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UW solution: a promising tool for cryopreservation of primarily isolated rat hepatocytes

Jun Arikura[1,2], Naoya Kobayashi[2,3], Teru Okitsu[2], Hirofumi Noguchi[2], Toshinori Totsugawa[2], Takamasa Watanabe[2], Toshihisa Matsumura[2], Masanobu Maruyama[2], Yoshikazu Kosaka[2], Noriaki Tanaka[2], Kazuhiko Onodera, and Shinichi Kasai[1]

[1] Second Department of Surgery, Asahikawa Medical College, 2-1-1-1 Midorigaoka-higashi, Asahikawa 078-8510, Japan
[2] Department of Surgery, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan
[3] Japan Health Sciences Foundation, Nihonbashi, Chuo-ku, Tokyo, Japan

Abstract Cryopreserved hepatocytes are a ready source of metabolic and synthetic functions for hepatocyte transplantation and bioartificial livers. In this study, we evaluated a cytoprotective effect of University of Wisconsin (UW) solution during cryopreservation of rat hepatocytes. We also investigated the feasibility of lentivirus-based gene transfer into thawed hepatocytes after cryopreservation. Primary rat hepatocytes were isolated using a two-step collagenase perfusion technique, and the resulting hepatocytes with more than 85% viability assessed by a trypan blue exclusion test were subjected to the present study. These cells were subjected to the present study. Cells were cryopreserved with UW solution containing 10% fetal bovine serum (FBS) with 12% dimethylsulfoxide (DMSO) (group 1, G1), Cellbanker solution (group 2, G2), and 10% FBS-containing Dulbecco modified Eagle medium (DMEM) with 12% DMSO (group 3, G3). After thawing the cryopreserved hepatocytes, cell viability, plating efficiency, morphological appearance, and ammonia clearance activity were determined for each group. The efficacy of lentivirus-mediated *Escherichia coli LacZ* gene delivery was evaluated. Hepatocyte viabilities after 3- and 7-day cryopreservation were 73.2% and 62.5% for G1, 57.5% and 46.5% for G2, and 57.3% and 41.5% for G3, respectively. Plating efficiency and ammonia clearance activity were improved in G1 hepatocytes compared to G2 and G3 cells. Lentiviral transfer of a *LacZ* gene was confirmed in the thawed hepatocytes after cryopreservation by an X-gal stain assay.

Key words Cryopreservation · Hepatocyte · UW solution · Lentiviral transduction

Introduction

Hepatocyte-based biological therapies, such as hepatocyte transplantation and a bioartificial liver, are promising tools for the treatment of patients with liver failure.[1–3] Cryopreserved hepatocytes are a ready source of metabolic and synthetic functions for such treatments. However, the conventional cryopreservation technique is often associated with a loss of hepatocyte viability and subsequent inadequate plating efficiency.[4–8] It is of extreme importance to establish an efficient cryopreservation method for primarily isolated hepatocytes. Development of efficient genetic manipulation of thawed hepatocytes after cryopreservation would facilitate the use of such cells in research and clinical applications. University of Wisconsin (UW) solution is currently used for cryopreservation of dog pancreatic islets, with considerably attractive results.[9] Lentivirus-mediated gene transfer is currently thought to be useful means for transducing terminally differentiated nondividing cells, such as hepatocytes and pancreatic islet cells.[10] Thus, in the current work we evaluated the cytoprotective effect of UW solution on hepatocyte cryopreservation and lentiviral transduction efficiency in thawed hepaocytes after cryopreservation.

Materials and methods

Animal experiments

All procedures performed on animals were approved by the Institutional Animal Care and Use Committee and were thus within the guidelines for humane care of laboratory animals.

Hepatocyte isolation

Hepatocytes were harvested from male Wistar rats (Shimizu Laboratory Supplies, Kyoto, Japan) 8–10 weeks old using a modification of the two-step collagenase perfusion technique introduced by Berry and Friend[11] and modified by Seglen.[12] Resulting hepatocytes of more than 85% viability (assessed by a trypan

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blue exclusion test) were subjected to the present study. Freshly isolated hepatocytes were plated on collagen-coated dishes (Biocoat, Becton Dickinson Labware) at densities of 5×10^5 cells/35 mm well in CS-C medium (Dainihon Pharmaceuticals, Osaka, Japan) with 10% fetal calf serum (FCS) and antibiotics.

