NOTE *Surgery*

High-Molecular-Weight Polyethylene Glycol Enhances Hypothermic Storage of Feline Kidney Cells

Masaaki KATAYAMA[1)]*, Shinobu TSUCHIAKA[1)], Tomoki MOTEGI[1)], Masao MIYAZAKI[2)], Tetsuro YAMASHITA[2)], Shunsuke SHIMAMURA[3)], Yasuhiko OKAMURA[1)] and Yuji UZUKA[1)]

[1)]*Division of Small Animal Surgery, Co-Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka, Iwate 020–8550, Japan,*
[2)]*Department of Biological Chemistry and Food Sciences, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka, Iwate 020–8550, Japan*
[3)]*Division of Small Animal Internal Medicine, Co-Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka, Iwate 020–8550, Japan*

(Received 11 November 2013/Accepted 18 February 2014/Published online in J-STAGE 5 March 2014)

ABSTRACT. Phosphate-buffered sucrose (PBSc) solution is effective for short-term hypothermic preservation of tissue during feline kidney transplantation. A high-molecular-weight polyethylene glycol (35,000 Da, PEG35) reportedly enhanced the protective effects against cold-induced tubular injuries in animal kidney transplantation models. We investigated the ability of PBSc solution containing PEG35 to preserve cultured feline kidney cells using *in vitro* WST-8 cell proliferation assays. PEG35 significantly improved cell viability during 24 hr of cold preservation. PBSc containing 20 g/*l* PEG35 achieved an effect almost equal to that of University of Wisconsin (UW) solution, the gold standard preservation solution used in human clinical kidney transplantation, for up to 24 hr of preservation. Our results suggest that PBSc containing PEG35 provides an excellent medium for graft cold storage during feline kidney transplantation.
KEY WORDS: cold storage, feline kidney transplantation, kidney cell, polyethylene glycol.

doi: 10.1292/jvms.13-0565; *J. Vet. Med. Sci.* 76(6): 923–926, 2014

Hypothermic storage using a preservation solution is effective for preventing ischemia and reperfusion injuries of the donor graft. In small animal surgery, this may allow sequential donor organ harvesting and transplant surgeries by a single surgical team. Phosphate-buffered sucrose (PBSc) solution achieves superior results in short- and medium-term renal preservation (up to 5 hr) in cats [18]. However, PBSc may not effectively protect against cold ischemic injury in a feline renal autotransplantation model [26]. University of Wisconsin (UW) solution, used as the gold standard preservation solution in human clinical kidney transplantation, may be applied [18]. UW solution is high in potassium ions and low in sodium ions and maintains the intracellular ionic balance. High potassium contents in the solution may increase the risk of cardiac arrhythmias after reperfusion of the graft in small animals, such as the cat. In addition, an intracellular type solution including high potassium levels may induce vasoconstriction which impairs organ perfusion during washout and reperfusion [22–24]. Therefore, an alternative storage solution with a simple extracellular composition should be developed for use in feline kidney transplantation.

*CORRESPONDENCE TO: KATAYAMA, M., Division of Small Animal Surgery, Co-Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka, Iwate 020–8550, Japan. e-mail: masaaki@iwate-u.ac.jp

Polyethylene glycol (PEG) is a neutral, water-soluble, non-antigenic polymer that serves as a colloid in organ preservation solutions [10]. PEG can protect renal tubule cells against cold injury by reducing osmotic cell swelling and prevent lipid peroxidation [4, 12, 16]. PEG also prevents ischemia and reperfusion injury induced inflammation by creating a barrier that prevents recognition of allogenic sites on cell membranes by the immune system [10, 21]. Fuller *et al.* [8] reported that PEG supplementation to sucrose based cold storage solution could reduce ischemia and reperfusion injury in a rabbit renal transplantation model. Protective effects of high molecular weight PEG 20,000 Da (PEG20) against cold ischemia and reperfusion injury were revealed in animal kidney transplant models [5–7, 11, 28]. PEG20 supplemented low potassium extracellular type solution was reported to preserve the kidney from inflammatory cell infiltrates, MHC class II and VCAM-1 overexpressions and occurrence of renal interstitial fibrosis in a pig kidney autotransplantation model [11]. Dutheil *et al.* [4] demonstrated that higher-molecular-weight PEG 35,000 Da (PEG35) compared with PEG20 enhances the protective effects against cold-induced tubular injuries in pig kidney cells and transplantation models.

