— **Research Letter** —

Phosphoenolpyruvic Acid, an Intermediate of Glycolysis, Attenuates Cellular Injury Induced by Hydrogen Peroxide and 2-Deoxy-D-glucose in the Porcine Proximal Kidney Tubular Cell Line, LLC-PK_1

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This study was conducted to clarify whether phosphoenolpyruvate (PEP), an intermediate substance of glycolysis, has the potential to attenuate cellular injury induced by oxidative stress or dysfunctions in energy metabolism *in vitro*. PEP (0.5–10 mM) attenuated hydrogen peroxide (H_2O_2)-induced cellular injury in the porcine proximal kidney tubular cell line, LLC-PK_1 in a dose-dependent manner. PEP also prevented cellular injury in LLC-PK_1 cells induced by the glycolysis inhibitor, 2-deoxy-D-glucose (2-DG). In addition, PEP significantly enhanced the degradation of H_2O_2. The prevention of H_2O_2-induced cellular injury mediated by PEP was more potent than that of the carbohydrates, glucose and trehalose, which are used as components of organ preservation solutions for clinical transplantation. In conclusion, we demonstrated that PEP is a bifunctional carbohydrate with anti-oxidant properties and suggest that PEP is potentially useful as an organ preservation agent in clinical transplantation.

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INTRODUCTION

Ischemia/reperfusion injury, which results from the interruption of blood flow and subsequent organ reperfusion, contributes to graft dysfunction and acute graft rejection; it is, therefore, a major problem in clinical organ transplantation.[1–3] Oxidative stress and compromised energy metabolism are associated, at least in part, with ischemia/reperfusion injury.[4–7] To inhibit this, organ preservation solutions such as Euro-Collins solution and University of Wisconsin solution are used.[8–10] These solutions are generally composed of electrolytes and buffers, with carbohydrates such as glucose and trehalose added to prevent energy loss. In addition, to inhibit the generation of reactive oxygen species during cold preservation and ischemia/reperfusion, the anti-oxidant glutathione (GSH) is added to the University of Wisconsin solution.[11–13] Although they are essential for clinical transplantation, there are several disadvantages to these solutions, including chemical instability and high viscosity. Also, they are not cost-effective for short-term storage.[14] Therefore, a new, effective reagent for organ preservation is critical.

Phosphoenolpyruvate (PEP) is an intermediate of glycolysis and gluconeogenesis that contains a high-energy phosphate bond. PEP can penetrate the cell membrane and transfer its high-energy phosphate group to adenosine 5′-diphosphate (ADP) to replenish intracellular ATP levels.[15,16] It has been reported that PEP improves the energy status of the heart[17,18] and skeletal muscle[19] after ischemia, and of the liver after ischemia-reperfusion.[15] Hojo *et al.*[20] demonstrated that the survival of transplanted kidney grafts was improved by Euro-Collins solution containing 10 mM PEP in a canine experimental kidney transplantation model. However, little is known about the precise mechanism underlying the protective effects of PEP and there is no evidence that PEP has the potential to attenuate cellular injury induced by oxidative stress, or dysfunctions

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in energy metabolism, which play important roles in ischemia/reperfusion injury.

Based on these facts, we designed an *in vitro* experiment to examine the effects of PEP on cellular injury induced by hydrogen peroxide (H_2O_2) or the glycolysis inhibitor, 2-deoxy-D-glucose (2-DG), in the porcine proximal kidney tubular cell line, LLC-PK_1. To evaluate the usefulness of PEP as an organ preservation agent, we compared the anti-oxidative effects of PEP with that of other components used in organ preservation solutions, such as glucose, trehalose and GSH.

MATERIALS AND METHODS

Materials —— Sodium phosphoenolpyruvate monohydrate was kindly donated by Ube Kousan (Yamaguchi, Japan). H_2O_2 and 2-DG were purchased from Wako Pure Chemicals (Osaka, Japan). The Cell Counting Kit was obtained from Dojindo Laboratories (Kumamoto, Japan). Medium 199 Earle's (M199) was obtained from Invitrogen Japan (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Thermo Scientific HyClone (Logan, UT, U.S.A.). All other reagents and solvents were of reagent grade. De-ionized and distilled bio-pure grade water was used throughout the study.