Cryopreservation of hepatocytes

Freshly isolated hepatocytes were cryopreserved in the following three groups. Cells were treated with the UW solution containing 10% fetal bovine serum (FBS) with 12% dimethylsulfoxide (DMSO) (group 1, G1), Cellbanker solution (Dia-Itron, Tokyo, Japan) (group 2, G2), and Dulbecco modified Eagle medium (DMEM) containing 10% FBS with 12% DMSO (group 3, G3). Hepatocytes in G1 and G3 were cooled to −80 °C by means of programmed freezing and stored at −80 °C. Cells in G2 were directly placed in −80 °C and maintained.

Cell viability

Hepatocytes in each group were thawed 3 and 7 days after cryopreservation. Cells were suspended in 10 ml DMEM with 10% FCS and centrifuged at 50 *g* for 3 min. The supernatant was aspirated, and cell pellets were suspended in CS-C medium. The cells were then inoculated on collagen-coated dishes at a density of 5×10^5 cells/35-mm well. Cell viability was determined by a trypan blue exclusion test via hemocytometer under a phase-contrast microscope and compensated with that of primarily isolated hepatocytes.

Assessment of plating efficiency

The plating efficiency of primarily isolated hepatocytes and thawed hepatocytes after cryopreservation was analyzed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. MTT (1 mg) dissolved in PBS was added to the culture medium, and cells were incubated at 37 °C for 3 h. The supernatant was aspirated, and isopropanol was added to the samples for measuring relative percentage viability using the Multiskan MS (Lifescience International, Japan) according to the manufacturer's instructions.

Ammonia clearance test

Ammonia (NH_3) clearance was measured in hepatocyte cultures. $(NH_4)_2SO_4$ was added to the culture medium at a final concentration of 0.56 mM. Twenty-four hours after loading $(NH_4)_2SO_4$, NH_3 levels were measured with FujiDrychemo (Fuji, Tokyo, Japan). Ammonia removal activity was compensated with the viable hepatocyte number assessed by the trypan blue exclusion test at the time of cell inoculation in each culture.

Leakage of hepatic enzymes

Levels of released hepatic enzymes, including lactate dehydrogenase (LDH), glutamate pyruvate transaminase (GPT), and glutamate oxaloacetate transaminase (GOT) were analyzed in the culture medium of hepatocytes as one of the hallmarks of hepatocyte damage. Samples obtained from each culture were preserved at −20 °C prior to measurement.

Morphology

Hepatocyte cultures were carefully examined at regular intervals using a phase-contrast microscope. Twenty-four hours after inoculation of cryopreserved hepatocytes, micrographs were obtained to assess the morphological appearance of cultured hepatocytes in each group.

Lentiviral gene transfer

The efficacy of lentivirus-mediated gene transfer was evaluated in cryopreserved hepatocytes. The vesicular stomatitis virus G-protein (VSV-G)-pseudotyped lentiviral vector, LtV-NLS/LacZ, encoding *Escherichia coli* was used. The *LacZ* gene tagged with nuclear localizing signal (NLS) was produced using HPV289, HPV274, and YN15 plasmids, as previously reported.[13,14] Twenty-four hours after inoculation of cryopreserved hepatocytes from G1, G2, and G3 for 7 days, cells were transduced with LtV-NLS/LacZ, as previously described; 24 h later the culture medium was replaced with fresh CS-C medium. Cells were maintained in the culture for other 48 h, and expression of NLS/LacZ activity was detected using an X-gal assay, as previously reported.[13,14]

Transplantation experiment

The in vivo transplantation effect of thawed hepatocytes (G1) cryopreserved with UW and 12% DMSO for 7 days was evaluated in a rat model of acute liver failure induced by D-(+)-galactosamine (Sigma). Intrasplenic injection of 0.5 ml CS-C medium was performed as a control in this experiment. Twenty-four hours before hepatocyte transplantation or medium injection D-(+)-galactosamine (0.85 g/kg) was administered intraperitoneally. UW-cryopreserved hepatocytes (1×10^7) suspended in 0.5 ml CSC medium were injected into the inferior pole of the spleen for 5 min, as previously reported.[15] During the procedure the splenic vessels were

clamped to prevent immediate passage of the transplanted cells. The survival rates were compared between rats with transplantation of cryopreserved hepatocyte (group A, $n = 5$) and rats with medium injection (group B, $n = 9$). As a positive control, 1×10^7 of primarily isolated rat hepatocytes were transplanted into D-(+)-galactosamine-treated rats (group C, $n = 5$). A postmortem examination was performed on each animal to determine the cause of death.