The aim of this study was to evaluate the effect of high-molecular-weight PEG35 on hypothermic storage of feline kidney cells. We investigated whether the addition of PEG35 to a simple extracellular type storage solution, PBSc, affected cell viability following cold preservation up to 24 hr compared to PBSc alone and the standard UW solution.

Crandell-Reese feline kidney (CRFK) cells [3] were grown in Dulbecco's modified Eagle's medium (D-MEM;

Wako, Osaka, Japan) with 10% fetal bovine serum, 100 U/m*l* penicillin, 100 μg/m*l* streptomycin and 0.25 μg/m*l* amphotericin B in a humidified atmosphere of 5% carbon dioxide at 37°C. CRFK cells (4.0×10^5/m*l*) in 10% fetal bovine serum-supplemented D-MEM were plated on 96-microwell plates (Corning Inc., Corning, NY, U.S.A.) and incubated until confluence in a humidified atmosphere of 5% carbon dioxide at 37°C. The culture medium was then discarded. After 2 washes with warm phosphate-buffered saline (Wako, Osaka, Japan) solution, cells were incubated for 3, 6, 9, 12, 15 and 24 hr at 4°C in 100 μ*l* of UW (Viaspan®, Astellas, Tokyo, Japan) and PBSc containing 0–40 g/m*l* PEG35 (Sigma-Aldrich Co., St. Louis, MO, U.S.A.). The PBSc storage solution contained 1,000 U/*l* heparin, 53.6 mM Na_2HPO_4, 15.5 mM NaH_2PO_4 and 140 mM sucrose (pH 7.2). The UW solution consisted of 25 mM KH_2PO_4, 5 mM $MgSO_4$, 100 mM lactobionate, 30 mM raffinose, 3 mM glutathione, 5 mM adenosine, 1mM allopurinol and 50 g/*l* hydroxyethylstarch (pH 7.4).

In this study, cell viability was assessed using a 4-[3-(2-methoxy-4-nitrophenyl)-2-(−4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium (WST-8) Cell Counting Kit (Dojindo, Osaka, Japan), which is a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method. WST-8, as compared to the conventional MTT method, produces a highly water soluble formazan dye and is stable and sensitive for measuring cell viability. For the WST-8 assay, 10 μ*l* of cell Counting Kit solution were added to 100 μ*l* of medium per well on the assay plate and incubated for 4 hr at 37°C. Sample absorbance at 450 nm was measured using a microplate reader (ARVO™MX, 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, U.S.A.). The blank value was the absorbance of the solution without the sample. Cell viability in each well was expressed as the percentage of viable cells compared to the warm control. Three wells were used for each sample solution per experiment, and each experiment was repeated three times.

All data are presented as means ± standard deviation (SD) and compared for statistical significance using variance analysis followed by Tukey-Kramer test for multiple comparison tests. A *P* value less than 0.05 was considered to indicate a significant difference.

Various doses of PEG35 in PBSc were tested to investigate the effect on cell preservation. The proportions of viable cells after 24 hr of cold storage were 6.3 ± 1.3%, 18.6 ± 5.5%, 42.0 ± 5.3%, 38.7 ± 3.7%, 40.1 ± 10.4%, 54.7 ± 6.2% and 45.2 ± 6.2% in PBSc supplemented with 0, 0.5, 1, 5, 10, 20 and 40 g/*l* PEG35, respectively (Fig. 1). The addition of PEG35 significantly improved cell viability compared with PBSc alone ($P<0.01$). The addition of 20 g/*l* PEG35 significantly prolonged cell survival compared with 0.5, 1, 5 and 10 g/*l* PEG35 ($P<0.01$). The effect of supplementation with 40 g/*l* PEG35 was not significantly different from the effects of 1, 5, 10 and 20 g/*l* PEG35.