Cell Culture —— The LLC-PK_1 cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). LLC-PK_1 cells were cultured in M199 supplemented with 3% FBS, 100 IU/ml of penicillin, 100 μg/ml streptomycin at 37°C and 5% CO_2. Confluent cultures were washed with phosphate-buffered saline (PBS) pH 7.4, detached with EDTA and trypsin, centrifuged and subcultured in 96-well plates. Cells were seeded at a density of 1×10^4 cells/well and grown on culture dishes for 24 hr before use in the cell viability experiments.

Cell Viability Assays —— To evaluate the viability of LLC-PK_1 cells, mitochondrial dehydrogenase activity was measured using a modified MTT assay (the WST-1 assay) and the Cell Counting Kit according to the manufacturer's protocol. Cells were incubated with either H_2O_2 or 2-DG at 37°C and 5% CO_2 and the cell viability was assessed after 24 hr. In a preliminary study, we determined the minimum concentrations of H_2O_2 and 2-DG that induced a decrease in cell viability with high reproducibility and optimal concentrations were chosen for treatment. Based on the results, we used H_2O_2 or 2-DG at concentrations of 0.1 mM and 15 mM, respectively. To evaluate the effects of PEP on cell viability, PEP was used at doses up to 10 mM, as the maximum non-cytotoxic dose in LLC-PK_1 cells was 20 mM (data not shown).

Measurement of Hydrogen Peroxide Decomposition —— We prepared a reaction mixture containing: 0.1 mM H_2O_2, 120 mM KCl and 50 mM Tris-HCl, pH 7.4 and then added PEP (0.1–10 mM) to the mixture. The reaction was allowed to proceed for 30 min at 37.8°C and terminated by the addition of a stop solution (25 mg/ml potassium biphthalate, 2.5 mg/ml NaOH, 82.5 mg/ml potassium iodide, and 0.25 mg/ml ammonium molybdate). The absorbance of the mixture was measured at 350 nm in a spectrophotometer (V-530; Jasco, Tokyo, Japan) and the concentration of H_2O_2 was determined by interpolation of the data using a standard curve.

Statistical Analysis —— Results were expressed as mean ± S.D. Multiple comparisons were made to examine the statistical significance of the data. When uniform variance was identified by Bartlett's analysis ($p < 0.05$), one-way analysis of variance (ANOVA) was used to test for statistical significance. When significant differences ($p < 0.05$) were identified the data were further analyzed using Tukey's multiple range test.

RESULTS

Effects of PEP Treatment on the Decrease in Cell Viability Induced by H_2O_2 or 2-DG in LLC-PK_1 Cells

LLC-PK_1 cells treated with 0.1 mM H_2O_2 for 24 hr showed a 67% decrease in cell viability compared with the control (Fig. 1A). When 0.5–10 mM PEP was added to the culture medium just after H_2O_2 treatment, the decrease in cell viability induced by H_2O_2 was significantly attenuated in a dose-dependent manner (Fig. 1A).

As shown in Fig. 1B, treatment with 15 mM 2-DG for 24 hr significantly reduced cell viability (to approximately 60% of the value of the control group). This decrease in viability was significantly attenuated by PEP (10 mM, to approximately 87% of the value of the control group).