Statistical analysis

Data were reported as the mean ± SE. Statistical differences were determined by ANOVA and Bonferroni tests in nonrepeated measures obtained by viability, plating efficiency, ammonia clearance, and hepatic enzyme leakage assays. The log-rank test was used to determine statistical significance in the transplantation experiment. Differences in P values that were <0.05 were considered significant.

Results

Yield and viability of primarily isolated hepatocytes

The yield of freshly isolated hepatocytes averaged 3.3×10^8 cells per whole liver (range 2.7×10^8 to 4.0×10^8). The average initial cell viability assessed by the trypan blue exclusion test was 85.9% (range 85.2%–88.7%).

Viability of cryopreserved hepatocytes

The viability of thawed hepatocytes that had been cryopreserved for 3 days was 73.2% for G1, 57.5% for G2, and 57.3% for G3, respectively, in a trypan blue exclusion assay ($P < 0.05$ for G1 vs G2 and G3) (Fig. 1A). The viability of the 7-day cryopreserved hepatocytes was 62.5% for G1, 46.5% for G2, and 41.5% for G3 ($P < 0.05$ for G1 vs G2 and G3) (Fig. 1B). The use of UW solution significantly improved the viability of cryopreserved hepatocytes.

Plating efficiency of cryopreserved hepatocytes

We examined the plating efficiency of primarily isolated hepatocytes and cryopreserved hepatocytes with an MTT assay. The plating efficiency of primary hepatocytes was 69.3% for day 1 after inoculation and 50.6% for day 2. Plating efficiencies of thawed G1 hepatoyctes cryopreserved for 3 and 7 days were 44.4% and 41.2%, respectively, for day 1 after culture (Fig. 2A,B) and 33.3% and 30.8%, respectively, for day 2 (Fig. 2C,D). Thawed G2 hepatocytes that had been cryopreserved for 3 and 7 days showed plating efficiencies of 33.0% and 25.8%, respectively, for day 1 after culture (Fig. 2A), and 17.7% and 29.7%, respectively, for day 2 (Fig. 2C,D). The plating efficiencies of thawed G3 hepatocytes cryopreserved for 3 and 7 days were 18.1% and 6.6%, respectively, for day 1 (Fig. 2A,B) and 12.0% and 3.8%, respectively, for day 2 (Fig. 2C,D). These findings indicated that UW was efficient in terms of plating efficiency of cryopreserved hepatocytes.

Ammonia clearance

Ammonia clearance, one of the most important hepatic functions, was evaluated in freshly isolated hepatocytes and cryopreserved hepatocytes in 24-h cultures. The activity was compensated with the number of viable haptocytes at the time of inoculation in each culture. Primary hepatocytes removed 72.8% of the loaded ammonia (Fig. 3). Thawed G1 hepatocytes cryopreserved for 3 and 7 days removed 51.7% and 38.5% of the loaded ammonia, respectively (Fig. 3). G2 hepatocytes cryopreserved for 3 and 7 days metabolized 30.0% and 33.1% of the added ammonia, respectively (Fig. 3). The 3- and 7-day cryopreserved G3 hepatocytes eliminated

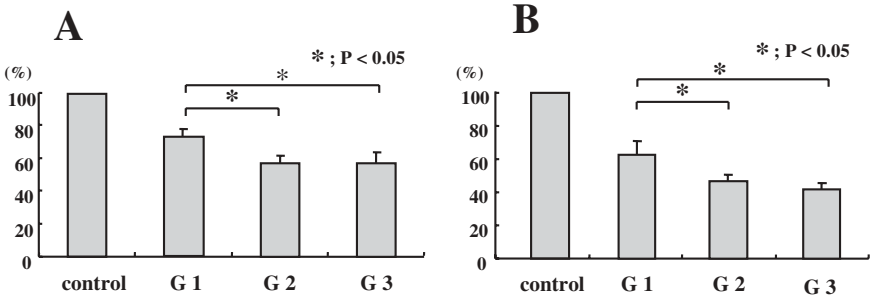

Fig. 1. Viability of cryopreserved hepatocytes. University of Wisconsin solution (UW)-based cryopreservation significantly improved the viability of cryopreserved hepatocytes. **A** Three-day cryopreservation. **B** Seven-day cryopreservation. Hepatocytes were cryopreserved with UW + 12% dimethylsulfoxide (DMSO) (*G1*); with Cellbanker (*G2*); or with Dulbecco modified Eagle medium (DMEM) with + 12% DMSO (*G3*). $P < 0.05$ for G1 vs G2 and G3. These data were obtained from five independent experiments

