An Effective New Cryopreservation Procedure for Pancreatic Islets Using Hollow Fiber Vitrification

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Abstract
The present study aimed at establishing a new cryopreservation method for mouse pancreatic islets by vitrification using hollow fibers as a container. A unique feature of the hollow fiber vitrification (HFV) method is that this method achieves stable vitrification using a minimum volume of cryoprotectant (CPA) solution, thereby ensuring high viability of the islets. The cytotoxicity, optimum composition, and concentration of the CPAs for vitrifying islets were examined. The viability, functional-integrity of vitrified islets were evaluated in comparison with those vitrified by conventional methods. Insulin secretion was measured in vitro by a static incubation assay and the metabolic functions was tested after transplantation into Streptozotocin-induced diabetic mice. The combination of 15% dimethyl sulfoxide + 15% ethylene glycol resulted in the best CPA solution for the HFV of islets. HFV showed the highest viability in comparison to 2 vitrification methods, open pulled straws and vitrification with EDT324 solution. The vitrified islets stably expressed β-cells markers NeuroD, Pancreatic and duodenal homeobox-1, and MafA. Transplantation of the vitrified islets achieved euglycemia of the host diabetic mice and response to an intraperitoneal glucose tolerance test to a similar extent as non-vitrified transplanted islets. The HFV method allows for efficient long-term cryopreservation of islets.

Supporting Information
This article is available online at http://www.thieme-connect.de/products

Introduction
Islet transplantation is considered as a viable option for the treatment of type 1 diabetes [1]. To achieve insulin-sufficiency by islet transplantation in diabetic patients, 5000 islet equivalents (IEQs) per kilogram of body weight are required [2]. However, at present, islet isolation is still complicated and technically difficult in some cases [3]. Therefore, islets with a low yield at isolation are not transplanted and distributed for basic research. To allow the unlimited collection of islets, islet cryopreservation methods need to be optimized. The development of islet cryopreservation methods was initiated over 20 years ago [4] and since then, many freezing methods have been investigated [5, 6]. However, the existing cryopreservation methods have an islet survival rate of only about 50% [7]. Effective low temperature storage is beneficial to buy time for sterility and viability testing of the islet preparation. Endocrine function of the islets can be examined before transplantation, for example by static incubation [8]. A library of cry-

Key words
- islet transplantation
- pancreatic islet
- vitrification
- cryopreservation
- hollow fiber

Received 10.02.2016
Accepted 09.05.2016

Bibliography
DOI http://dx.doi.org/10.1055/s-0042-102628
Published online: June 24, 2016
Horm Metab Res 2016; 48: 540–549
© Georg Thieme Verlag KG Stuttgart · New York
ISSN 0018-5043

Abbreviations
- HBSS Hank’s balanced salt solution
- RPMI Roswell Park Memorial Institute
- CPA Cryoprotective agent
- DMSO Dimethyl sulfoxide
- EG Ethylene glycol
- EtOH Ethanol
- PROH Propanediol
- PI Propidium iodide
- HF Hollow fiber
- HFV Hollow fiber vitrification
- OPSV Open-pulled straw vitrification
- MVC Minimum volume cooling
- IPGTT Intraperitoneal glucose tolerance test
- SI Stimulation index
- Pdx-1 Pancreas duodenum homeobox-1
- Maf A V-maf avian musculoaponeurotic fibrosarcoma oncogene homologue A

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opreserved islets allows for selection based on HLA tissue type and creates more flexibility with regard to the total amount of transplantable mass [1,2,9].

Cryoprotectants (CPAs) are neutral solutes with a low molecular weight (allowing cell penetration), low toxicity, and high solubility in water. They are tolerated in sufficiently high concentrations and can significantly reduce the amount of ice crystals that form at any given subzero temperature [10, 11]. Dimethyl sulfoxide (DMSO) is a widely used CPA that has been reported to cryopreserve human islets [6]. Several other compounds, including ethylene glycol (EG) [12], and polyethylene glycol [13], have also been used for the cryostorage of islets. Nevertheless, current freezing methods have limitations and the formation of intracellular ice crystal is unavoidable. Ice crystals disrupt the integrity of the capsule membrane and hence impaired insulin secretion [14,15]. If the drawbacks of cryopreservation can be overcome, banking of islets will become realistic and will greatly benefit islet transplantation treatments for diabetic patients.