The protective effect of PBSc supplemented with 20 g/*l* PEG35 was compared to those of PBSc alone and UW solution for up to 24 hr (Fig. 2). No differences were observed

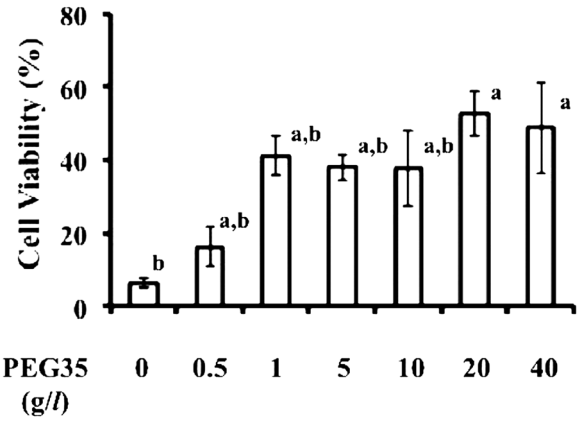

Fig. 1. Dose effect of PEG35 supplemented in PBSc on 24 hr cold preservation of feline kidney cells. Values are presented as means ± SD. a) Significantly different from PBSc alone ($P<0.01$). b) Significantly different from PBSc supplemented with 20 g/*l* PEG35 ($P<0.01$).

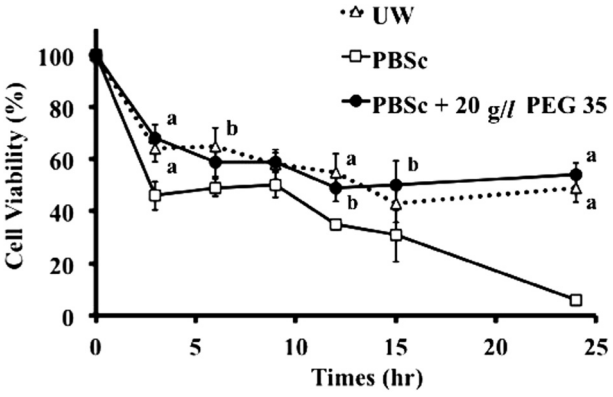

Fig. 2. Cell protective effect of PBSc supplemented with 20 g/*l* PEG35 compared with PBSc alone and UW solution for up to 24 hr. Values are presented as means ± SD. a) $P<0.01$, b) $P<0.05$ compared with PBSc alone.

between PBSc with 20 g/*l* PEG35 and UW solution. The addition of 20 g/*l* PEG35 to PBSc significantly increased cell viability at 3, 12, 15 and 24 hr compared with PBSc alone ($P<0.01$, $P<0.05$, $P<0.05$ and $P<0.01$, respectively). UW solution also significantly improved cell viability compared to PBSc alone at 3, 6, 12 and 24 hr ($P<0.01$, $P<0.05$, $P<0.01$ and $P<0.01$, respectively).

Cold storage of organs at 4°C plays an important role in organ transplantation. However, cold storage allows the prolongation of ischemic condition which seriously damages organs. Cold ischemic injury is known to be associated with malfunction of osmoregulation, energetics and metabolism [1]. Both intra- and extra-cellular type solutions have been developed to reduce this serious injury. UW solution is the current gold standard commercial storage solution used for human transplantion and is classified as an intracellular so-

lution because of the high potassium concentration, which prevents the increase in intracellular Ca^{2+} during ischemia [1, 17]. It was reported that UW solution could preserve allograft kidneys for up to 7 hr in cats [18]. However, a high potassium concentration induces cellular depolarization, accelerates the decrease in cellular ATP level and activates voltage-dependent channels, such as calcium channels. The consecutive calcium influx results in cellular damages [22, 24]. A potassium concentration greater than 20 mM/*l* is a potent stimulus for vasoconstriction, impairing organ perfusion during washout and reperfusion [23, 25].

