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Original Article

Diverting the food-freezing technology improves the cryopreservation efficiency of induced pluripotent stem cells and derived neurospheres

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ABSTRACT

Introduction: Recent advances in induced pluripotent stem (iPS) technology and regenerative medicine require effective cryopreservation of iPSC-derived differentiated cells and three-dimensional cell aggregates (eg. Spheroids and organoids). Moreover, innovative freezing technologies for keeping food fresh over the long-term rapidly developed in the food industry. Therefore, we examined whether one of such freezing technologies, called "Dynamic Effect Powerful Antioxidation Keeping (DEPAK)," could be effective for the cryopreservation of biological materials.

Methods: We evaluated the efficiency of cryopreservation using DEPAK and Proton freezers, both of which are used in the food industry, compared with conventional slow-freezing methods using a programmable freezer and a cell-freezing vessel. As they are highly susceptible cells to freeze-thaw damage, we selected two suspension cell lines (KHYG-1 derived from human natural killer cell leukemia and THP-1 derived from human acute monocyte leukemia) and two adherent cell lines (OVMANA derived from human ovarian tumors and HuH-7 derived from human hepatocarcinoma). We used two human iPS cell lines, 201B7-Ff and 1231A3, which were either undifferentiated or differentiated into neurospheres. After freezing using the above methods, the frozen cells and neurospheres were immediately transferred to liquid nitrogen. After thawing, we assessed the cryopreservation efficiency of cell viability, proliferation, neurosphere formation, and neurite outgrowth after thawing.

Results: Among the four cryopreservation methods, DEPAK freezing resulted in the highest cell proliferation in suspension and adherent cell lines. Similar results were obtained for the cryopreservation of undifferentiated human iPS cells. In addition, we demonstrated that the DEPAK freezing method sustained the neurosphere formation capacity of differentiated iPS cells to the same extent as unfrozen controls. In addition, we observed that DEPAK-frozen neurospheres exhibited higher viability after thawing and underwent neural differentiation more efficiently than slow-freezing methods.

Conclusions: Our results suggest that diversifying food-freezing technologies can overcome the difficulties associated with the cryopreservation of various biological materials, including three-dimensional cell aggregates.

1. Introduction

Cell cryopreservation is essential for basic biology, drug discovery, and regenerative medicine. These can be classified into two main methods: slow freezing and vitrification [1–3]. Slow freezing is a simple and versatile method in which cells are frozen by cooling at −1 °C/min in the presence of a low concentration of cryoprotectant, such as dimethyl sulfoxide (DMSO) or glycerol [4–9]. In contrast, vitrification is an ultrafast cooling method in which the cells are transferred to liquid nitrogen immediately after treatment

Abbreviations: BDNF, brain-derived neurotrophic factor; CAS, cells alive system; DAPI, 4′,6-diamidino-2-phenylindole; dbcAMP, dibutyryl cyclic AMP; DEPAK, dynamic effect powerful antioxidation keeping; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FGF8, Fibroblast growth factor 8; GDNF, glial cell line-derived neurotrophic factor; G-MEM, Glasgow Minimum Essential Medium; iPSC, induced pluripotent stem cells; rIL-2, recombinant interleukin-2; JCRB, Japanese collection of research bioresources; TUBB3, tubulin beta III.

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with a high cryoprotectant concentration [10–12]. Unlike the conventional slow-freezing method, vitrification enables the preservation of early rodent embryos and fertilized eggs of livestock [2,3]. However, its usage is limited due to the requirement for a high level of manipulation, such as rapid and strict control [1–3,13–15]. Recently, with the development of iPS cell technology, the need to preserve pluripotent and differentiated single cells and three-dimensional cell aggregates, such as spheroids and organoids, has grown rapidly. However, these two methods have limitations, and new technologies must be developed.

Meanwhile, in the food industry, innovative technologies for maintaining freshness of foodstuffs are being developed and are now in the practical stage. The first practical freezer developed by ABI CO., Ltd. has the Cells Alive System (CAS) engine that generates a magnetic field inside the freezer and applies a weak electric current to food [16]. This quick-freezing technology prevents the formation of larger ice crystals that causes cell destruction in fresh food. Consequently, it minimizes drip loss (water and nutrients flow) during thawing. Recently, the RYOHO FREEZE SYSTEMS CO., Ltd. offered a hybrid freezer called the “Proton freezer” that combines an environment of evenly distributed electromagnetic waves with cold air [17]. On the other hand, the DEPAK freezer, developed by SANTETSU ENGINEERING Inc., is equipped with a high-voltage electrostatic induction system that suppresses oxidation during long-term storage by applying a high-voltage weak current and powerful cold air to food [18]. It should be noted that, unlike the CAS and Proton freezers, DEPAK is dedicated to not “freezing,” but “uniform defrosting,” by which food can be thawed evenly while controlling drip loss.