An alternative approach for cryopreserving living cells was described by Rall and Fahy [16]. This process is known as vitrification [7,17,18] and is based on the increased viscosity of a highly concentrated aqueous solution of CPAs with decreasing temperature until an amorphous glass-like solid forms. Vitrification procedures have been developed and shown to effectively preserve a variety of cells, embryos, and tissues [19–22]. The efficacy of vitrification has been demonstrated for embryos of various mammalian species [23]. The vitrification procedure involves ultra-rapid cooling of embryos in a solution containing a high concentration of CPAs, thereby preventing ice crystal formation. Established protocols that allow for high survival of the vitrified embryos include the solid surface [24], cryoloop [25], open-pulled straw (OPS) [26], and cryotop [27] methods. These methods are commonly characterized by the minimum-volume cooling (MVC) concept [28], in which embryos are vitrified in a minimal amount of solution to maximize the cooling rate.

Recently, we have developed a new vitrification method using hollow fiber (HF) as a device for loading embryos [29,30]. The hollow fiber vitrification (HFV) method has been demonstrated to have superior performance to cryopreserve cryosensitive embryos such as porcine in vitro-produced embryos [30]. In addition, the HFV method allows the vitrification of large numbers of embryos at the same time, while still following the MVC principle. This is a distinctive difference from the previous methods, which are suitable only to vitrify a small number of embryos in a single device. In this study, we set out to apply the improved HFV method to cryopreservation of pancreatic islets. Adapting our baseline vitrification protocols to islets led to the identification of a variety of specific technical challenges. Nonetheless, we found that cryopreservation using the HFV technique is a practical method for long-term storage of islets.

Islet isolation
Mice were anesthetized by a mixture of ketamine (Fujita Pharmaceutical Co., Ltd, Tokyo, Japan) and xylazine (Bayer Yakuhin, Ltd, Osaka, Japan). After laparotomy, the pancreas was distended with 2 ml of cold HBSS (Life Technologies, Carlsbad, CA) containing 1000U/ml of Collagenase-Yakult (Yakult Pharmaceutical Industry Co., Ltd, Tokyo, Japan) through the common bile duct, and subsequently excised and incubated in a stationary bath at 37 °C. Islets were separated by density gradients (Histopaque-1077; Sigma, St. Louis, MO, USA) and handpicked under a stereomicroscope. The isolated islets were cultured 24–36h in RPMI1640 medium (Life Technologies, Carlsbad, CA, USA) with 10% inactivated fetal bovine serum (FBS, Life Technologies) and antibiotics in an Ultra-Low Attachment flask (Corning Incorporated, New York, NY, USA) at 37 °C in a humidified air atmosphere containing 5% CO₂. The islets were used for further experiments and some of them were assigned to the non-vitrified group.

Evaluation of islet viability
The viability of the islets was determined by staining with propidium iodide (PI; Molecular Probes, Eugene, OR). PI-positive islets cells were defined as dead cells and counted by visual inspection of PI staining under a fluorescent microscope. The isolated islets were cultured 24h in RPMI1640 medium (Life Technologies, Carlsbad, CA, USA) with 10% inactivated fetal bovine serum (FBS, Life Technologies) and antibiotics in an Ultra-Low Attachment flask (Corning Incorporated, New York, NY, USA) at 37 °C in a humidified air atmosphere containing 5% CO₂. The islets were used for further experiments and some of them were assigned to the non-vitrified group.

Cryopreservation of the islets
We first examined the sensitivity of mouse islets to various CPAs and then determined the optimum CPA composition and concentration for vitrification. For each series of experiments, the viability and/or function of frozen/vitrified islets were evaluated 3 h after thawing and compared to non-vitrified controls. Heps (20mM)-buffered tissue culture medium 199 (Nissui Pharmaceutical) supplemented with 20% calf serum was used as the basal solution (BS) to prepare equilibration solution (ES), vitrification solution (VS), thawing solution (TS), and dilution solutions (DS). BS was also used as washing solution (WS).

