An Improved Encapsulation Method for Cryopreserving Hepatocytes for Functional Transplantation Using a Thermo-reversible Gelation Polymer

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Abstract. *Background/Aim:* Thermo-reversible gelation polymer (TGP) can be converted into a gel state upon warming and liquid upon cooling. The present study aimed to demonstrate a new method for cryopreservation and encapsulation of rat hepatocytes using a TGP and their successful transplantation. *Materials and Methods:* The isolated rat hepatocytes were microencapsulated using TGP, and stored in liquid nitrogen. After cryopreservation, hepatocytes were cultured. Moreover, hepatocytes were transplanted into the spleen without a TGP capsule. *Results:* The viability of hepatocytes that were cryopreserved in TGP was $71.2 \pm 2.3\%$. The hepatocytes demonstrated adequate survival, maintained their hepatic function in culture, and expressed albumin after transplantation to the rat spleen. *Conclusion:* We demonstrated a cryopreservation method of rat hepatocyte encapsulation using a TGP gel in the hydrogel state which subsequently allowed successful transplantation of unencapsulated hepatocytes in a sol state TGP gel at low temperature.

The number of liver transplantation in patients with end-stage liver disease has been increasing. Unfortunately, there

is a shortage of organ donors for these patients, which prevents treatment via transplantation.

A potential alternative to liver transplantation for patients with severe liver disease is hepatocyte transplantation (HT) (1-7). However, the transplantation of allogeneic hepatocytes is also limited due to a shortage of liver donors and poor proliferative potential of hepatocytes *in vitro*. Therefore, a method whereby large quantities of hepatocytes can be preserved for a long period, thereby providing a convenient and easily accessible supply, is highly desirable.

Cryopreservation of hepatocytes is a standard method for long-term preservation (8, 9). However, standard cryopreservation procedures can severely damage hepatocytes. Therefore, new strategies for long term storage with adequate protection of hepatocytes are needed (8, 9). Although utilizing a programmable freezer with a special medium is effective for cryopreservation of hepatocytes, new methods are still needed to maximize cell viability and maintain normal hepatic function. Our group has previously demonstrated an encapsulation technique as a new method for long-term cryopreservation of rat and human hepatocytes (10).

Although the encapsulation technique was effective for cryopreserving hepatocytes, post-transplantation was problematic because the capsule can easily rupture in the spleen, resulting in leakage of the donor cells. Moreover, the capsular membrane inhibits cell-to-cell physical contact, which may impact normal cellular function.

To address this issue, we used a thermo-reversible gelation polymer (TGP) (Mebiol Gel® Mebiol Inc., Hiratsuka, Japan). TGP is a unique polymer that remains in solution (sol) state at low temperatures (0°C-15°C), but can change into a gel state at temperatures higher than 25°C. This polymer enables encapsulation during cryopreservation to

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Figure 1. Microencapsulation technique.

protect the hepatocytes from cryoinjury, and easy removal of the capsule after transplantation of the cryopreserved cells.

The present study provides a cryopreservation method of rat hepatocytes encapsulated using TGP in the gel state, and viable recovery of unencapsulated hepatocytes in the sol state at a low temperature with subsequent functional transplantation.

Materials and Methods

Animals. Sprague-Dawley (SD) rats (250-350 g, Saitama Experimental Animals Supply, Saitama, Japan) were housed under 12-hour light/dark cycles for over 7 days before surgery. Animal studies were performed at the Laboratory Animal Center of Showa University School of Medicine and approved by the Showa University Ethics Committee for Animal Care and Use. All aseptic operations were under general anesthesia.

Rat hepatocytes. Rat liver cells were harvested by a two-step EDTA/collagenase digestion *in situ* as previously described (11). Following enrichment through a Percoll density gradient, cell viability was determined to be greater than 95%, assessed using the trypan-blue dye-exclusion test.

Preparation of thermo-reversible gelation polymer (TGP). Under a clean-air laminar-flow hood, a flask containing 10 ml of sterile Mebiol gel (supplied by Mebiol Gel Inc., Ltd, Hiratsuka, Japan from Nichi In Drugs & Devices (Pvt) Ltd, Chennai, India) was opened and 10 ml Eagle's Minimum Essential Medium (MEM) containing gentamicin 50 mg/ml ciprofloxacin 10 mg/ml and 10% fetal bovine serum (FBS) (all obtained from HiMedia, Mumbai, India) were included. The gel was dissolved in the medium at 4 to 8°C within 72 h.