Interestingly, recent studies attempted to divert food-freezing and preservation technologies into the field of life sciences. Nishiyama et al. have used CAS to cryopreserve human induced pluripotent stem cell (iPSC)-derived neural stem or progenitor cells and found that this technology increases cell viability after thawing and has less impact on cellular proliferation and differentiation [19]. Hiramatsu et al. have reported that iPSC-derived dopaminergic neurospheres cryopreserved in a Proton freezer show favorable viability after thawing and displayed equivalent secretion of dopamine and electrophysiological activity compared with fresh spheres [20]. Based on this background, we examined whether these food-freezing and food-preservation technologies could overcome the difficulties of cryopreservation of various biological materials, including cell lines, iPS cells, and neurospheres, by comparing them with conventional slow-freezing methods. This was the first attempt to use a DEPAK freezer to freeze and preserve biological materials.

2. Materials and methods

2.1. Cells

KHYG-1 (JCRB0156), THP-1 (JCRB0112), OVMANA (JCRB01045), and HuH-7 (JCRB0403) cells were obtained from the JCRB Cell Bank (Osaka, Japan). KHYG-1 cells were grown in RPMI1640 (Thermo Fisher Scientific K.K., Tokyo, Japan) supplemented with 10% (v/v) FBS (BioWest SAS, Nuaillé, France) and 100 U/mL of rIL-2 (GC Lymphotec Inc., Tokyo, Japan). THP-1 cells were grown in RPMI1640 supplemented with 5% (v/v) FBS. OVMANA cells were grown in RPMI1640 supplemented with 10% (v/v) FBS. HuH-7 cells were grown in DMEM (Thermo Fisher Scientific K.K.) supplemented with 10% (v/v) FBS. Human iPSC lines, 1231A3 and 201B7-Ff, were obtained from the RIKEN Bioresource Center (Tsukuba, Japan) and grown on an iMatrix-511 (Matrixome, Inc., Osaka, Japan) layer in StemFit AK02N medium (Ajinomoto Co., Inc., Tokyo, Japan) to maintain the cells in an undifferentiated state. Before this study, KHYG-1, THP-1, OVMANA, and HuH-7 cells were cryopreserved and stored in Bambanker™ freezing medium (GC Lymphotec Inc.). Before this study, undifferentiated human iPSC lines 1231A3 and 201B7-Ff were cryopreserved and stored in Bambanker™ hRM freezing medium (GC Lymphotec Inc.).

2.2. Culture and cryopreservation of KHYG-1, THP-1, OVMANA and HuH-7 cells

KHYG-1 and THP-1 cells cultured in suspension and OVMANA and HuH-7 cells cultured in adhesion were collected through centrifugation at 190×*g* for 3 min. Pellets of these cell lines were resuspended in pre-chilled Bambanker™ at a final concentration of 2.0×10^6 cells/mL, the 1 ml of the cell suspensions were transferred to cryotubes, and stored in CoolBox™ 2XT (Corning Inc., New York, US) until freezing. For cryopreservation, the cryotubes were transferred into a cell-freezing container BICELL (Nihon Freezer Co., Ltd., Tokyo, Japan), programmable freezer PF-NP-200 (Nepa Gene Co., Ltd., Chiba, Japan), Proton freezer (Ryoho Freeze Systems Co., Ltd., Nara, Japan), or DEPAK freezer (Santetsu Engineering Inc., Kobe, Japan). The BICELL was stored in a deep freezer at −80 °C overnight. In the cases of the programmable freezers, the cryotubes were frozen under two conditions: the PF-NP-200 lowers the temperature from 4 °C to −80 °C at −1 °C/min. In the cases of the Proton and the DEPAK freezers, cryotubes were kept in each chamber at −35 °C for 30 min until completely frozen. After freezing, each cryotube was stored in the vapor phase in a liquid nitrogen tank for approximately one month. The frozen cells were quickly thawed at 37 °C, and then the cell suspension was transferred to 7 mL of culture medium. This suspension was centrifuged at 190×*g* for 3 min to obtain a pellet, which was resuspended in the culture medium. Cell viability and viable cells number were determined using the trypan blue exclusion method with a Vi-CELL BLU (Beckman Coulter K.K., Tokyo, Japan).