Experiment 1: Exploration of CPAs for the vitrification of mouse islets
A pilot study was undertaken to examine the toxicity of different CPAs towards mouse islets under hypothermic conditions. Various permeating CPAs, including DMSO (Nacalai Tesque, Inc., Kyoto, Japan), EG (Nacalai Tesque), propanediol (PROH), or glycero1, were tested at a concentration of 10% (v/v). A group of 12 islets was exposed for 30 min on ice to BS containing each of the CPA. Subsequently, islets were loaded with the CPA solution (30μl) in a 0.25 ml plastic straw (IMV Technologies, L'Aigle), followed by programmed cooling at a rate of ~0.5 °C/min to ~20 °C
using a Freeze Control CL-863 (CryoLogic Pty Ltd, Australia). Thawing was performed by exposing the straws to air (room temperature, RT) for 3 s prior to immersion of the straw directly in warm water at 37 °C. Recovered islets were treated with a stepwise dilution of CPA and washed as summarized in Table 1. The same post-thaw procedure was used in experiments 1–3.

Experiment 2: Optimization of CPA composition and concentration for islet vitrification
The viability of islets was assessed after exposure to 7 types of VSS: 30 % DMSO, 30 % EG, 35 % EG, 40 % EG, 15 % DMSO + 15 % EG, 17.5 % DMSO + 17.5 % EG, 20 % DMSO + 20 % EG. Solutions with higher DMSO concentrations (35 and 40 %) were omitted as they showed evident toxicity to the islets in a preliminary test. A group of 10–12 islets was exposed on ice for 25 min to ES containing respective CPA(s) at a concentration of 15 %, and then kept in VS for 2 min. When VS with ≥ 35 % CPA(s) was used, islets were placed in 30 % CPA solution for 2 min before being exposed to the VS. Procedures for dilution and removal of CPA were the same as those in experiment 1.

Experiment 3: Comparison of 3 different vitrification methods
The open pulled straw vitrification (OPSV) method originally developed by Vajta et al. [26] has been widely used and docu-
Table 1 Composition and osmolarity of each solution, and the time and temperature of each step.

<table>
<thead>
<tr>
<th>Step</th>
<th>TCM199 + 20% FCS</th>
<th>DMSO % (v/v)</th>
<th>EG % (v/v)</th>
<th>Sucrose (mol)</th>
<th>mOsm/l</th>
<th>Time (min)</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cooling</strong></td>
<td></td>
<td>7.5</td>
<td>7.5</td>
<td>2.938</td>
<td>25</td>
<td>on ice</td>
<td></td>
</tr>
<tr>
<td>Vitrification solution</td>
<td></td>
<td>15</td>
<td>15</td>
<td>0.5</td>
<td>6345</td>
<td>2</td>
<td>on ice</td>
</tr>
<tr>
<td><strong>Thawing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thawing solution (TS)</td>
<td></td>
<td>1</td>
<td>1</td>
<td>2211</td>
<td>1</td>
<td>37 °C</td>
<td></td>
</tr>
<tr>
<td>Dilution solution (DS)</td>
<td></td>
<td></td>
<td></td>
<td>968</td>
<td>5</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>DS2</td>
<td></td>
<td></td>
<td></td>
<td>581</td>
<td>5</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>DS3</td>
<td></td>
<td></td>
<td></td>
<td>435</td>
<td>5</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>Washing solution (WS)</td>
<td></td>
<td>1</td>
<td>1</td>
<td>292</td>
<td>5</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>WS2</td>
<td></td>
<td></td>
<td></td>
<td>292</td>
<td>5</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>Basal solution (BS)</td>
<td></td>
<td>1</td>
<td></td>
<td>292</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FCS: Fetal calf serum; DMSO: Dimethyl sulfoxide, EG: Ethylene glycol; RT: Room temperature

HFV protocol

Islets underwent HFV according to the methods originally developed by our groups for mammalian embryos, after some modifications as shown in Table 1 [29, 30]. Based on the result of experiment 2, we chose DMSO and EG as permeable CPAs. First, a group of 25–35 islets was placed in 4 ml of ES in a 35 mm dish. The CPAs were diluted and removed in a stepwise manner by transferring the HF from the TS to WS. In WS 2, the islets were expelled from the HF by gently squeezing the fiber from one end to the other (Fig. 2f).