Hepatocyte isolation and microencapsulation. Figure 1 illustrates the microencapsulation technique. Hepatocytes (1×10^7) suspended in a small amount of Dulbecco's MEM (DMEM) were

mixed with 5 ml of TGP/MEM at 4°C. Droplets containing hepatocytes in TGP/MEM formed microcapsules by extrusion through a 21 G needle, followed by placing them in a beaker containing 10 ml paraffin oil (PO) (No. 7162, Merck, Darmstadt, Germany) and rotation at 4°C. This resulted in the solidification of the microcapsules, which became further water-insoluble after being heated above the soluble-gel transition temperature (SGTT) for 10 min. The beaker containing 10 ml DMEM was immersed in a 37°C water bath and shaken gently to precipitate the microcapsules. The microcapsules were then washed in DMEM, and those with diameters between 150 and 300 µm were spontaneously formed.

Cryopreservation of hepatocytes. Isolated encapsulated rat hepatocytes were placed in a cryopreservation medium consisting of 10% dimethyl sulfoxide (DMSO), 10% FBS and 80% Dulbecco's Modified Eagle Medium (DMEM). Aliquots of encapsulated hepatocytes were distributed in 1.8 ml freezing vials (Nunc, Denmark), which were immediately frozen in liquid nitrogen for storage.

In order to thaw the encapsulated hepatocytes, the vials were placed in a 37°C water bath until the liquid in the tube was melted. The encapsulated hepatocytes were pipetted out and were washed with DMEM containing 10% FBS. The aliquots were then transferred to DMEM culture medium in dishes.

Culture of cryopreserved hepatocytes. Cryopreserved hepatocytes (1×10^7) were placed in DMEM enriched with 0.2% bovine serum albumin, 10% FBS, 10 mM nicotinamide, 25 mM NaHCO₃, 20 ng/ml EGF, 10^{-7} M dexamethasone, 30 µg/ml L-proline, 0.5 mM glutamine, 1 mg/ml galactose, 20 mM HEPES, 0.1 mM L-ascorbic acid 2-phosphate (Acs-2P), 0.5 µg/ml insulin-transferrin-selenium-X 51500 (ITS 51500, Life Technologies, Rockville, MD, USA), 50 µg/ml streptomycin and, 100 µg/ml penicillin, and transferred to a suspension-culture dish (60 mm×15 mm, Corning Incorporated, Corning, NY, USA) and cultured for 28 days. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂, and the medium was changed every other day.

Cell viability assays. The trypan-blue exclusion test was used to examine the viability of hepatocytes before and at specific times after cryopreservation as previously described (12).

Measurement of albumin production in culture. Albumin production and urea synthesis were measured, in the 3 groups below, in order to evaluate hepatic function in freeze-thaw and post-thaw culture conditions. The groups were: Group A: Non-cryopreserved rat hepatocytes; Group B: Non-cryopreserved rat hepatocytes with TGP; Group C: Cryopreserved rat hepatocytes with TGP.

Albumin production was analyzed in the medium on days 1, 3, 5, 7, 10, and 14 of culture, by utilizing a rat albumin ELISA kit (Shibayagi Co. Ltd., Gunma, Japan). The albumin levels in each of the samples in the medium was also determined.

Measurement of urea nitrogen synthesis in culture. Ammonium chloride (2.0 mM) was added in the culture medium on days 1, 3, 5, 7, 10, and 14 after plating. Six hours following the addition of ammonium chloride, the urea nitrogen concentration was measured using a urea-nitrogen diagnostic kit (Bioparmigen, San Diego, CA, USA). The extinction coefficient was measured with a spectrophotometer for measuring urea synthesis (UV-1200, Shimadzu Co., Ltd, Kyoto, Japan).

Histological assessment. Transplanted microencapsulated hepatocytes were fixed in 10% formaldehyde. Immunocytochemical staining of intracellular albumin in encapsulated rat hepatocytes was performed on paraffin sections. Immunocytochemical staining of intracellular albumin was demonstrated in cultured hepatocytes fixed in 10% formaldehyde. Briefly, the slides were incubated with a peroxidase blocking reagent (DAKO, Milan, Italy) for 10 min, washed, and incubated with a primary rabbit polyclonal antibody against rat albumin (Omega Scientific, Inc. Tarzana, CA, USA) for 30 min at room temperature. Subsequently, a secondary antibody (Omega Scientific, Inc. Tarzana, CA, USA) and a visualization reagent (DAKO) were added to the slide, which was incubated at room temperature for 30 min. Diaminobenzidine (DAB) was used as the chromogen.

