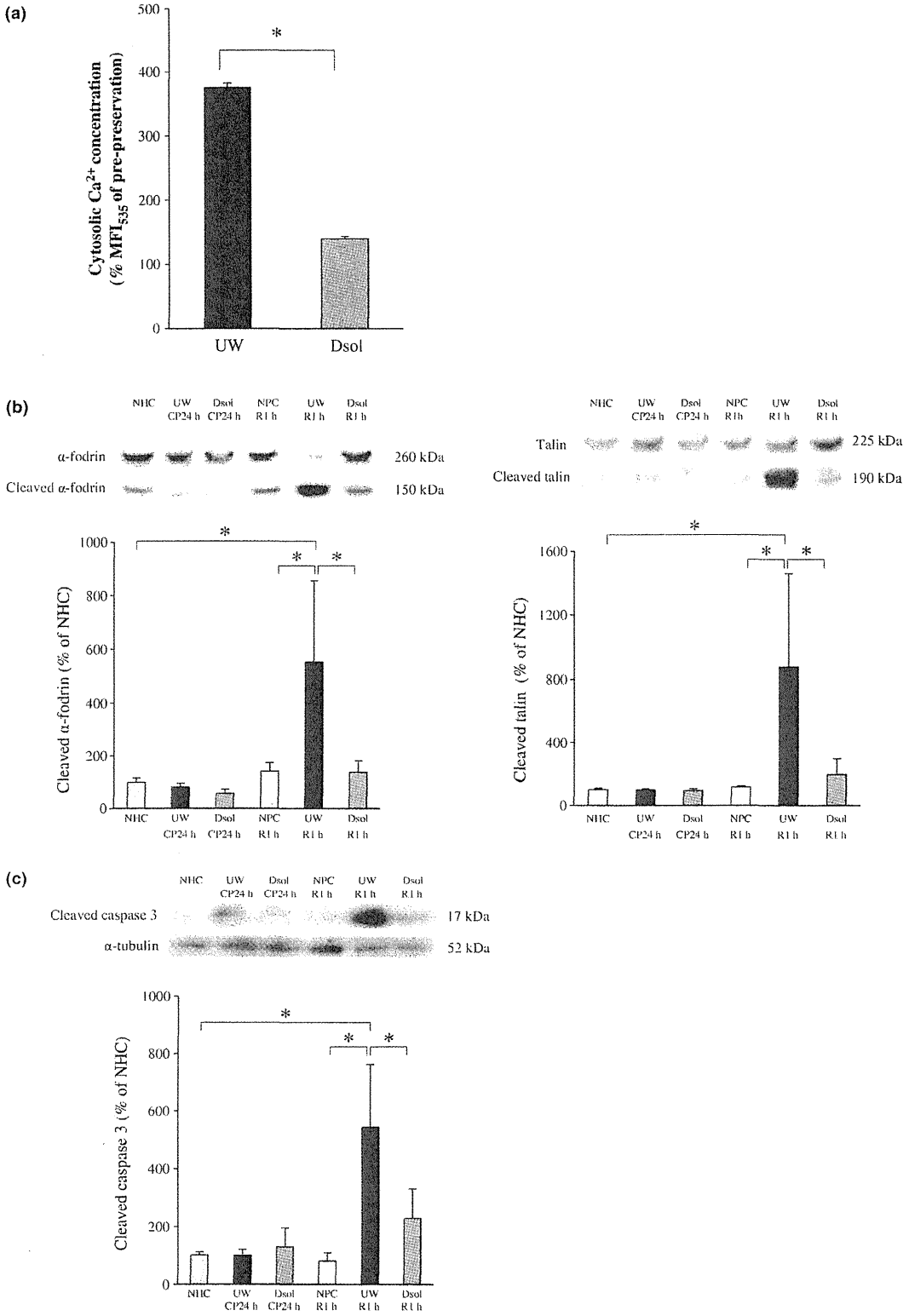
The calpain-specific substrates, talin and α-fodrin, were not cleaved at the end of the 24-h cold preservation period in either the UW or Dsol group (Fig. 4b). At R1h, they showed a significantly greater amount of cleavage in the UW group compared to the normal heart control group (NHC). Calpain-mediated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

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Professional Education and Hospital Development for Organ Donation

N. Fukushima, S. Konaka, O. Kato, and J. Ashikari

ABSTRACT

Because of the strict Organ Transplantation Act, only 81 brain dead (BD) organ donations had been performed in Japan for 13 years since 1997. The Act was revised on July 17, 2010, allowing, organs to be donated after BD with consent from the family, if the subject had not denied organ donation previously. This act has lead to an expectation of a 6–7-fold increase in BD donation. The 82 organ procurement coordinators (OPC) in Japan include 32 belonging to the Japanese Organ Network (JOT) and the others to each administrative division. JOT has guideline manuals of standard roles and procedures of OPC during organ procurement from BD and cardiac death donors.