Measurement of insulin secretory activity

Glucose-stimulated insulin release was measured in a static incubation assay. In one experiment, 20 islets from the non-vitrified and vitrified (OPSV or HFV method) groups were plated in the wells of a 48-well culture plate (SUMILON, Sumitomo Bake-lite Co., Ltd, Tokyo, Japan) and were subjected to static incubation in Krebs-Ringer bicarbonate buffer (KRB; 115 mM NaCl, 5 mM KCl, 25 mM HEPES, 2.5 mM CaCl2·2H2O, 24 mM NaHCO3, 1 mM MgCl2·6H2O) supplemented with 2.8 or 28 mM glucose and 0.1 % BSA. First, islets were immersed into KRB with low glucose. They were further incubated in another well containing KRB with low glucose (2.8 mM), followed by incubation in KRB with high glucose (28 mM). Each incubation was for 1 h at 37 °C. The supernatants were collected and stored at –80 °C. The insulin concentration was assessed using an enzyme-linked immunosorbent assay kit (Shibayagi, Gunma, Japan). The stimulation index (SI) was calculated by dividing the insulin level in response to 28 mM glucose by that in response to 2.8 mM glucose.

Transplantation

Diabetes was induced by intraperitoneal injection of 180 mg/kg Streptozocin (STZ, Sigma) freshly dissolved in an equivalent volume of 0.05 M citrate buffer (pH 4.5) into 7-week-old ICR SCID mice. Blood glucose was measured using a Glucosecard™+ meter (ARKRAY, Inc., Kyoto, Japan) using whole blood samples obtained by tail puncture. Body weight was also monitored. Diabetes was confirmed by the presence of hyperglycemia with fed blood glucose levels higher than 350 mg/dl. Islet transplantation was carried out 7 days after STZ injection. Recipients were anesthetized with isoflurane (Abbott Japan Co., Ltd, Tokyo, Japan). The left kidney was exposed through a lumbar incision. Three hundred IEQs of the non-vitrified or HFV groups were packed in a capillary tube and were transplanted under the kidney capsule of diabetic mice. After transplantation, the blood glucose levels were monitored 3 times per week. We needed at least 300 islets to achieve euglycemia even if we used non-vitrified islets. The mice were considered to be euglycemic when the blood glucose levels were below 200 mg/dl. On day 25, an intraperitoneal glucose tolerance test (IPGTT) was performed in the fasting (8 h) state, using a 10% glucose solution (2 g/kg body weight). Blood glucose levels were measured at 0, 15, 30, 60, and 120 min on
day 29 or 30, the mice underwent nephrectomy to remove the transplant-bearing kidneys and were subsequently monitored.

Histology
The islets from the non-vitrified or HFV group were fixed in absolute ethanol. The kidney tissue was fixed in 4% paraformaldehyde, then embedded in paraffin and sliced into 4μm sections. After deparaffinization and blocking, diluted primary antibodies were added to the sections, which were incubated overnight at 4°C. For amplification, biotinylated anti-rabbit antibodies (Life Technologies) were used, followed by incubation with Alexa Fluor® 488 Streptavidin Conjugates (Life Technologies). The antibodies used in this study were: mouse anti-NeuroD (Abcam, Plc., Cambridge, UK), rabbit anti-Pancreatic and duodenal homeobox (pdx)-1 (Transgenic Inc., Kobe, Japan), rabbit α-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (MafA) (Bethyl Laboratories, Inc. Montgomery, TX, USA), Glucose transporter, type 2 (GLUT2) (Millipore, Billerica, MA), mouse anti-glucagon (Sigma), guinea pig anti-insulin (Linco Research Immunoasay, St. Charles, MO, USA). The reason why we selected these antibodies was as follows. The 3 transcription factors, NeuroD, Pdx1, and MafA, bind the insulin promoter region and regulate glucose-responsiveness of insulin [31, 32]. The expression of these transcriptional factors in islets is crucial for β-cell specificity. Glucose transporter 2 (GLUT2) is a transmembrane carrier protein that enables protein-facilitated glucose movement across cell membranes [33]. Immunocytochemistry shows that GLUT2 is localized in β-cells [34]. In mice, the few glucagon-immunoreactive islets are localized to the periphery of the islets [35]. Rabbit anti-C-peptide (Cell Signaling Technology, Inc., Danvers, MA) and Alexa Fluor®-conjugated and Alexa 488-conjugated antibodies (Molecular Probes, Eugene, OR) were used as secondary antibodies. The methods used for immunohistochemistry and immunocytochemistry were previously described [36]. Cell nuclei were counter-stained with DAPI (VECTASHIELD Mounting Medium with DAPI) or Hoechst 33342. As a negative control, only secondary antibodies were applied. Islets and pancreas sections were stained as positive controls. Sections were imaged using a confocal microscope (FV-1000, Olympus, Tokyo, Japan).