RT-PCR analysis of albumin mRNA. Total RNA was isolated from hepatocytes of five different SD rats by using the acid guanidinium thiocyanate (GTC)-phenol-chloroform extraction method. The liver tissue was homogenized in GTC solution (4.0 M GTC containing 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol in a ratio of 10 ml GTC solution per 100 mg liver tissue). The RNA was extracted twice into phenol and chloroform (1 volume phenol/0.2 volumes chloroform/1 volume GTC solution) and precipitated with isopropanol at room temperature. The precipitated pellet was then dissolved in 0.3 ml of GTC solution and precipitated with isopropanol at -20°C , washed with ice-cold 80% ethanol and dissolved in an appropriate volume of diethylpyrocarbonate-treated water. The RNA yield, purity, and integrity were analyzed by the 260 nm/280 nm absorbance ratio (>1.6), which was confirmed by electrophoresis on 1.0% agarose/formamide gels.

One μg sample of total RNA was used for RT-PCR. The nucleotide sequences of the RT-PCR primers and the length of the amplified products were as follows: rat albumin, (CTGATATCTGCAC ACTCCCA and TCAGTGGCGAAGCAGTTATC, 181 bp). RT-PCR was performed under the following conditions according to the manufacturer's instructions (Toyobo, Osaka, Japan): 1 cycle at 60°C for 30 min, 1 cycle at 94°C for 2 min, 40 cycles at 91°C for 30 s,

annealing at 62°C for 30 s, 72°C for 30 s, which was followed by a final extension at 72°C for 10 min. Amplified PCR products obtained from five different rats were loaded onto a 2.0% agarose gel.

Hepatocyte transplantation (HT) study. Hepatocytes from male SD rats were used as transplant donor cells, and male Nagase analbuminemic (NA) rats were used as recipients. With the rats under general anesthesia (achieved with diethyl ether), a small left subcostal incision was made to expose the spleen in each NA rat. Approximately 2×10^7 cryopreserved unencapsulated hepatocytes in TGP, suspended in 1.5 ml of phosphate-buffered saline, were injected into the spleen through an 18-gauge needle. Hemostasis at the injection site was achieved with 2-0 silk sutures. Each group was subcutaneously injected with 1 mg/kg FK506 (Astellas Pharmaceutical Co., Ltd, Tokyo, Japan) daily for one week before and two weeks after HT, then once a week until the end of the experiment. Rats were sacrificed at various times following HT, and the spleen was collected.

Measurements of albumin production in NA Rats. In order to evaluate the efficacy of HT, the levels of albumin were determined in serum. Samples were measured using a standard blood chemistry method, automatic analyzer.

Morphologic studies of transplanted hepatocytes in the spleens of NA rats. The spleens that underwent HT were processed for histology. Paraffin-embedded sections (4 μm) were stained with H&E, PAS, and immunocytochemistry for albumin.

Periodic acid-Schiff's (PAS) staining. Transplanted hepatocytes were fixed in 10% formaldehyde for 30 min, oxidized in 10 g/l periodic acid for 10 min and rinsed three times in distilled water (dH_2O). The cells were then placed in Schiff's reagent for 10 min, rinsed in dH_2O for 10 min, and stained with H-E for 2 min. After mixing with 1% alcohol/HCL, the cells were rinsed in dH_2O and examined using a light microscope.

Statistical analysis. All values are presented as mean $\pm$ standard deviation (SD). The Student's *t*-test was performed. $p < 0.05$ was considered significant.

Results

Viability of cryopreserved rat hepatocytes. The viability of hepatocytes in Group A, B, and C before cryopreservation was $86.7 \pm 4.5\%$, $85.3 \pm 5.1\%$ and $85.3 \pm 5.1\%$ respectively, and more than 80% of the cryopreserved microencapsulated rat hepatocytes were viable 48 hours after cryopreservation. The viability of cryopreserved unencapsulated hepatocytes in the sol state TGP gel at low temperature was $71.2 \pm 2.3\%$. Comparing the viability between before cryopreservation and several points after cryopreservation, no significant differences were noted.