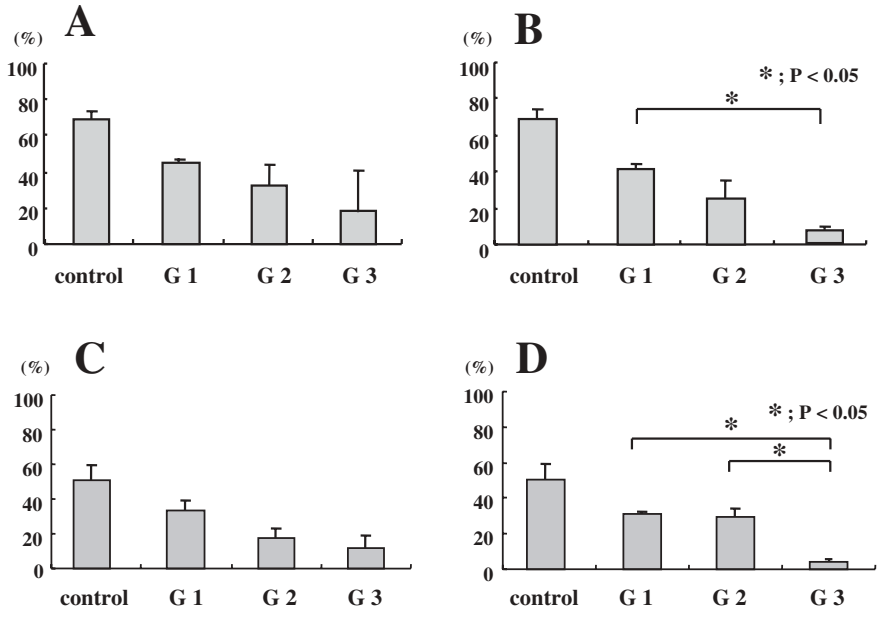

Fig. 2. Plating efficiency of cryopreserved hepatocytes. The use of UW solution significantly maintained plating efficiency of cryopreserved hepatocytes. **A**,**C** Three-day cryopreserved hepatocytes. **B**,**D** Seven-day cryopreserved hepatocytes. **A**,**B** Day 1 after culture. **C**,**D** Day 2 after culture. $P < 0.05$ for G1 vs G3 on day 1; $P < 0.05$ for G1 vs G3 and for G2 vs G3 on day 2. These data were obtained from four independent experiments

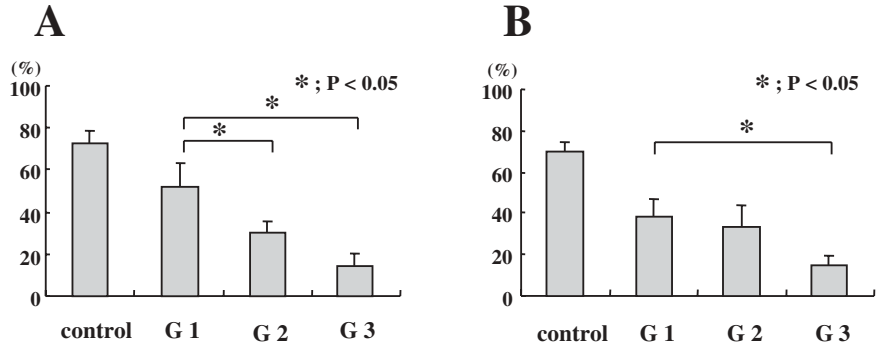

Fig. 3. NH_3 clearance. UW-based cryopreserved hepatocytes (*G1*) significantly metabolized the loaded ammonia compared to hepatocytes treated with Cellbanker (*G2*) and with DMEM + 12% DMSO (*G3*) in a three-day cryopresrved experiment. $P < 0.05$ for G1 vs G2 and G3. Hepaocytes cryopreserved for 7 days with UW (G1) significantly removed the loaded ammonia compared to those with DMEM (G3). $P < 0.05$ for G1 vs G3. **A** Three-day cryopreserved hepatocytes. **B** Seven-day cryopresreved hepatocytes. These data were obtained from five independent experiments