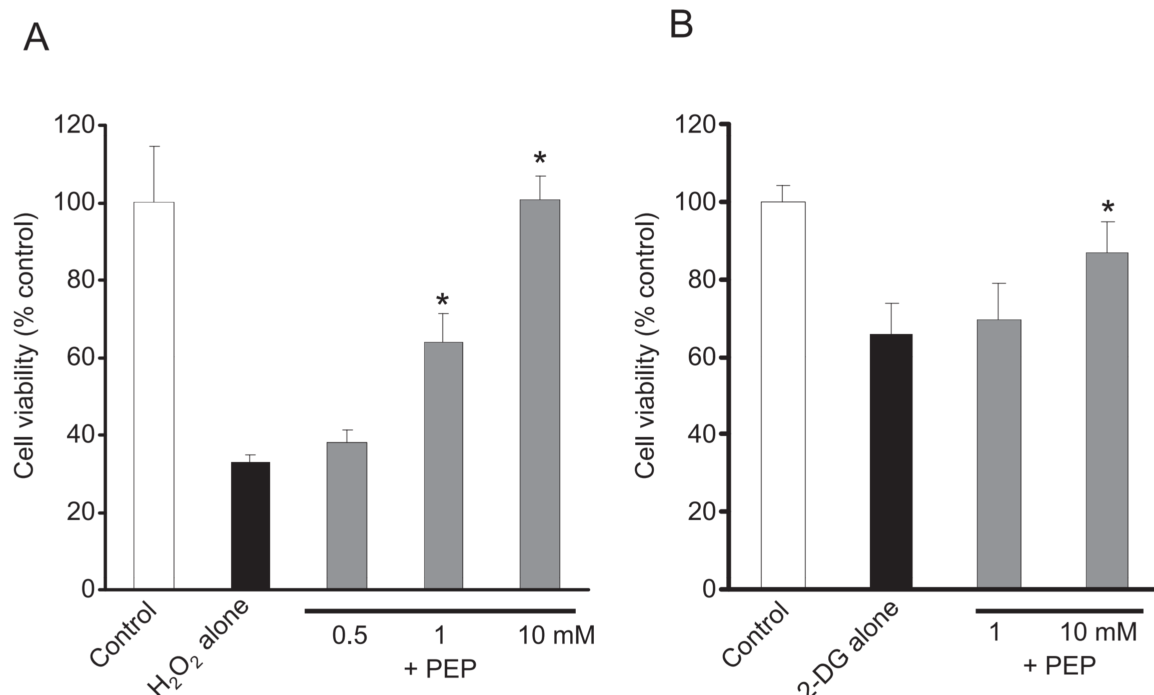

Fig. 1. PEP Abrogates the Decrease in Cell Viability Induced by H_2O_2 or 2-DG in LLC-PK_1 Cells

LLC-PK_1 cells exposed to 0.1 mM H_2O_2 (A) or 15 mM 2-DG (B) in either the presence or absence of PEP at different concentrations (0.5–10 mM). Cell viability was measured 24 hr after the addition of H_2O_2 or 2-DG using the WST-1 assay. Data are expressed as percent cell survival relative to the control group. Each bar represents mean ± S.D. ($n = 8$). $^{*}p < 0.01$ compared with the H_2O_2-treated or 2-DG-treated group.

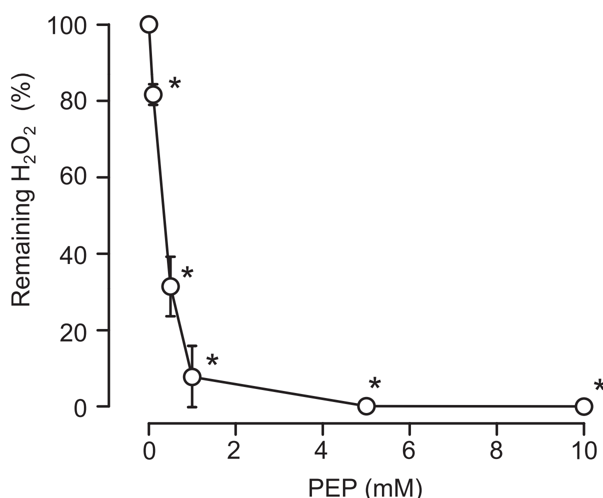

Fig. 2. Effects of PEP on H_2O_2 Decomposition

Data are expressed as percent H_2O_2 relative to the PEP-free control. Each point represents mean ± S.D. ($n = 4$). $^{*}p < 0.01$.

Evaluation of PEP-mediated H_2O_2 Decomposition

As shown in Fig. 2, treatment with PEP significantly reduced the H_2O_2 content of the reaction mixture, which was completely abolished by PEP at concentrations ≥ 5 mM. To compare the ability of PEP to degrade H_2O_2 with that of the components of Euro-Collins or University of Wisconsin organ preservation solutions (such as glucose, trehalose and GSH) we determined the concentration of each reagent required to yield 50% decomposition of 0.1 mM H_2O_2 (50% effective concentration, EC_{50}). The EC_{50} value for PEP was 0.26 mM compared with 200 and 120 mM (close to the concentrations used for organ preservation) for glucose and trehalose, respectively. The EC_{50} of GSH and N-acetylcysteine (NAC), also used as antioxidants for organ preservation, were 0.07 and 0.02 mM, respectively.

Effects of PEP and Other Components in Organ Preservation Solutions on H_2O_2-induced Cellular Injury in LLC-PK_1 Cells

Glucose (194 mM) and trehalose (120 mM, the concentrations used in organ preservation solutions) had little effect in preventing the H_2O_2-mediated decrease in cell viability (Fig. 3). However, this decrease in viability was significantly abrogated by PEP (10 mM), GSH (3 mM) and NAC (10 mM, Fig. 3).