Measurement of albumin production. Albumin production by the hepatocytes in each group was analyzed throughout the cell culture period ($n=5$ in each group) (Figure 2). In Groups B and C, the albumin levels were maintained at a high level, with no statistically-significant differences between them. In

Figure 2. Albumin production by cultured hepatocytes at various time points during cell cultivation (n=5 in each group).

Figure 3. Urea synthesis by cultured hepatocytes at various time points during cell cultivation (n=5 in each group).

group A, albumin production was lower than in the other groups throughout the experimental period, except for one day. Following five days of culture, the albumin levels in all groups gradually decreased and stabilized at a low plateau. At that time point, no statistical differences between the groups were noted.

Measurement of urea synthesis. Urea synthesis by hepatocytes was evaluated throughout the cell culture period (n=5) (Figure 3). In Groups A and B urea synthesis peaked at 3 days after culture began and then gradually decreased. No statistically-significant differences were observed between groups A and B at any time point. Urea synthesis in Group C reached a maximum level 3 days after culture began and then gradually decreased.

Morphological findings. Before cryopreservation and at various times after cryopreservation, rat hepatocyte morphology was well-preserved. After encapsulation, the capsules retained their round shape, and the hepatocytes inside the capsules had a normal appearance as observed by phase-contrast microscopy, just before cryopreservation (Figure 4). The presence of albumin was demonstrated using a specific cytoplasmic stain. The entrapped hepatocytes stained strongly positive for albumin, and this persisted after 48 hours of cryopreservation.

Culture of cryopreserved rat hepatocytes. The cryopreserved rat hepatocytes were functional after one week of culture. This was confirmed through immunocytochemistry, which showed that hepatocytes were strongly positive for albumin, throughout the study (Figure 5A and B).

Figure 4. Morphology of isolated rat hepatocytes with TGP before cryopreservation (original magnification $\times 200$).

RT-PCR analysis of cultured hepatocytes. The albumin mRNA levels in cultured hepatocytes of Groups A, B, and C after 0, 1, 3, 5, 7, 10, and 14 days in culture are shown in Figure 6. The measured liver-specific markers including albumin, were expressed at high levels in Groups A, B, and C, at all points throughout the culture period (Figure 6).

Intrasplenic transplantation of cryopreserved hepatocytes. The serum albumin levels were measured at various time points after HT to the spleen of NA rats (Figure 7). Before HT, the baseline levels of serum albumin in each group were at a minimum, with no statistically-significant differences

Figure 5. Immunocytochemical staining for rat albumin in cryopreserved hepatocytes with TGP (Group C) 7 days after cultivation [original magnifications $\times 100$ (A) and $\times 200$ (B)].

Figure 6. RT-PCR analysis of each group after 0, 1, 3, 5, 7, 10, and 14 days of culture. (A); Group A. (B); Group B. (C); Group C. Lane S: Standard 200 bp. Lanes 1-7: Hepatocyte albumin mRNA from 0, 1, 3, 5, 7, 10, and 14 days of culture.

between them ($n=5$). After HT, the serum albumin level increased significantly in all groups, and was maintained at a high plateau for at least 10 days. Although there were statistically-significant differences on day 3 and 10, as well as 2 weeks after HT, no statistically-significant differences were noted between groups at the peak point on day 7 or at 8 weeks after HT (Figure 7).

Morphologic studies of transplanted hepatocytes in the rat spleens of NA rats. The transplanted cryopreserved hepatocytes in the spleen parenchyma of the NA rats were observed to be microscopically viable 56 days after HT in every group (Figure 8A-I). PAS staining analysis showed that the engrafted hepatocytes in Groups A, B, and C were positive for PAS 56 days after HT, demonstrating that the engrafted

Figure 7. Albumin production at various time points after HT of hepatocytes to NA rats ($n=5$ in each group). * $p<0.05$ (Group A versus Group C). ** $p<0.05$ (Group B versus Group C). HT: Hepatocyte transplantation.

hepatocytes were producing glycogen, an important function specific to hepatocytes. Immunocytochemical analysis of albumin also demonstrated that the engrafted hepatocytes in all groups were positive for albumin, which was seen 56 days after HT.