13.8% and 17.5% of the loaded ammonia, respectively (Fig. 3). UW-based cryopreserved hepatocytes (G1) significantly metabolized the loaded ammonia compared to hepatocytes with Cellbanker (G2) or DMEM (G3) in a 3-day cryopreservation experiment ($P < 0.05$ for G1 vs G2 and G3). G1 hepatocytes removed significantly more loaded ammonia than did the G3 hepatocytes in a 7-day cryopreservation assay ($P < 0.05$ for G1 vs G3). These data were obtained from five independent experiments.

LDH, GPT, and GOT levels in culture medium

Release of LDH, GPT, and GOT was measured in the culture medium of thawed hepatocytes after cryopreservation. As shown in Fig. 4, the 3-day cryopreserved G1 hepatocytes showed lower levels of such enzymes than did the G2 and G3 hepatocytes, but the difference was not statistically significant. GPT release was significantly decreased in 7-day cryopreserved hepatocytes (G1) compared to G2 and G3 hepatocytes. These findings indicated that UW solution reduced the adverse effects of cryopreservation on hepatocytes.

Morphological appearance of thawed hepatocytes after cryopreservation

At 3 and 7 days after cryopreservation, hepatocytes were thawed and inoculated into collagen-coated

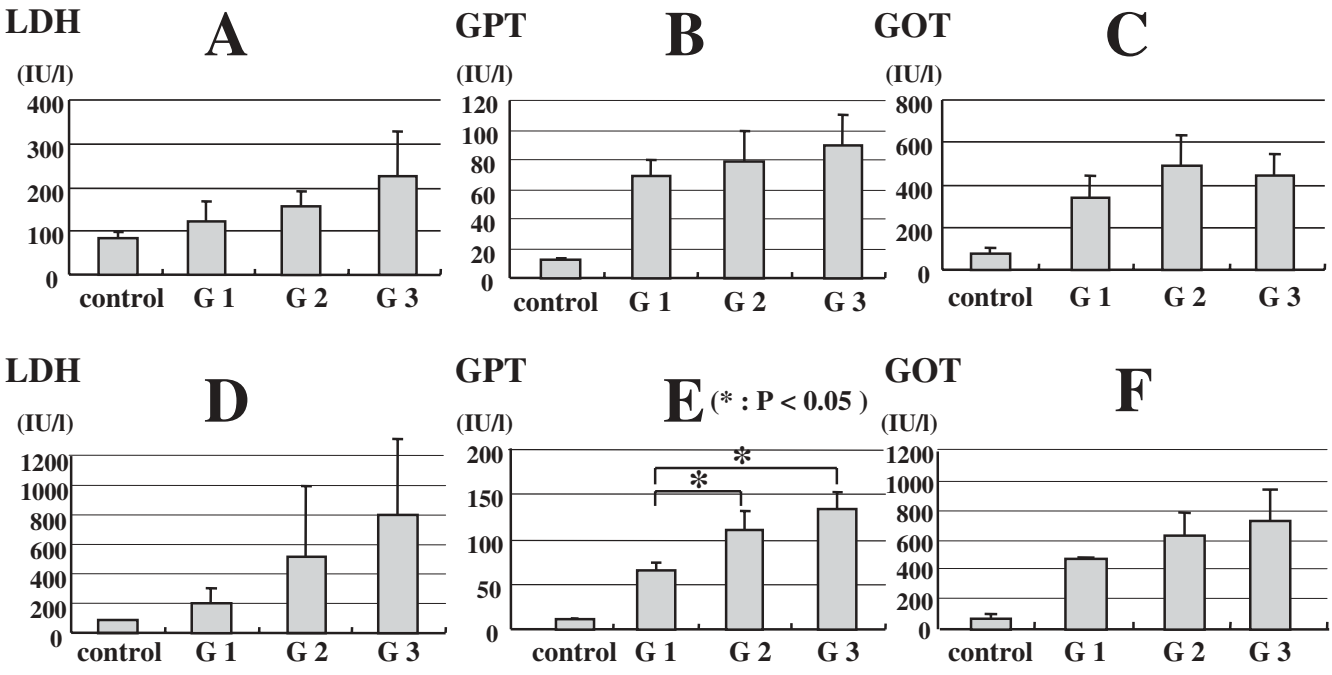

Fig. 4. Lactate dehydrogenase (*LDH*), glutamates pyruvate transaminase (*GPT*), and glutamate oxaloacetate transaminase (*GOT*) levels in culture medium. Reduced release of hepatic enzymes, including LDH, GPT, and GOT, into the culture medium was