2.3. Culture and cryopreservation of undifferentiated human iPSCs

Human iPSC lines 1231A3 and 201B7-Ff were dissociated from the iMatrix-511-coated plastic dish with 0.5 × TrypLE™ Select CTS™ (Thermo Fisher Scientific K.K.). The 0.5 × TrypLE™ Select CTS™ was prepared by mixing TrypLE™ Select CTS™ and 0.5 mM EDTA/PBS. Following cell counting, the human iPSC suspension was centrifuged at 190×*g* for 3 min to obtain a pellet. Human iPSC lines 1231A3 and 201B7-Ff were resuspended in pre-chilled Bambanker™ hRM at final concentrations of 2.0×10^6 cells/mL, respectively. The 0.2 ml of the cell suspensions were immediately transferred to cryotubes and stored in CoolBox™ 2XT until freezing. Cryopreservation and cell thawing were performed as described in 2.2. Cell viability and viable cells number were determined by using the trypan blue exclusion method with a Vi-CELL BLU. The undifferentiated state of human iPSCs was confirmed by immunostaining. The cultured iPSCs were fixed in 4% paraformaldehyde for 10 min at 4 °C. After 2 washes with PBS/Tween20, the cells were permeabilized with 0.2% Triton X-100 for 10 min, blocked with 5% normal goat serum for 1 h at room temperature, and then incubated with primary antibodies (anti-Oct-3/4 (Becton, Dickinson and Company, NJ, USA), anti-Nanog (Abcam, Cambridge, UK), anti-Sox2 (Merck Ltd., Tokyo, Japan) for 2 h at room temperature. After 3 washes with PBS/Tween20, secondary antibodies conjugated to Alexa Fluor® 488 or Alexa Fluor® 555 (Thermo Fisher Scientific K.K.) were incubated for 30 min at room temperature in the dark. After nuclei were stained with DAPI (Biotium, Inc., CA, USA) for 10 min, and the cells were washed 4 times with PBS/Tween20, micrographs were taken with a

fluorescence microscope (BZ-X710; KEYENCE CORPORATION, Osaka, Japan).

2.4. Cryopreservation of human iPSCs cultured in neural differentiation for 13 days

Human iPSCs 1231A3 and 201B7-Ff were maintained and differentiated as previously described [21]. For neural differentiation, iPSCs were dissociated into single cells after 10 min of incubation with 0.5 × TrypLE™ Select CTS™ and were seeded onto iMatrix511 coated plates with differentiation media containing G-MEM (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) supplemented with 8% StemSure serum replacement (FUJIFILM Wako Pure Chemical Corporation), 0.1 mM MEM nonessential amino acids (Thermo Fisher Scientific K.K.), 1 mM sodium pyruvate (Thermo Fisher Scientific K.K.), and 0.1 mM 2-mercaptoethanol (Merck Ltd.). To efficiently induce neuronal differentiation, we added 100 nM LDN193189 (STEMGENT, Cambridge, MA, USA) and 500 nM A83-01 (FUJIFILM Wako Pure Chemical Corporation) [22]. We added 2 μM purmorphamine (FUJIFILM Wako Pure Chemical Corporation) and 100 ng/mL Fibroblast growth factor 8 (FGF8) (FUJIFILM Wako Pure Chemical Corporation) from days 1–7 and 3 μM CHIR99021 (FUJIFILM Wako Pure Chemical Corporation) from day 3 to induce floor plate cells [23]. On day 13, differentiated and cultured day 13th cells were dissociated using Accumax™ (Innovative Cell Technologies, Inc., San Diego, CA, USA). Following cell counting, the human iPSC suspension was centrifuged at 190×*g* for 3 min to obtain the pellet and then resuspended in pre-chilled Bambanker™ hRM at a final concentration of 2.5 × 10^6 cells/mL. The 200 μL of the cell suspension was immediately transferred to a cryotube and stored in a CoolBox™ 2XT until freezing. The cryotubes were frozen in a BICELL, a programmable freezer (PF-NP-200), a Proton freezer or a DEPAK freezer for cryopreservation. The BICELL was transferred into a deep freezer at −80 °C overnight. In the case of the programmable freezer, the cryotubes were frozen from 4 °C to −80 °C at −1 °C/min. In comparison, cryotubes in the Proton freezer and DEPAK freezer were kept in the chamber at −35 °C for 30 min until completely frozen. After freezing, each cryotube was stored in the vapor phase in a liquid nitrogen tank. The frozen cells were quickly thawed at 37 °C, and then the cell suspension was transferred to 7 mL of culture medium. The suspension was centrifuged at 190×*g* for 3 min to obtain a pellet, which was resuspended in neural differentiation medium containing neurobasal medium (Thermo Fisher Scientific) supplemented with 2% B27 (Thermo Fisher Scientific), 2 mM L-glutamine (FUJIFILM Wako Pure Chemical Corporation), 10 ng/mL glial cell line-derived neurotrophic factor (GDNF) (FUJIFILM Wako Pure Chemical Corporation), 200 μM ascorbic acid (FUJIFILM Wako Pure Chemical Corporation), 20 ng/mL brain-derived neurotrophic factor (BDNF) (FUJIFILM Wako Pure Chemical Corporation), 400 μM dibutyryl cyclic AMP (dbcAMP) (Merck Ltd., Tokyo, Japan) and 30 μM Y27632 (FUJIFILM Wako Pure Chemical Corporation). Cell viability and viable cells number were determined by using the trypan blue exclusion method with a Vi-CELL BLU. Cells were seeded in low cell adhesion U-bottom 96-well plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and on the fourth day neurosphere formation of the cultured cells were observed using the phase-contrast microscope (BZ-X710; KEYENCE CORPORATION, Osaka, Japan).