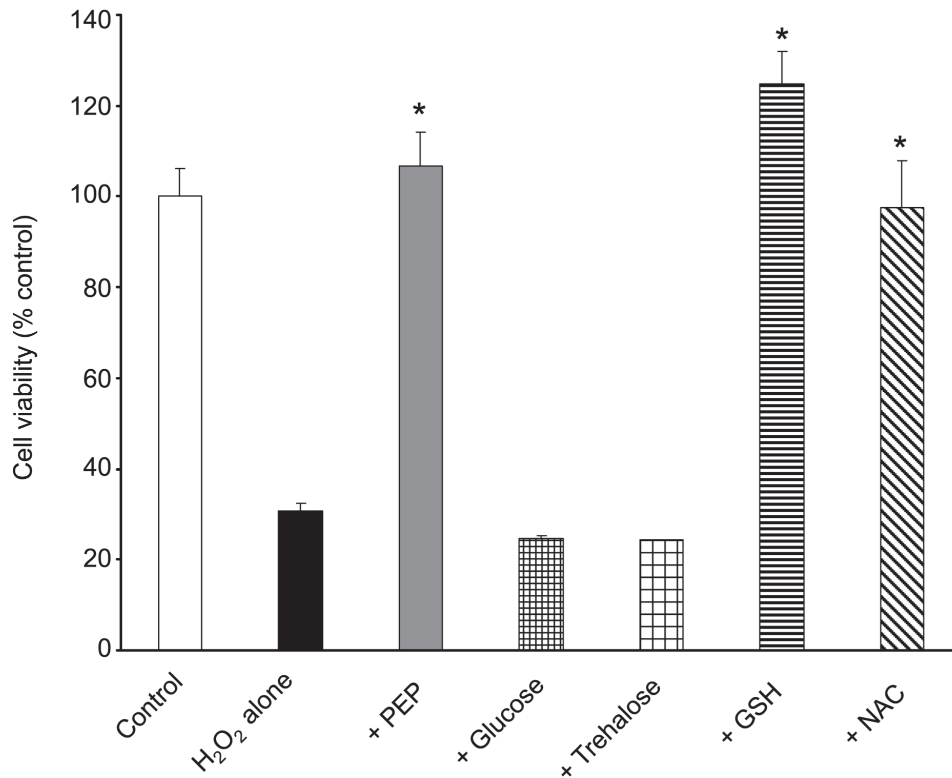

Fig. 3. Effects of PEP or Other Components of Organ Preservation Solutions on the Decrease in Cell Viability Induced by H_2O_2

LLC-PK_1 cells exposed to H_2O_2 in either the presence or absence of PEP (10 mM), glucose (194 mM), trehalose (120 mM), glutathione (3 mM) or NAC (10 mM). Cell viability was estimated 24 hr after the addition of H_2O_2. Data are expressed as percent cell survival relative to the control group. Each bar represents mean ± S.D. ($n = 6$). $^*p < 0.01$ compared with the H_2O_2-treated group.

DISCUSSION

In this study, we showed that PEP, an intermediate of glycolysis, protected LLC-PK_1 cells from injury induced by H_2O_2 and 2-DG. PEP was also able to mediate the decomposition of H_2O_2 These results indicate that PEP protects against cellular injury induced by oxidative stress and against dysfunctions in carbohydrate metabolism through, at least in part, an antioxidant effect.

Ischemia/reperfusion of allografts triggers oxidative stress leading to dysfunction and acute rejection.[2] The xanthine oxidase system is activated during ischemia/reperfusion and increased levels of H_2O_2 and hydroxyl radicals (decomposition products of H_2O_2) can injure the allograft; thus, H_2O_2-induced renal cell injury has frequently been used as an *in vitro* model of ischemia/reperfusion injury in the kidney.[21, 22] The results of our current *in vitro* study support those from *in vivo* studies showing that PEP improves energy status and improves the function and survival of allografts.[20] Our results also indicate that PEP protects allografts from acute rejection, not only through improved ATP levels, but also via its antioxidant effects.