observed in UW-cryopreserved and thawed hepatocytes. Significantly decreased GPT release was observed in G1 hepatocytes cryopreserved with UW + 12% DMSO for 7 days compared to G2 and G3 hepatocytes (*G1*: UW + 12% DMSO; *G2*: Cellbanker; *G3*: DMEM with + 12% DMSO). **A–C** Three-day cryopreserved hepatocytes. **D–F** Seven-day cryopreserved hepatocytes. These data were obtained from five independent experiments

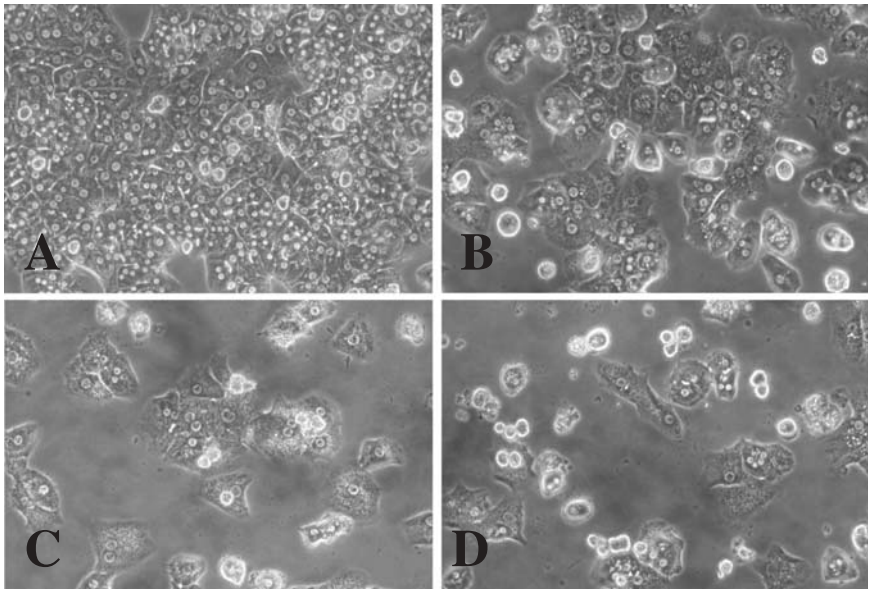

Fig. 5. Morphological appearance of thawed hepatocytes after cryopreservation. G1 hepatocytes, cryopreserved with UW + 12% DMSO, had a cobblestone-like architecture that was one of the characteristics of the primarily cultured hepatocytes. In contrast, G2 and G3 hepatocytes showed inadequate attachment in culture dishes. **A** Primary hepatocytes. **B** UW-cryopreserved hepatocytes. **C** Cellbanker-cryopreserved hepatocytes. **D** DEME-based cryopreserved hepatocytes. These data are representative of five independent experiments. **A–D**: original magnification ×100

dishes. The morphological appearance was carefully examined by phase-contrast microscopy. Seven-day cryopreserved G1 hepatocytes showed a cobble stone-like architecture, similar to that of primary hepatocytes (Fig. 5A,B). In contrast, 7-day cryopreserved G2 and G3 hepatocytes showed inadequate attachment in culture dishes (Fig. 5C,D). The well-maintained morphological findings of G1 hepatocytes were consistent with adequate ammonia clearance and low leakage of hepatic enzymes.