2.5. Cryopreservation of human iPSCs cultured in neural differentiation for 27 days

Human iPSCs 1231A3 and 201B7-Ff cultured in neural differentiation medium for 13 days were dissociated using Accumax™ and reseeded onto low-cell-adhesion U-bottom 96-well plates at a density of 2–5 × 10^4 cells per well and in neurobasal medium containing 2% B27 supplement, 2 mM L-glutamine, 10 ng/mL GDNF, 200 μM ascorbic acid, 20 ng/mL BDNF and 400 μM dbcAMP. The medium was changed every three days, and 30 μM Y27632 was added to the first medium. Spheres collected on day 27 were placed in cryotubes with 0.2 mL pre-chilled Bambanker™ hRM and kept in CoolBox™ 2XT until freezing. Cryopreservation was performed according to the methods described in 2.4. The frozen cells were quickly thawed in 7 mL neurobasal medium at 37 °C. After removing the supernatant, the cells were rinsed with the neurobasal medium and used for neurite extension assays.

2.6. Neurite extension assay

Neurospheres frozen on day 27 were thawed, seeded in 24-well plates coated with iMatrix511 for three days, and fixed with 4% paraformaldehyde. The neurospheres were stained with an anti-tubulin beta III (TUBB3) antibody (BioLegend, Inc., San Diego, CA, USA) and an Alexa 488-conjugated anti-rabbit IgG secondary antibody (Thermo Fisher Scientific), followed by counterstaining of the nucleus with 4',6-diamidino-2-phenylindole (DAPI) (Biotium Inc., Fremont, USA). A fluorescence microscope (BZ-X710; KEYENCE CORPORATION, Osaka, Japan) was used for visualization, and the area covered by neurites was defined as the TUBB3-positive area minus the DAPI-positive area using the BZ-H3M measurement module (KEYENCE CORPORATION).

2.7. Statistical analysis

The significance of differences between groups was determined using a paired *t*-test and the statistical processing software GraphPad Prism 8 J (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Evaluation of cryopreservation methods for cell lines susceptible to freezing damage

First, we selected two suspension cell lines: KHYG-1 and THP-1 and two adherent cell lines, OVMANA and HuH-7, all of which are known to be highly susceptible to freeze-thaw damage during cryopreservation. As a recent freezing technology in the food industry, we tested two different types of freezers: the DEPAK freezer, which is used primarily to preserve foodstuffs at −35 °C while generating minute alternating currents and powerful cold air, and the Proton freezer, which freezes foodstuffs at −35 °C by combining uniform magnetic flux, electromagnetic waves, and powerful cold air. After freezing the cells by the above methods or the conventional slow-freezing method at a cooling rate of −1 °C/min to −80 °C using a programmed freezer or a cell-freezing vessel (BICELL), the frozen cells were immediately transferred to the vapor phase in liquid nitrogen and preserved for approximately one month (Fig. 1). To evaluate the cryopreservation efficiency of these four methods, we counted the viability of cells suspended in the culture medium immediately after thawing and the number of cells cultured for 3–5 days after thawing. The cell viability of KHYG-1 cryopreserved in a DEPAK freezer immediately after thawing was similar to that of cells cryopreserved in a proton freezer, but were higher than that of cells cryopreserved in the slow-freezing methods (Fig. 2A). On the other hand, the cell viability of THP-1, OMVANA, and HuH-7 cells cryopreserved in the DEPAK freezer was almost same to the other three methods (Fig. 2B–D). Both KHYG-1 and OVMANA cells cryopreserved in the DEPAK freezer showed more than twice the number of cells compared with those cryopreserved in the Proton freezer or the slow-freezing methods