To manage the increased organ donations after the revision of the act, we have modified the education system. First, we modified the guideline manuals for OPC to correspond to the revised Transplant Act and governmental guidelines. Second, all OPC gathered in a meeting room to learn the new organ procurement system to deal with the revised Transplant Act and guidelines. Third, a special education program for 2 months was provided for the 10 newcomers. Last, the practical training in each donor case for newcomers was performed by older OPC.

Topics of the education program were the revised transplant act and guidelines, family approach to organ donation, BD diagnosis, donor evaluation and management, organ procurement and preservation, allocation system, hospital development and family care.

In the future, each OPC will be divided into special categories, such as the donor family OPC, the donor management OPC, and the operating room OPC. Therefore, we need to construct separate special education programs for each category.

TO DISCUSS PROFESSIONAL education and hospital development for organ donation, we need to develop the following programs in each country: organ transplant legislation, network for organ allocation and sharing, public awareness, basic education for medical students (physicians, nurses, and medical engineers, etc), and for transplant professionals. Since 1978, the donation of kidneys after cardiac death (DCD) has been legally accepted in Japan, if family consent was obtained. Small children have been able to donate their kidneys after cardiac death. The Japanese Organ Transplantation Act for brain dead (BD) donation was issued in October 1997. The act required a living written consent for BD and organ donation; it did not allow BD donation from children <15 years of age. For these reasons, only 81 BD organ donations were performed in Japan over 13 years after the Act was issued in October 1997.

Finally, the act was revised on July 17, 2010, to allow organ donation after BD with consent from the family,[1] if the patient has not rejected organ donation. By this renewal, we expected a 6 to 7-fold increase in BD donations.

There are 82 governmentally authorized organ procurement coordinators (OPC) in Japan: 32 OPC belong to The Japan Organ Network (JOT) and the others to each administrative division (prefectural OPC). About 400 donor hospitals hire in-hospital OPC individually. The JOT OPCs play a role in organ allocation and sharing, informed consent, and care of the donor family, donor evaluation and management, control of organ retrieval in the operating

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room, public awareness, and hospital development. The prefectural OPCs play a role in public awareness and hospital development in their district and assisting JOT OPC in organ donation and allocation. In hospitals, OPCs play a role in awareness of organ donation among the hospital staff and assisting authorized OPCs in donation processes.

The Department of Coordinators and the coordinator committee in The JOT play the main roles to educate these OPC. JOT has published guideline manuals for the standard roles and procedures of OPC during organ procurement in BD and DCD.

To manage increased organ donations after the revision of the act, we sought to modify the education system. First, we modified OPC guideline manuals to correspond with the revised Transplant Act and governmental guidelines. Second, all OPCs gathered in a room to learn the new organ procurement system, including the revised Transplant Act and the guidelines. Third, a special 2-month educational program was provided for 10 newcomers as well as practical training in individual donor cases by older OPCs.

EDUCATION OF THE JOT OPCs

For the rookie JOT OPCs, we provided a special 2-month education programs. The topics of the classroom lectures were an overview of transplant medicine, of the previous and revised organ Transplant Acts, organ transplant history in Japan and in the world, of the JOT and organ transplant network system, as well as the roles and tasks of OPO in organ donation (BD and DCD), the current status and problems in organ transplantation (heart, lung, liver, pancreas, and kidney), therapies for end-stage organ failure, process and roles of OPCs in BD organ donation under the revised act, donor evaluation and management, and family consent at BD donation and DCD. After 2 months of classroom lectures, they were assigned to the JOT branch for the on-job-the-training by older OPCs.

For the older JOT OPCs, we taught details of BD organ donation under the revised act, details of pediatric organ donation, such as pediatric emergency therapy, care of the pediatric family, and organ procurement in children. Simulations of family consent in special conditions, such as, how to address refusal of the person for organ donation, child abuse, consent of the child's family, and consent of priority organ donation to relatives.

The leader JOT OPCs were trained in how to supervise family consent and care, coordination of organ procurement surgery and control of the an OPC team in BD donation and DCD.

EDUCATION FOR THE PREFECTURAL OPCs

A 2-day seminar is held every February for the prefectural OPCs. The topics of the program are the processes of DCD and BD organ donations (initial action, family consent, donor evaluation), the roles of OPC, especially prefectural OPC in DCD and BD donation, the standard process of priority organ donation to relatives, information disclosure to the media, family consent at BD donation as well as case studies of DCD and BD donations.

Each JOT branch conducts its own classroom lectures 3–5 times a year. The topics include the current status of DCD and BD donation in each branch or in the special prefectures with case studies, how to proceed with pediatric organ donation, family care during and after donation, case studies of DCD and BD donations, public awareness, and hospital development.