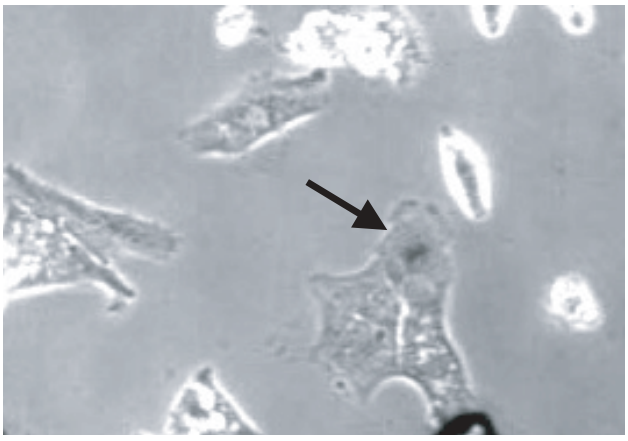

Fig. 6. Transduction of cryopreserved hepatocytes with Lt/NLS-LacZ. After Lt/NLS-LacZ transduction, 5% of hepatocytes were positive for NLS/LacZ activity in an X-gal assay, as indicated by the *arrow*. These data are representative of three independent experiments. (Original magnification ×100)

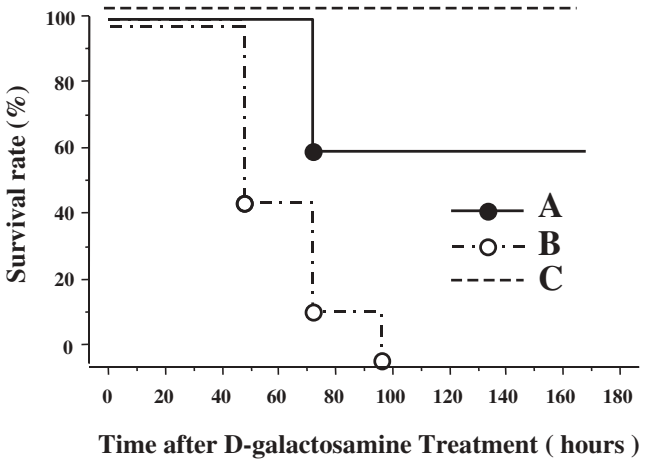

Fig. 7. Transplantation experiment. Intrasplenic transplantation of hepatocytes cryopreserved with UW + 12% DMSO for 7 days significantly improved the survival of rats with D-(+)-galactosamine treatment. *A*, rats with cryopreserved hepatocyte transplantation; *B*, control rats; *C*, rats with freshly isolated hepatocyte transplantation. $P = 0.0088$ for *A* vs *B*; $P = 0.1336$ for *A* vs *C*; $P = 0.0004$ for *B* vs *C*

Lentiviral transduction

To evaluate the efficacy of lentiviral gene transfer, thawed hepatocytes from the 7-day cryopreserved G1, G2, and G3 groups were subjected to Lt/NLS-LacZ transduction. Approximately 5% of hepatocytes in each group were positive for NLS/LacZ in an X-gal staining assay (Fig. 6). There was no difference in lentiviral transduction efficiency among the groups.

In vivo effect of cryopreserved hepatocytes

Intrasplenic transplantation of thawed G1 hepatocytes cryopreserved for 7 days was performed in a rat model of acute liver failure induced by D-(+)-galactosamine. The 1-week survival rate was 60% for rats with transplantation of G1 hepatocytes (group A) and 100% for rats with transplantation of freshy isolated hepatocytes (group C). In contrast, control rats with injection of medium (group B) died within 96h after D-(+)-galactosamine treatment due to hemorrhage and jaundice (Fig. 7). The *P* values were determined for A vs B, A vs C, and B vs C, respectively. A significant difference in survival was observed between A and B ($P = 0.0088$). There was no significant difference between A and C ($P = 0.1336$).

Discussion

Serious liver diseases are still associated with high mortality despite various means of intensive care.[16] Orthotopic liver transplantation (OLTX) is the only curative therapy for patients with decreased liver function, but this procedure is extremely costly, complex, and limited by the scarcity of donor livers.[17] Thus, there is a growing and compelling need to develop an attractive alternative to OLTX for sustaining patients with liver insufficiency. Since Berry and Friend established a method to isolate hepatocytes from the liver in 1969,[11] animal studies have shown that hepatocyte transplantation (HTX) can be used to treat liver failure and inborn errors of liver-based metabolism.[18–20] In addition, current clinical reports have suggested that HTX is a considerably promising therapy for a wide spectrum of liver diseases. Treatment of liver diseases with HTX has significant implications for organ replacement. Compared to OLTX, HTX is a technically simple procedure.