Recently, extracellular solutions have shown equal or greater preservative effects compared to intracellular solutions [15]. Faure *et al.* [7] demonstrated that extracellular type solution greatly improved the glomerular filtration rate of the autotransplanted pig kidney. Although simple extracellular type PBSc solution has been used to reduce cold ischemic injury of allograft kidney in clinical feline renal transplantation [18], our findings showed that PBSc was much less effective than UW solution even for short-term cold storage (3 hr). We report here that supplementation of PEG35 in PBSc protects cultured feline kidney cells against damage caused by hypothermic storage by mimicking organ preservation conditions. The WST-8 assay demonstrated that PBSc containing 20 g/*l* PEG35 was at least as effective as UW solution. Therefore, simple extracellular solutions, such as PBSc containing PEG35, represent an alternative for cold storage of grafts for feline kidney transplantation.

The PEG molecules has interesting properties in the context of organ preservation. PEG increases oncotic pressure, limiting the deleterious effects of edema [9, 10]. It is sufficiently adsorbed to the cell membrane surface to stabilize membrane lipids and induce immunocamouflage of antigenic sites, enhancing the immunoprotection of donor tissues and organs [21]. PEG inhibits or reduces oxidative stress by preserving and restoring cell membrane integrity, resulting in protection against reactive oxygen species (ROS) produced during ischemia [4]. Dutheil *et al.* [4] demonstrated that ROS generation was significantly reduced by the addition of high-molecular-weight PEG35 at concentrations greater than 1 g/*l* during cold storage of porcine kidney cells. In this study, PEG35 concentrations greater than 1 g/*l*, especially 20 g/*l*, significantly increased the viability of feline kidney cells. In addition, PEG significantly reduced MHC class II expression in epithelial tubule cells and the number of CD4+T cell infiltrates and limited the infiltration of macrophages/monocytes and progression of interstitial fibrosis in the 8 to 12 weeks after surgery in a pig renal autotransplantion model [11]. Further investigations regarding the mechanisms of the protective effects of PEG on feline kidney cells are required.

The effects of storage solutions must be confirmed by evaluating its ability to preserve real organs in animal models. However, the study using a monolayer cell model may be recommended before trials using experimental animals for humane reasons and the possibilities of unidentified factors influencing the results. The advantage of a monolayer cell model is the generation of highly reproducible data due to the identical characteristics of the cultured cells. Also, it is easier to analyze and readjust the solution composition. Cultured cells have been used in many studies [4, 13, 20]. Therefore, a feline kidney cell monolayer model was adopted in this report.

Although our results demonstrated the possibility of 24-hr cold preservation of kidney cells using PEG35 in PBSc, the feasible duration of hypothermic preservation of real feline kidneys remains to be determined. Estimation of preservation time from other animal species should be avoided, because of interspecies differences [2, 14, 19, 27].

In conclusion, PEG35 significantly improved feline kidney cell viability in a dose-dependent manner. PBSc containing 20 g/*l* PEG35 exhibited a cold preservation effect almost equal to that of UW solution. Further research should focus on confirming the effects of PBSc supplemented with PEG35 on ischemia and reperfusion injury in a feline kidney transplant model.