EDUCATION FOR THE IN-HOSPITAL OPCs

The JOT OPCs and the prefectural OPCs conduct classroom lectures 1–3 times a year. The topics are legislation of organ transplantation/donation in Japan, the current status of DCD and BD organ donation in Japan, the standard process of DCD and BD organ donation (initial action, family consent, donor evaluation, etc), the roles of in-hospital OPCs in DCD and BD organ donations, information disclosure to Media and family consent at BD donation as well as case studies in DCD and BD donation.

A simulation with classroom lectures of DCD or BD donation in each hospital was effective in the education of in-hospital OPCs as well as hospital development. To perform the simulation, the in-hospital OPC and medical staffs needed to establish their own organ donation system, to make their own guidelines and manuals of organ donation and to determine the roles of each hospital staff member in organ donation.

EDUCATION OF MEDICAL STAFF IN A DONOR HOSPITAL

The JOT OPCs and the prefectural OPCs conduct a classroom lecture for the medical staff in each donor hospital: physicians, nurses, medical engineers, and medical examiners. The topics are the organ transplantation/donation legislation in Japan, the current status of DCD and BD organ donation in Japan, the process of DCD and brain dead organ donation (initial action, family consent, donor evaluation, etc), and the roles of medical staff. Especially for physicians in the emergency department and in the intensive care unit as well as anesthesiologists, donor evaluation and management, multiple organ retrieval procedures in the operating room and donor management during surgery are important topics to increase the number of organs transplanted per donor and to improve their graft functions.[2] These lectures and simulations of organ donation are important for hospital development.

EDUCATION OF MEDICAL STAFF PERFORMING BD DETERMINATION IN A DONOR HOSPITAL

The JOT, the academic societies (Japan Neurosurgical Society, Japanese Association for acute medicine, Japanese Society of Emergency Pediatrics, etc), and the research groups supported by governmental grants conduct seminars

on BD determination for medical examiners, physicians and medical staffs in donor hospitals.

The topics are the definition of brain death, its determination, performance of the electroencephalogram, and options for organ donation.

HOSPITAL DEVELOPMENT

To increase organ donation, the recognition of importance of organ transplantation and donation by medical staffs in donor hospitals is important. Frequent visits to donor hospitals by OPCs are difficult, but represent the most effective method for hospital development. Frequent visits allow OPCs to find a key person for organ donation in each hospital, establishing a good relationship between OPOs and the medical staff. Moreover, seminars for the medical staff performing BD determinations or evaluating and managing donors until and during the organ procurement surgery are important for hospital development.

In the future, each OPC will be divided into special categories, such as those responsible to obtain informed consent and care donor families until the subject leaves the donor hospital, to evaluate organs and provide care until procurement surgery and to participate in organ procurement procedures.

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ELSEVIER

A Newly Developed Container for Safe, Easy, and Cost-effective Overnight Transportation of Tissues and Organs by Electrically Keeping Tissue or Organ Temperature at 3 to 6°C

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ABSTRACT

Background. As there is only one skin procurement organization in Japan the Japan Skin Bank Network (JSBN), all skin grafts procured in Japan are sent by a commercialized delivery system. Preliminarily, bottles containing saline were transported in a cardboard box using a so-called "cooled home delivery service" using a truck with a refrigerated cargo container. During transportation the temperature in the cardboard box increased to 18°C in summer and decreased to −5°C in winter. For these reasons, we investigated whether a newly developed container "Medi Cube" would be useful to transport skin grafts.

Objectives. Four bottles with a capacity of 300 mL containing 150 mL of saline in a Medi Cube container were transported from Osaka to the JSBN in Tokyo between 4 PM and 10 AM using a commercialized cooled home delivery service. Two bottles were transported in a Medi Cube container without phase change materials (PCM) in winter and summer, respectively. Another two bottles were transported in the Medi Cube with PCMs in winter. The temperatures inside saline, inside a transportation container, and outside the container, and air temperature were monitored continuously with a recordable thermometer.

Results. The temperatures inside saline and inside a Medi Cube container were maintained between 3 and 6°C, even when the temperature outside the container increased during parking. The temperature inside a Medi Cube container without PCM decreased to −3°C when the inside of the cargo container was overcooled in winter. However, the temperatures inside saline and inside a Medi Cube container with PCM were between 3 and 6°C, even when the temperature outside the container decreased to below 0°C in winter.

Conclusion. A Medi Cube container with PCM provided a safe, easy, and cost-effective method for overnight transportation of skin grafts.

IN Japan, only the the Japan Skin Bank Network (JSBN) performs frozen preservation and supplies skin. For example, when the skin sheets are procured from locations near Osaka, they are cooled temporarily at our bank overnight, and then transported using a commercial delivery system to the JSBN in Tokyo.[1] Although most Japanese delivery companies advertise that they can maintain the temperature of goods to

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