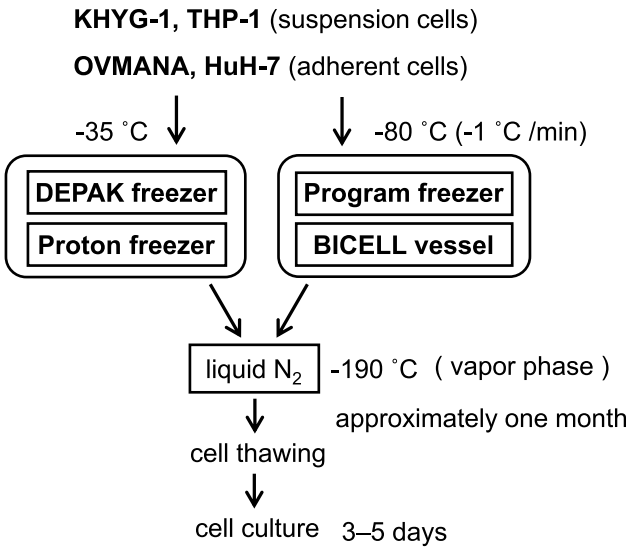

Fig. 1. Schematic overview of the cryopreservation protocol for cancer cell lines. Each of the two lines of suspension and adherent cancer cells was frozen with food-freezing methods (DEPAK and Proton freezers) by cooling rapidly at −35 °C or slow freezing methods (Program freezer and BICELL vessel) by cooling at −1 °C/min, followed by storing in the vapor phase in a liquid nitrogen tank. Frozen cells were thawed and seeded onto plates.

(Fig. 2E and G). In addition, the cell counts of THP-1 and HuH-7 cells cryopreserved in the DEPAK freezer were the highest among the four methods (Fig. 2F and H). These results demonstrate that the DEPAK freezing method improves the cryopreservation efficiency of suspensions and adherent cancer cell lines susceptible to freeze-thaw damage.

3.2. Evaluation of cryopreservation methods for undifferentiated human iPS cells

Next, we investigated whether the DEPAK freezing method was effective for the cryopreservation of undifferentiated human iPS cells. To this end, we cultured two iPS cell lines, 201B7-Ff and 1231A3, both of which were derived from distinct healthy individuals, and froze them using the four freezing methods including DEPAK freezer, followed by preservation in the vapor phase in liquid nitrogen for one week. Cell viability was measured immediately after thawing and the cells were cultured for 4–5 days, and the number of viable cells was counted. Fig. 3A and B showed that the viability immediately after thawing using the DEPAK freezer was comparable to the other three methods, but Fig. 3C and D showed that cryopreservation using the DEPAK freezer was equivalent to proton and resulted in a slight but significant increase in 201B7-Ff and 1231A3 lines compared with those in the slow-freezing methods. The effect of DEPAK in freezing and thawing human iPS cells was reproducible under two identical conditions (data not shown). Furthermore, both frozen undifferentiated iPS cell lines remained undifferentiated even after thawing and culture (Fig. 3E and F). These data indicate that the DEPAK freezing method was optimal for the cryopreservation of undifferentiated human iPS cells.

3.3. Evaluation of cryopreservation methods for iPSC-derived neural cells

Based on the positive results obtained with undifferentiated human iPS cells (Fig. 3), we assessed the potential effect of the DEPAK freezing method on the cryopreservation of iPSC-derived neural cells. The two iPS cell lines used in Fig. 3 were induced to differentiate into neural cells for 13 days in an adherent culture and