REFERENCES

1. Belzer, F. O. and Southard, J. H. 1988. Principles of solid-organ preservation by cold storage. *Transplantation* **45**: 673–676. [Medline] [CrossRef]
2. Collins, G. M., Bravo-Shugarman, M. and Terasaki, P. I. 1969. Kidney preservation for transportation. Initial perfusion and 30 hours' ice storage. *Lancet* **2**: 1219–1222. [Medline] [CrossRef]
3. Crandell, R. A., Fabricant, C. G. and Nelson-Rees, W. A. 1973. Development, characterization, and viral susceptibility of a feline (Felis catus) renal cell line (CRFK). *In Vitro* **9**: 176–185. [Medline] [CrossRef]
4. Dutheil, D., Rioja-Pastor, I., Tallineau, C., Goujon, J. M., Hauet, T., Mauco, G. and Petit-Paris, I. 2006. Protective effect of PEG 35,000 Da on renal cells: paradoxical activation of JNK signaling pathway during cold storage. *Am. J. Transplant.* **6**: 1529–1540. [Medline] [CrossRef]
5. Faure, J. P., Hauet, T., Han, Z., Goujon, J. M., Petit, I., Mauco, G., Eugene, M., Carretier, M. and Papadopoulos, V. 2002. Polyethylene glycol reduces early and long-term cold ischemia-reperfusion and renal medulla injury. *J. Pharmacol. Exp. Ther.* **302**: 861–870. [Medline] [CrossRef]
6. Faure, J. P., Jayle, C., Dutheil, D., Eugene, M., Zhang, K., Goujon, J. M., Petit-Paris, I., Tillement, J. P., Touchard, G., Robert, R., Wahl, A., Seguin, F., Mauco, G., Vandewalle, A. and Hauet, T. 2004. Evidence for protective roles of polyethylene glycol plus high sodium solution and trimetazidine against consequences of renal medulla ischaemia during cold preservation and reperfusion in a pig kidney model. *Nephrol. Dial. Transplant.* **19**: 1742–1751. [Medline] [CrossRef]
7. Faure, J. P., Petit, I., Zhang, K., Dutheil, D., Doucet, C., Favreau, F., Eugène, M., Goujon, J. M., Tillement, J. P., Mauco, G., Vandewalle, A. and Hauet, T. 2004. Protective roles of polyethylene glycol and trimetazidine against cold ischemia and reperfusion injuries of pig kidney graft. *Am. J. Transplant.* **4**: 495–504. [Medline] [CrossRef]
8. Fuller, B. J., Shurey, C., Lane, N., Petrenko, A. and Green, C. 2006. Hypothermic renal preservation with a sucrose/polyethylene glycol solution in a rabbit renal transplant model. *Cryo Letters* **27**: 127–132. [Medline]
9. Ganote, C. E., Worstell, J., Ianotti, J. P. and Kaltenbach, J. P. 1977. Cellular swelling and irreversible myocardial injury. *Am. J. Pathol.* **88**: 95–118. [Medline]
10. Hauet, T. and Eugene, M. 2008. A new approach in organ