PEP contains a high-energy phosphate group and efficiently supplies energy to ischemic/reperfused organs *in vivo* and *in situ*[15, 17–20] Our current results suggest that the *in vitro* protective effects of PEP against cellular injury induced by the glycolysis inhibitor, 2-DG, used as an *in vitro* model of ischemic organ injury due to energy metabolism dysfunction,[21] may be due to the fact that PEP replenishes cellular ATP and acts as a cytoprotective agent against energy metabolism dysfunctions.

The protective effects of PEP against cellular injury mediated by oxidative stress and dysfunctions in carbohydrate metabolism protect allografts and organs from ischemia/reperfusion injury. Antioxidants are frequently used in organ preservation solutions to protect tissues from oxidative stress induced during preservation and ischemia/reperfusion.[8, 12] University of Wisconsin solution contains glutathione and allopurinol, whereas ET-Kyoto solution contains NAC. These preservation solutions

seem to achieve better results than other preservation solutions.[23,24] In addition, carbohydrates such as glucose and trehalose are added to preservation solutions to reduce energy depletion from organs/cells during preservation. PEP may be an attractive bifunctional carbohydrate with the potential to act as both energy supplier and antioxidant. To establish the efficacy of PEP as a component of organ preservation solutions, it is important to identify its effects on other causes of allograft dysfunction, such as cold preservation and rewarming of the cells and organs and immune responses/inflammatory reactions.[25] In addition, it is important to clarify whether PEP-containing organ preservation solutions are safe for both the allograft and the recipient. Further studies are therefore warranted.

In summary, we have shown that PEP protected LLC-PK_1 cells against injury induced by H_2O_2 and 2-DG and facilitated the decomposition of H_2O_2. The results suggest that PEP is a bifunctional carbohydrate with antioxidant effects and has potential for use as an agent for organ preservation in clinical transplantation.

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REFERENCES

1) Guan, X., Dei-Anane, G., Liang, R., Gross, M. L., Nickkholgh, A., Kern, M., Ludwig, J., Zeier, M., Buchler, M. W., Schmidt, J. and Schemmer, P. (2008) Donor preconditioning with taurine protects kidney grafts from injury after experimental transplantation. *J. Surg. Res.*, **146**, 127–134.
2) Kosieradzki, M. and Rowinski, W. (2008) Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. *Transplant. Proc.*, **40**, 3279–3288.
3) Macedo, F. I. and Miranda, L. E. (2010) Role of ischemic preconditioning in liver transplant: a review of literature. *Exp. Clin. Transplant.*, **8**, 1–3.
4) Yang, C. C., Lin, L. C., Wu, M. S., Chien, C. T. and Lai, M. K. (2009) Repetitive hypoxic preconditioning attenuates renal ischemia/reperfusion induced oxidative injury via upregulating HIF-1 alpha-dependent bcl-2 signaling. *Transplantation*, **88**, 1251–1260.
5) Li, Z., Nickkholgh, A., Yi, X., Bruns, H., Gross, M. L., Hoffmann, K., Mohr, E., Zorn, M., Buchler, M. W. and Schemmer, P. (2009) Melatonin protects kidney grafts from ischemia/reperfusion injury through inhibition of NF-kB and apoptosis after experimental kidney transplantation. *J. Pineal Res.*, **46**, 365–372.
6) Evans, Z. P., Mandavilli, B. S., Ellett, J. D., Rodwell, D., Fariss, M. W., Fiorini, R. N., Schnellmann, R. G., Schmidt, M. G. and Chavin, K. (2009) Vitamin E succinate enhances steatotic liver energy status and prevents oxidative damage following ischemia/reperfusion. *Transplant. Proc.*, **41**, 4094–4098.
7) Jassem, W. and Heaton, N. D. (2004) The role of mitochondria in ischemia/reperfusion injury in organ transplantation. *Kidney Int.*, **66**, 514–517.
8) Muhlbacher, F., Langer, F. and Mittermayer, C. (1999) Preservation solutions for transplantation. *Transplant. Proc.*, **31**, 2069–2070.
9) Schneeberger, S., Biebl, M., Steurer, W., Hesse, U. J., Troisi, R., Langrehr, J. M., Schareck, W., Mark, W., Margreiter, R. and Konigsrainer, A. (2009) A prospective randomized multicenter trial comparing histidine-tryptophane-ketoglutarate versus University of Wisconsin perfusion solution in clinical pancreas transplantation. *Transpl. Int.*, **22**, 217–224.
10) Opelz, G. and Dohler, B. (2007) Multicenter analysis of kidney preservation. *Transplantation*, **83**, 247–253.
11) de Boer, J., De Meester, J., Smits, J. M., Groenewoud, A. F., Bok, A., van der Velde, O., Doxiadis, N. I. I. and Persijn, G. G. (1999) Eurotransplant randomized multicenter kidney graft preservation study comparing HTK with UW and Euro-Collins. *Transpl. Int.*, **12**, 447–453.
12) Ploeg, R. J., Goossens, D., McAnulty, J. F., Southard, J. H. and Belzer, F. O. (1988) Successful 72-hour cold storage of dog kidneys with UW solution. *Transplantation*, **46**, 191–196.
13) Ringe, B., Braun, F., Moritz, M., Zeldin, G., Soriano, H. and Meyers, W. (2005) Safety and efficacy of living donor liver preservation with HTK solution. *Transplant. Proc.*, **37**, 316–319.
14) Feng, L., Zhao, N., Yao, X., Sun, X., Du, L., Diao, X., Li, S. and Li, Y. (2007) Histidine-tryptophan-ketoglutarate solution vs. University of Wisconsin solution for liver transplantation: a systematic review. *Liver Transpl.*, **13**, 1125–1136.
15) Saiki, S., Yamaguchi, K., Chijiiwa, K., Shimizu, S., Hamasaki, N. and Tanaka, M. (1997) Phospho-