Statistical analysis
Experimental results were expressed as the means ± standard error of mean (SEM). Student’s t-test, ANOVA, and Fisher’s protected least significant difference test were used, and p<0.05 was considered statistically significant.

Results

Cryptopreservation of the islets
Experiment 1: Exploration of CPAs for the vitrification of mouse islets
It was clear that slow-rate freezing of islets with 10% PROH or 10% Glycerol resulted in a much less compact gross structure. The average score of the viability in both of these groups is significantly lower than that of the 10% DMSO and/or 10% EG groups (Fig. 1b). On the other hand, the islets in the DMSO and EG groups showed a regular periphery with a continuous layer.

Experiment 2: Optimization of CPA composition and concentration for islet vitrification
The islets in all groups retained gross structural integrity, indicating that the permeable CPAs in the compositions and concentrations tested were less toxic. Among the CPAs tested, the highest viability scores were obtained for the groups with 30% EG (8.3±0.2), 35% EG (7.8±0.3), and 15% DMSO+15% EG (8.2±0.3) (Fig. 1c). These 3 types of CPA solutions were therefore selected for subsequent evaluation of the HFV method.

When the islets were vitrified using these 3 CPA solution conditions, the highest viability score was obtained in the group of 15% DMSO + 15% EG (7.7±0.3, Fig. 1d).

Experiment 3: Comparison of different vitrification methods
In the SSV-EDT324 group, the islets were mostly disrupted and the viability score was low (Score: 1.5±1.2, Fig. 3a). The islets in the OPSV and HFV groups retained gross structural integrity with a high proportion of the cells maintaining membrane integrity. Hence, we used OPSV as the control method to compare the efficacy of the HFV method. The viability of islets cryopreserved using the HFV method was the highest of all 3 methods.

Insulin release assay
All islets of the OPSV group were disrupted during the static incubation assay. Therefore, the islets of the HFV group were compared to non-vitrified islets (Fig. 3b, left). The SIs of the non-vitrified and vitrified islets were 27.8±8.2 and 3.5±0.6, respectively (p<0.05, Fig. 3b, right).

Immunostaining of islets after HFV
The expression of the markers in the appropriate locations demonstrates that the vitrification does not affect the cells. Islets were stained with indicated antibodies that recognize NeuroD, Pdx1, and MafA on nuclei (green nuclear), or hormone insulin or C-peptide-positive cells (green cytoplasm) in cytoplasm (red). DAPI: blue on the nuclei). GLUT2 expression in insulin-producing cells and only a few glucagon-positive cells (Fig. 4d, d′) was strongly positive (green cytoplasm) in insulin-producing cells, and only a few glucagon-positive cells (Fig. 4e, e′ and g, g′) surrounded the insulin- or C-peptide-positive cells (Fig. 4g, g′). There were no apparent differences in the expression patterns for non-vitrified islets and islets treated by HFV.

Islet transplantation
All the mice in non-vitrified and HFV groups were euglycemic within 4–8 days after transplantation and throughout the further follow-up period (Fig. 5a–f). On day 29 or 30, removal of the graft-bearing kidney in both groups was followed by a rapid return to hyperglycemia, indicating that the islets grafts were responsible for the euglycemic state. Results of the IPGTTs showed similar glycemic values at all-time points in both groups (Fig. 5c–f). There were no differences in the mean blood glucose levels at 15, 30, and 60 min. Analysis of immunohistochemical staining confirmed the presence of Pdx-1, insulin-, and C-peptide-positive cells in the renal capsule space in both the non-frozen and HFV groups (Fig. 5d–f and d′–f′). Pdx1-positive cells are restricted to the insulin producing cells and c-peptide-positive cells (Fig. 5e′, f′ and h′, f′).
Successful islet banking is required to optimize and modernize islet transplantation techniques. However, there are several drawbacks to preservation of the islets using conventional freezing procedures. The most critical cryo-injuries are undoubtedly caused by intra- and extra-cellular ice formation, which may irreversibly damage the cells. Taylor et al. showed that tissue and organ damage after freeze-thawing are caused by accumulating ice crystals formed in vascularized tissues that rupture the capillaries [37]. Islets have a complex cellular composition, with no less than 5 different cell types. The cells and vessels are well cross-linked to maintain polarity and the capillary vessels construct a frame of islets [38]. Therefore, the islets may not maintain complete structural integrity if the capillary vessels are destroyed.