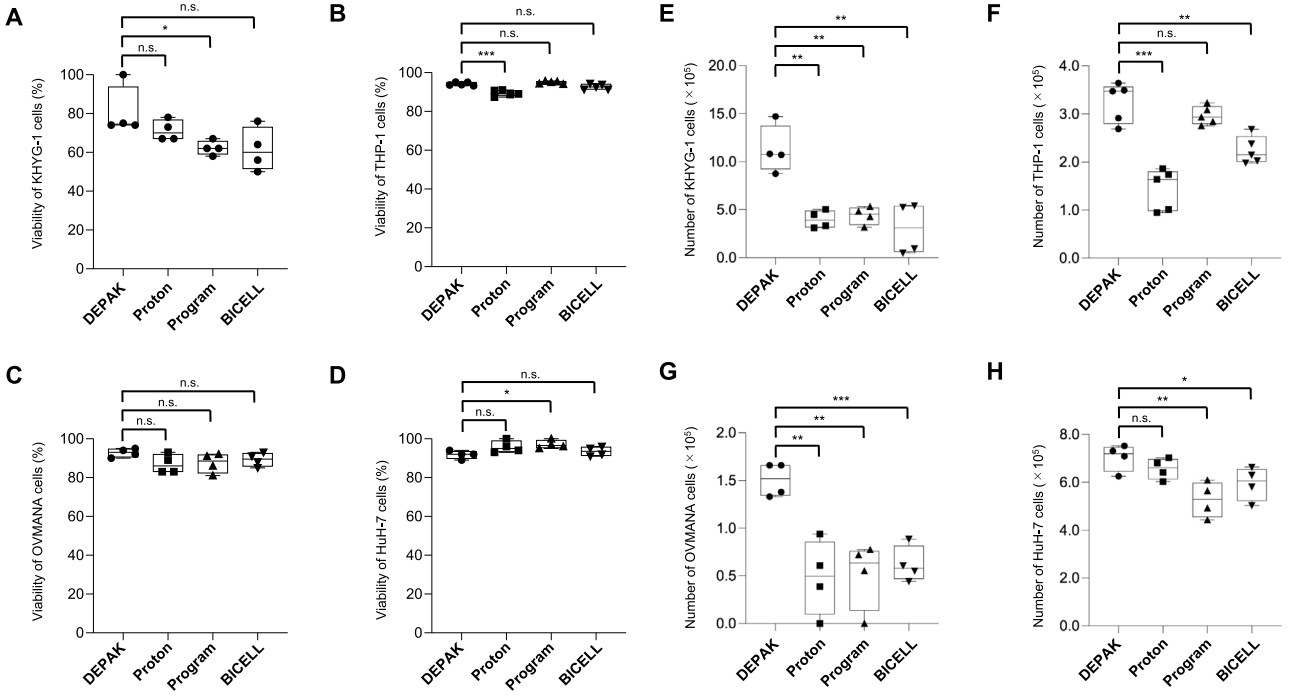

Fig. 2. Cryopreservation efficiencies of four freezing methods on the proliferation of KHYG-1, THP-1, OVMANA, and HuH-7 cells. (A–D) Each cell line was cryopreserved using the four freezing methods as indicated and then resuspended in the culture medium. Cell viability was counted immediately after resuspension. (A): KHYG-1 cells (n = 4); (B): THP-1 cells (n = 5); (C): OVMANA cells (n = 4); (D): HuH-7 cells (n = 4). Data are shown as mean ± SD, $*p < 0.05$, $***p < 0.005$. n.s.; not significant. (E–H) Each cell line was cryopreserved using the four freezing methods as indicated and then resuspended in the culture medium. Cells were counted when cultured for three to five days after cell thawing. (E): KHYG-1 cells (on day 5, n = 4); (F): THP-1 cells (on day 3, n = 5); (G): OVMANA cells (on day 3, n = 4); (H): HuH-7 cells (on day 3, n = 4). Data are shown as mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. n.s.; not significant.