preservation: potential role of new polymers. *Kidney Int.* **74**: 998–1003. [Medline] [CrossRef]
11. Hauet, T., Goujon, J. M., Baumert, H., Petit, I., Carretier, M., Eugene, M. and Vandewalle, A. 2002. Polyethylene glycol reduces the inflammatory injury due to cold ischemia/reperfusion in autotransplanted pig kidneys. *Kidney Int.* **62**: 654–667. [Medline] [CrossRef]
12. Hauet, T., Mothes, D., Goujon, J. M., Carretier, M. and Eugene, M. 2001. Protective effect of polyethylene glycol against prolonged cold ischemia and reperfusion injury: study in the isolated perfused rat kidney. *J. Pharmacol. Exp. Ther.* **297**: 946–952. [Medline]
13. Isowa, N., Hitomi, S. and Wada, H. 1996. Trehalose-containing solutions enhance preservation of cultured endothelial cells. *Ann. Thorac. Surg.* **61**: 542–545. [Medline] [CrossRef]
14. Kehrer, G., Blech, M., Kallerhoff, M., Kleinert, H., Langheinrich, M. and Bretschneider, H. J. 1990. Glucose content and efficiency of glycolysis in protected ischemic kidneys of different species. *J. Invest. Surg.* **3**: 147–168. [Medline] [CrossRef]
15. Maathuis, M. H., Leuvenink, H. G. and Ploeg, R. J. 2007. Perspectives in organ preservation. *Transplantation* **83**: 1289–1298. [Medline] [CrossRef]
16. Mack, J. E., Kerr, J. A., Vreugdenhil, P. K., Belzer, F. O. and Southard, J. H. 1991. Effect of polyethylene glycol on lipid peroxidation in cold-stored rat hepatocytes. *Cryobiology* **28**: 1–7. [Medline] [CrossRef]
17. McAnulty, J. F. 2010. Hypothermic organ preservation by static storage methods: Current status and a view to the future. *Cryobiology* **60**: S13–S19. [Medline] [CrossRef]
18. McAnulty, J. F. 1998. Hypothermic storage of feline kidneys for transplantation: successful ex vivo storage up to 7 hours. *Vet. Surg.* **27**: 312–320. [Medline] [CrossRef]
19. McAnulty, J. F., Vreugdenhil, P. K., Lindell, S., Southard, J. H. and Belzer, F. O. 1993. Successful 7-day perfusion preservation of the canine kidney. *Transplant. Proc.* **25**: 1642–1644. [Medline]
20. Moutabarrik, A., Mourid, M. and Nakanishi, I. 1998. The effect of organ preservation solutions on kidney tubular and endothelial cells. *Transpl. Int.* **11**: 58–62. [Medline] [CrossRef]
21. Murad, K. L., Gosselin, E. J., Eaton, J. W. and Scott, M. D. 1999. Stealth cells: prevention of majorhistocompatibility complex class II-mediated T-cell activation by cell surface modification. *Blood* **94**: 2135–2141. [Medline]
22. Rauen, U. and de Groot, H. 2004. New insights into the cellular and molecular mechanisms of cold storage injury. *J. Investig. Med.* **52**: 299–309. [Medline] [CrossRef]
23. Ryman, T., Brandt, L., Andersson, K.E. and MellergÅrd, P. 1989. Regional and species differences in vascular reactivity to extracellular potassium. *Acta Physiol. Scand.* **136**: 151–159. [Medline] [CrossRef]
24. Salahudeen, A. K. 2004. Cold ischemic injury of transplanted kidneys: new insights from experimental studies. *Am. J. Physiol. Renal Physiol.* **287**: F181–F187. [Medline] [CrossRef]
25. Sasaki, S., McCully, J. D., Alessandrini, F. and LoCicero, J. 1995. Impact of initial flush potassium concentration on the adequacy of lung preservation. *J. Thorac. Cardiovasc. Surg.* **109**: 1090–1095. [Medline] [CrossRef]
26. Schmiedt, C. W., Mercurio, A. D., Glassman, M. M., McAnulty, J. F., Brown, C. A. and Brown, S. A. 2009. Effects of renal autograft ischemia and reperfusion associated with renal transplantation on arterial blood pressure variables in clinically normal cats. *Am. J. Vet. Res.* **70**: 1426–1432. [Medline] [CrossRef]
27. Southard, J. H., Marsh, D. C., McAnulty, J. F. and Belzer, F. O. 1987. Oxygen-derived free radical damage in organ preservation: activity of superoxide dismutase and xanthine oxidase. *Surgery* **101**: 566–570. [Medline]
28. Thuillier, R., Renard, C., Rogel-Gaillard, C., Demars, J., Milan, D., Forestier, L., Ouldmoulene, A., Goujon, J. M., Badet, L. and Hauet, T. 2011. Effect of polyethylene glycol-based preservation solutions on graft injury in experimental kidney transplantation. *Br. J. Surg.* **98**: 368–378. [Medline] [CrossRef]