Langer et al. [39] reported that mouse islets could maintain their viability after vitrification. Since then, many methods have been tested to cryopreserve islets [4–8, 12–15, 18–20]. In the study of embryo cryopreservation, vitrification has been demonstrated to be more effective than conventional freezing [30]. In particular for porcine embryos, which are known to be highly cryosensitive, vitrification has become the standard option [30, 40]. Embryos or tissues having an intrinsic cryotolerance can survive cryogenic-stress regardless of the effectiveness of the cryopreservation method used. However, an authentically effective method is required to cryopreserve tissues with high cryosensitivity or structural fragility such as islets. In this regards, the HFV method may provide a promising option for cryopreserving islets. The
superior performance of the HFV method was previously demonstrated by the efficient production of offspring from porcine vitrified embryos derived from in vitro oocyte maturation followed by in vitro fertilization (IVM/IVF) [30]. This was a significant breakthrough in the development of embryo cryopreservation technology, because none of the previously reported methods enabled cryopreserving of in vitro produced pig embryos on a practical level. The effectiveness of the HFV method proven in the embryo cryopreservation was thus extrapolated to islets.

We therefore applied the HFV method for islet cryopreservation after several modifications. For example, porcine early embryos, which are composed of only 15–40 cells, can be equilibrate with permeable CPAs after 5–7 min [29, 30]. In contrast, islets contain an average of 2000 cells [41], requiring a longer equilibration time to allow the CPAs to permeate into each of the cells. The β-cell specificity and glucose-responsiveness of insulin expression are conferred by 3 conserved enhancer elements in the promoter region of the insulin gene. The functions of the islets were preserved after vitrification. This characteristic of the HFV method was clearly evident in the permeability property tests performed in this study (Fig. 2).

A second beneficial characteristic of the HFV method is its high thermal conductivity. The thin HF membrane permits ultra-rapid cooling and re-warming of the solution held inside, which is known to be critical for gaining a stable vitrified status. Generally, embryos are vitrified in a very small amount of solution to ensure an ultra-rapid cooling/re-warming rate. However, the drawback of such methods is the limited number of embryos that can be vitrified at one time. The HFV method can potentially be expanded to permit the vitrification of a large number of islets if longer HFVs are used. The development of a new automatic system is currently underway.

In vitro evaluations showed apparently lower indices for HFV islets. It was therefore assumed that the islets might have lost some cells after vitrification. However, all islets of the OPSV group were disrupted during the static incubation assay. The average score of OPSV 33 and 40% was 3.3 ± 0.4 and 3.8 ± 1.4, respectively (Fig. 3a). These scores reflected that those islets included more than 50% of dead cells. The structural integrity of those islets was assumed to be damaged, thereby became very fragile. To perform glucose-stimulated insulin release assay, islets needed to be transferred through different solutions using pipetting. During the steps of the incubation assay, the islets were disrupted one after another, so that the number of islets was remarkably reduced. In contrast, islets in HFV group showed higher score than other groups, indicating better structural integrity and functions. Sasamoto et al. [18] reported superior viability of islets after cryopreservation by the SSV-EDT324 and OPSV methods. This discrepancy from our data may be ascribed to the difference(s) in the condition of the islets used or we might have failed to reproduce special knacks that were not available in their report [18].

To confirm the vigorous function of the vitrified islets, we subsequently carried out in vivo evaluations. The transplantation of islet grafts vitrified using the HFV method behaved as non-vitrified islets even on IPGTTs. These results suggest that the functionality of the vitrified islets was comparable to that of fresh islets. Optimization of the in vitro insulin release assay may improve the measurement of function indices in the vitrified islets, as an increased insulin secreting ability was occasionally observed after prolonged post-thaw culture (data not shown). It is possible that the vitrified islets regain their competence under in vivo conditions following transplantation. Better criteria for the accurate in vitro evaluation of the quality of cryopreserved islets need to be developed.
The present study demonstrates the potential use of HFV for islet cryopreservation. Additional practical issues will need to be addressed while further developing this approach to a scale that allows for the processing of clinically relevant sample sizes.