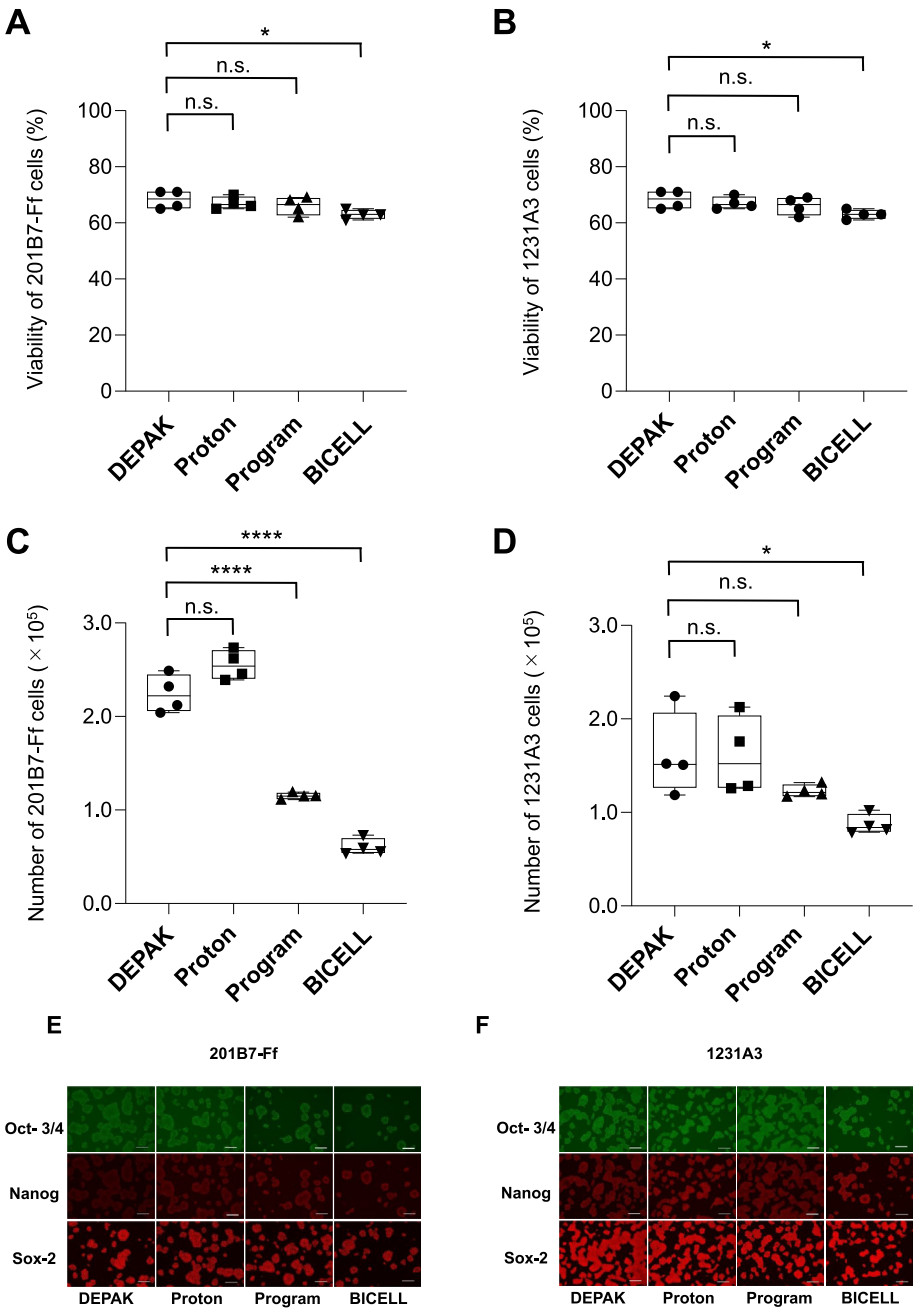

Fig. 3. Cryopreservation efficiencies of four freezing methods on the proliferation of human-induced pluripotent stem cells (iPSCs) 201B7-Ff and 1231A3. (A and B) Each cell line was cryopreserved using the four freezing methods as indicated and then resuspended in the culture medium. Cell viability was counted immediately after resuspension. (A): 201B7-Ff cells (n = 4); (B): 1231A3 cells (n = 4). Data are shown as mean ± SD, *$p < 0.05$, n.s.; not significant. (C and D) Each cell line was cryopreserved using the four freezing methods as indicated and then resuspended in the culture medium. Cells were counted when cultured for five days after cell thawing. (C): 201B7-Ff cells (on day 5, n = 4); (D): 1231A3 cells (on day 5, n = 4). Data are shown as mean ± SD, *$p < 0.05$, ****$p < 0.0001$, n.s.; not significant. (E and F) Immunostaining of cell lines frozen each four methods. (E): 201B7-Ff cells (on day 6); (F): 1231A3 cells (on day 4). Oct-3/4 (upper), Nanog (middle), and Sox-2 (lower). Scale bars, 500 μm.

subsequently frozen using DEPAK freezing, Proton freezing or the two slow-freezing methods (Fig. 4A). After one- or seven-days preservation in the vapor phase in liquid nitrogen, the frozen-thawed cells were cultured under floating conditions for 4 days, and a growing cell aggregate was observed as sphere formation (Fig. 4A). The viability of all four freezing methods from the 201B7-Ff line and the 1231A3 line immediately after thawing was the same for both one-day and seven-day freezing (Fig. 4B–E). In the case of slow-freezing methods using the Programmable freezer or BICELL, one-day cryopreservation of both neural cells differentiated from the 201B7-Ff and 1231A3 lines caused a decrease in sphere formation capacity compared with their unfrozen condition (Fig. 4F–H and J). In the case of the proton freezing, when neural cells differentiated from the 201B7-Ff lines were cryopreserved for one-day, the number and size of sphere formation decreased compared to the non-frozen state, and in the case of the 1231A3 lines, sphere formation decreased. (Fig. 4F–H and J). In contrast, one-day frozen-thawed cells displayed proper cell aggregates through the DEPAK freezing method, as did unfrozen cells in both 201B7-Ff and 1231A3 lines. Similar results were obtained even after