enolpyruvate prevents the decline in hepatic ATP and energy charge after ischemia and reperfusion injury in rats. *J. Surg. Res.*, **73**, 59–65.
16) Hamasaki, N., Hardjono, I. S. and Minakami, S. (1978) Transport of phosphoenolpyruvate through the erythrocyte membrane. *Biochem. J.*, **170**, 39–46.
17) Hultman, J., Ronquist, G., Forsberg, J. O. and Hansson, H. E. (1983) Myocardial energy restoration of ischemic damage by administration of phosphoenolpyruvate during reperfusion. A study in a paracorporeal rat heart model. *Eur. Surg. Res.*, **15**, 200–207.
18) Matsuzaki, K., Takano, H., Tokunaga, K., Inaba, S. and Hamasaki, N. (1993) Clinical application of phosphoenolpyruvate (PEP) to autologous transfusion of patients with open heart surgery. *Fukuoka Acta Med.*, **84**, 7–14.
19) Hickey, M. J., Knight, K. R., Hurley, J. V. and Lepore, D. A. (1995) Phosphoenolpyruvate/adenosine triphosphate enhances post-ischemic survival of skeletal muscle. *J. Reconstr. Microsurg.*, **11**, 415–422.
20) Hojo, M., Ito, H., Tsuji, Y., Ariyoshi, A. and Hamasaki, N. (1994) A study of preservation solution containing phosphoenolpyruvate in renal transplantation. *Med. Bull. Fukuoka Univ.*, **21**, 301–310.
21) Park, K. M., Kramers, C., Vayssier-Taussat, M., Chen, A. and Bonventre, J. V. (2002) Prevention of kidney ischemia/reperfusion-induced functional injury, MAPK and MAPK kinase activation, and inflammation by remote transient ureteral obstruction. *J. Biol. Chem.*, **277**, 2040–2049.
22) Harrison, R. (2002) Structure and function of xanthine oxidoreductase: where are we now? *Free Radic. Biol Med.*, **33**, 774–797.
23) Chen, F., Fukuse, T., Hasegawa, S., Bando, T., Hanaoka, N., Kawashima, M., Sakai, H., Hamakawa, H., Fujinaga, T., Nakamura, T. and Wada, H. (2004) Effective application of ET-Kyoto solution for clinical lung transplantation. *Transplant. Proc.*, **36**, 2812–2815.
24) Yoshida, H., Okuno, H., Kamoto, T., Habuchi, T., Toda, Y., Hasegawa, S., Nakamura, T., Wada, H., Ogawa, O. and Yamamoto, S. (2002) Comparison of the effectiveness of ET-Kyoto with Euro-Collins and University of Wisconsin solutions in cold renal storage. *Transplantation*, **74**, 1231–1236.
25) Inoue, K., Suzuki, S., Kubo, H., Ishida, I., Ueda, S. and Kondo, T. (2003) Effects of rewarming on nuclear factor-kappaB and interleukin 8 expression in cold-preserved alveolar epithelial cells. *Transplantation*, **76**, 409–415.