**Author Contributions**


**Acknowledgements**

This research was supported by grants from Grant-in-Aid for Scientific Research (25293279 to M.N) and Grant-in-Aid for Exploratory Research (15K15480 to M.N) and by the Meiji University International Institute for Bio-Resource Research (MUIBR to H.N). This research is partially supported by Otsuka Pharmaceutical Factory Inc. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Disclosure Statement**

Dr. Nagashima conducts research in collaboration with Otsuka pharmaceutical factory Inc. This research is partially supported.

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**Fig. 4** Immunofluorescence analysis of the islets after HFV: (Left) Non-vitrified islets a–g. (Right) HFV islets cultured for 3h after thawing a′–g′. a–g, a′–g′: The left panel in each staining shows the bright-field image of the islet. The right panel is the merged image of each staining. (a–c, a′–c′) Insulin staining (red). (f, g and f, g′) C-peptide staining (red). (a–c, f, and a–c, f) Nuclear staining (green) for NeuroD (a, a′ and f, f′), Pdx-1 (b, b′) and Maf A (c, c′). (d, d′ and g, g′): Immunocytochemistry shows that GLUT2 (cytoplasm; green) is localized in insulin-producing cells (d, d′) and C-peptide-positive cells (g, g′). (e, e′ and g, g′): Glucagon-producing cells (cytoplasm; green) surround the insulin-producing cells (e, e′) and C-peptide-positive cells (g, g′). Inset images are higher magnifications of the region indicated by a yellow square in each merged image. There were no apparent differences in the transcription factor expression pattern between non-vitrified and HFV islets. Scale bar: 100 μm.
Fig. 5  Islet transplantation into the left renal subcapsular space. Islet transplantation was carried out 7 days after STZ injection. Three hundred IEQs of non-vitrified \( (a'-c', \ n = 5) \) or HFV islets \( (a''-c'', \ n = 5) \) were transplanted under the left kidney capsule of diabetic syngeneic mice. Control mice were prepared as well \( (a-c, \ n = 5) \). Blood glucose levels \( a', a'' \) and body weight \( b', b'' \) of diabetic mice were measured after transplantation into the left renal subcapsular space. Mice transplanted with non-vitrified islets \( a' \) and HFV islets \( a'' \) were euglycemic within 4–8 days after transplantation. Nephrectomy was performed on day 29 or 30 after islet transplantation (blue arrows). There were no significant differences in blood glucose levels and body weight between both groups. An intraperitoneal glucose tolerance test (red arrows) was performed on day 25 after islet transplantation. Mice transplanted with non-vitrified islets \( d-f \) and \( d'-f' \) were euglycemic within 4–8 days after transplantation. Serial sections of each islet were prepared, and immunohistochemical staining was performed. (Left) Non-vitrified islets \( d-f \) and \( d'-f' \). (Right) Vitrified islets with HFV method \( g-i \) and \( g'-i' \). The panels of \( d-i \) are the same magnification. \( d'-f' \) and \( g'-i' \) Enlargement of the square shown in \( d \) and \( g \), respectively. \( d, d' \) and \( g, g' \): Hematoxylin and eosin staining of a kidney section with the transplanted islets. \( e, f, h, i \) and \( e', f', h', i' \) Immunofluorescence analysis of Pdx-1 (\( e, f, h, i \) and \( e', f', h', i' \), left), insulin (\( e, h \) and \( e', h' \), middle), and c-peptide (\( f, i \) and \( f', i' \), middle) expression. The right panel is the merged image of each staining. \( e, f, h, i \) and \( e', f', h', i' \) left: Fluorescence micrographs show the expression of Pdx-1 (Nuclear; green). Nuclei are stained in blue with Hoechst 33342. There was complete overlap between transcription factor expression and nuclear staining. \( e', f' \) and \( h', i' \): Inset images are higher magnifications of the region indicated by a white square in each merged image. There were no apparent differences in the transcription factor expression pattern between non-vitrified and HFV islets. Most of the islet cells in the graft area showed strong staining for all markers. Scale bar: 100 μm.
by Otsuka Pharmaceutical Factory Inc. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. Dr. Nagashima reports others from null, during the conduct of the study. The other authors declare no conflicts of interest.

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