To facilitate HTX, it would be of great value to develop an efficient cryopreservation technique for primarily isolated hepatocytes. Once such a technology is established, freshly isolated hepatocytes can be cryopreserved for future use. Toward this goal, we have focused on the utilization of UW solution,[21] as the data regarding cryopreservation of dog pancreatic islets and woodchuck hepatocytes with UW solution have been well documented.[9,22]

UW solution was originally developed for hypothermic preservation of organs and is now widely used in clinical practice for cardiac, hepatic, and renal transplantation.[23] The Na^+/K^+ and Ca^{2+}/Mg^{2+} ratios in UW solution are well adjusted to prevent passive diffusion of such ions at low temperatures when ionic pumps are inactivated.[24] UW solution is an intracellular-based preservative containing several important metabolic molecules, including hydroxyethyl starch, raffinose, and lactobiotine. Hydroxyethyl starch and raffinose elevate

the intracellular osmotic pressure to stabilize the cell membrane. Lactobionate prevents edema of cells and acidosis.[23] The buffered solution in UW solution is designed to work at a low temperature, leading to prevention of intracellular acidosis. Mannitol, one of the essential components of UW solution, is a hydroxy radical scavenger. Glutathione adenosine and allopurinol promote ATP synthesis and significantly reduce oxidant damage to cells.[21,24] Glutathione is also a cofactor for glutathione peroxidase, which breaks down lipid peroxides and hydrogen peroxide.[24] These are well-characterized mechanisms of UW solution that enhances preservation. Furthermore, our recent work regarding porcine hepatocyte cryopresrevation has demonstrated that use of UW solution decreased caspase-3 actiation during cryopreservation (unpublished data).

In the present study, hepatocytes cryopreserved with UW solution were significantly functional in terms of plating efficiency, ammonia metabolism, hepatic enzyme leakage, and in vivo transplantation. Notably, after thawing, only hepatocytes presrved in UW solution showed cobblestone structures, which were in vitro characteristics of primary isolated hepatocytes cultured. In contrast, cells in other groups lacked cell-to-cell attachment, resulting in low metabolism of ammonia. Further examinations, including electron microscopy and expression analysis of cell adhesive molecules, can provide answers about more detailed intracellular structures of UW-cryopreserved cells. Hepatocytes cryopreserved for 7 days showed worse outcomes than those cryopreserved for 3 days in terms of viability, plating efficiency, ammonia clearance activity, and release of hepatic enzymes. These findings were consistent with recent work indicating that apoptosis would progress during cryopreservation.[25] Cellular functions should be carefully examined in hepatocytes cryopreserved for much longer periods of time.

We also evaluated the efficacy of lentivirus-mediated gene delivery into cryopreserved hepatocytes. Lentivirus expressing NLS-LacZ was capable of transducing the cultured thawed hepatocytes after cryopreservation, suggesting that the utilization of immortalizing genes, such as simian virus 40 large T antigen and human telomerase reverse transcriptase (hTERT), could expand the populations of hepatocytes under such conditions.[26,27] Considerable leakage of hepatic enzymes occurred even with the use of UW solution. A recent report has indicated that apoptosis occurred during cryopreservation and thawing of hepatocytes and that introduction of caspase-3 inhibitor significantly reduced apoptosis by preventing mitochondria damage of porcine hepatocytes after cryopreservation.[6,25] Active lentivirus-mediated expression of antiapoptotic molecules would prevent apoptosis in such hepatocytes, leading to the development of cell therapies. Michalopoulos et al. reported that mixed cultures of hepatocytes and nonparenchymal cells in Matrigel increased hepatocellular function.[28] Such cell–cell interactions would be important for preserving the cellular function of thawed cryopreserved hepatocytes. Further modifications, including the use of caspase inhibitors, vitamin C supplementation, a co-culture system using nonparenchymal cells, or introduction of extracellular matrices would facilitate advances in the cryopreservation of hepatocytes.

Conclusions

We demonstrated the efficacy of UW-based cryopreservation of primarily isolated rat hepatocytes in in vitro studies and an in vivo transplantation experiment. We also showed that the lentivirus was capable of transducing cryopreserved hepatocytes.

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