Discussion

Koebe *et al.* developed a method that allows for cryopreservation of porcine hepatocytes by immobilizing them in a collagen gel on a tissue culture surface (13). Dixit (14) and Guyomard (15) described an encapsulation technique that utilizes an alginate-bead gel, which was found

Figure 8. Morphologic studies of transplanted hepatocytes in the spleens of NA rats 56 days after HT. (A-C); Group A. (D-F); Group B. (G-I); Group C. (A, D, and G); Hematoxylin and eosin staining (original magnification $\times 100$). (B, E, and H); Periodic acid-Schiff's (PAS) staining (original magnification $\times 100$). (C, F and I); Immunocytochemical staining for albumin (original magnification $\times 100$). HT: Hepatocyte transplantation.

to be useful and beneficial for cryopreservation of hepatocytes. When these encapsulated hepatocytes were thawed and transplanted into Gunn rats with hyperbilirubinemia, the rats demonstrated improvement in serum bilirubin levels, which was maintained for 30 days following HT (14). Guyomard *et al.* evaluated the survival function of calcium-alginate-bead-entrapped rat hepatocytes. They analyzed both phase I and phase II enzyme activities before and after cryopreservation and demonstrated that all functions tested were well-preserved (15). We have previously reported successful cryopreservation of human and rat hepatocytes after encapsulation in alginate/poly-L-lysine. Using a cryomicroscope to observe the response of the cells subjected to the freezing-thawing process (10), cell viability, organic-anion transporter expression, and expression of drug-metabolizing enzymes were shown to be well-preserved at different time points after cryopreservation. Morphologically, the entrapped hepatocytes retained their normal appearance and had well-preserved nuclei following 90 days of cryopreservation. Similar success was achieved with cryopreserved encapsulated human hepatocytes, which also retained viability and hepatic function (10). However, the use of encapsulated hepatocytes was generally limited by low mechanical strength, degeneration of capsules over a long-term period, and frequent induction of inflammatory

responses. In the present study, we described the development of a new and simple technique for the cryopreservation of hepatocytes after encapsulation using TGP. The capsule itself was removed to obtain excellent cell survival after transplantation. TGP is a chemically-synthesized biocompatible polymer material with unique chemical properties. Below a low critical solution temperature (LCST), it becomes a liquid, and above this temperature, it becomes a solid. Using TGP, we were able to cryopreserve encapsulated hepatocytes without causing hepatocyte dysfunction after capsule removal following thawing. Our results indicated that the viability of hepatocytes was maintained at 85.3% compared to 86.7% just after capsulation following their isolation. The decrease in viability was only 14.1% after cryopreservation. There were no apparent differences in cell morphology between Group B and Group C, and albumin production and urea synthesis were maintained at levels similar to those of non-cryopreserved hepatocytes. Shimizu *et al.* have demonstrated that TGP gel microencapsulated pancreatic islets (16) which showed excellent survival and function *in vitro*. Although several investigators have succeeded to use different materials to prepare microcapsules for cryopreserving cells (17, 18), no study on capsule removal after cryopreservation has been reported until the present study. It is not absolutely necessary to remove the TGP

capsule for transplantation of cells, because the capsule contains a membrane that has the principal characteristics of permeability, biocompatibility, and immunoprotection. However, in the clinical setting, encapsulated-cell transplantation has not been widely applied because of poor cell survival and other obstacles related to the material used for encapsulation. The present study indicated that TGP encapsulation has great potential for cryopreservation and transplantation of hepatocytes in the clinic.

Prior to clinical application, *in vivo* assessment of hepatic function after transplantation of the cryopreserved hepatocytes must be performed. The present study showed that cryopreserved hepatocytes demonstrated an increase in their albumin levels at least 7 days after transplantation in NA recipient rats, similar to that of transplanted non-cryopreserved hepatocytes. Refinements of transplantation conditions should improve hepatocyte survival, ensuring long-term viability.

In the near future, HT is anticipated to be a viable treatment option for various liver diseases. Accordingly, the demand for hepatocytes will increase further. Such a situation raises concerns regarding the shortage of fresh hepatocytes, which will lead to a rapid increase in the demand for frozen hepatocytes. Therefore, it is essential to develop methods for cryopreserving large quantities of hepatocytes without compromising specific hepatocyte functions. The present report describes a new technology for cryopreserving hepatocytes, which would ultimately be a critical step in achieving HT as a viable treatment option.

Conflicts of Interest

The Authors declare no competing financial interests in relation to this study.

Authors' Contributions

K.Y. and T.A. designed and performed experiments, analyzed data and wrote the paper; Y.E., Y.T., Z.Z., T.K., T.K., K.M., Y.W., H.S., K.T., K.S., R.M.H., M.M. gave technical support and conceptual advice.

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