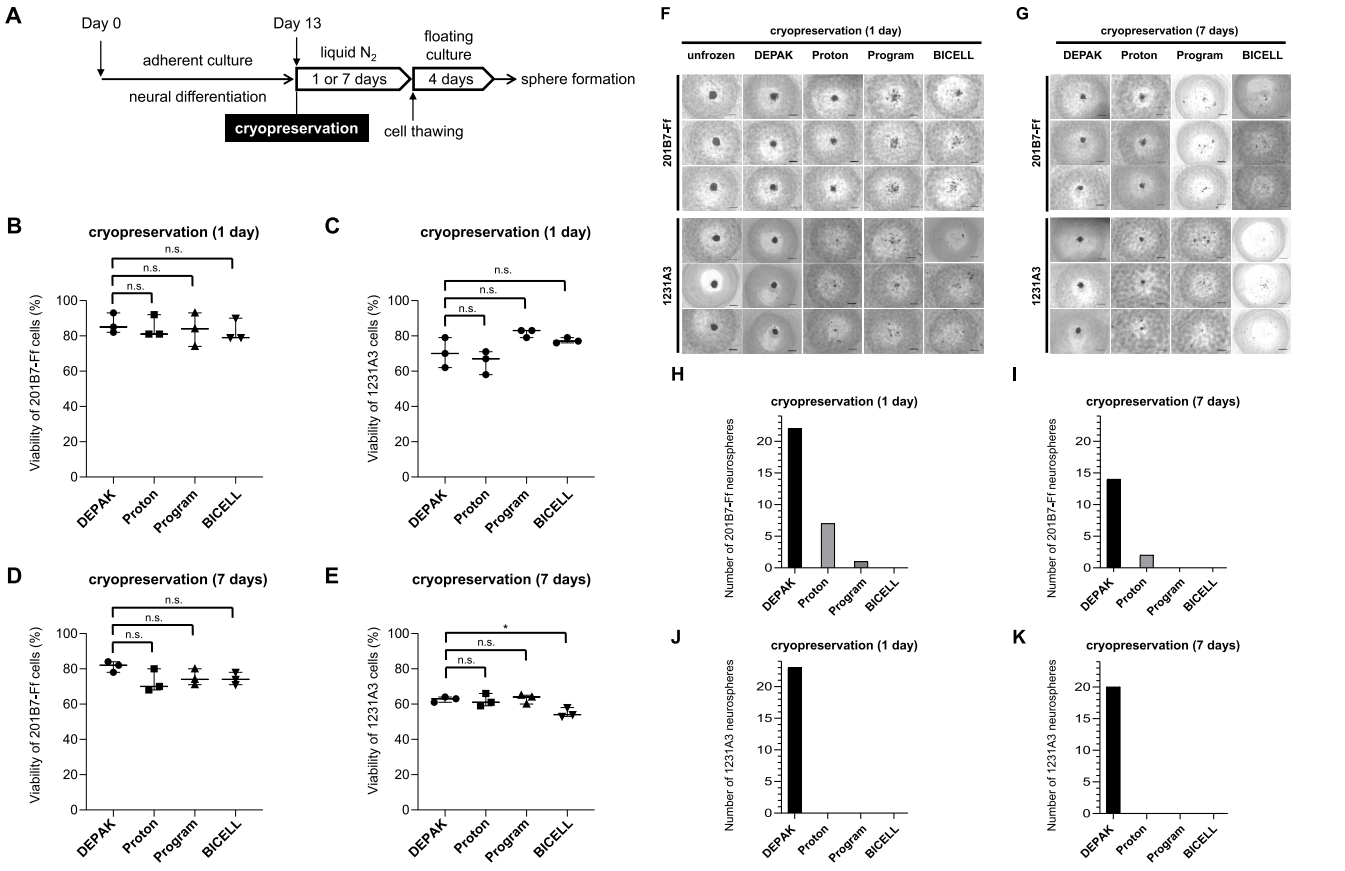

Fig. 4. Effects of four freezing methods on the sphere formation capacity of the two iPSC-derived neural cells. (A) Schematic overview of the protocol for testing the effects of four freezing methods on the sphere formation capacity of iPSC-derived neural cells. iPSC-derived neural cells in adherent culture were cryopreserved on day 13 and stored in the vapor phase in liquid nitrogen for one or seven days. After thawing, cells were grown in floating culture for four days and then sphere formation was observed under a microscope. (B–E) Each cell line was cryopreserved using the four freezing methods as indicated and then resuspended in the culture medium. Cell viability was counted immediately after resuspension. (B): 201B7-Ff cells (one-day, n = 3); (C): 1231A3 cells (one-day, n = 3); (D): 201B7-Ff cells (seven-days, n = 3); (E): 1231A3 cells (seven-days, n = 3). Data are shown as mean ± SD, *$p < 0.05$, n.s.; not significant. (F) The phase-contrast images of neurospheres derived from 201B7-Ff and 1231A3 cells, both of which were cryopreserved using the four freezing methods as indicated for one day (F) or seven days (G). Images of neurospheres without cryopreservation were shown as an unfrozen control in (F). Scale bars, 1 mm. (H–K) Effect of four different freezing methods on the number of neurosphere formed in 24 wells derived from each iPSC. (H): 201B7-Ff cells (one-day); (I): 201B7-Ff cells (seven-days); (J): 1231A3 cells (one-day); (K): 1231A3 cells (seven-days).

seven-day cryopreservation of iPSC-derived neural cells (Fig. 4F–K). Altogether, these data imply that the DEPAK freezing method is more appropriate for maintaining the sphere formation capacity after cryopreservation of iPSC-derived neural cells.

3.4. Evaluation of cryopreservation methods for neurospheres

Finally, to address whether the DEPAK freezing method improves the success rates of spheroid cryopreservation, we evaluated the viability and subsequent neurite outgrowth of frozen-thawed spheroids. Six floating neurospheres differentiated from the 201B7-Ff and 1231A3 lines were frozen on day 27 using four different freezing methods and preserved in cryotubes (six spheres per tube) under the vapor phase in liquid nitrogen for one week, after which viable spheres were further cultured in adherent culture for three days and then immunostained with an anti-TUBB3 antibody to visualize neurite outgrowth (Fig. 5A). The number of viable spheres out of the six in each cryotube was represented in the form of a stack of images. In the case of 201B7-Ff-derived neurospheres, cryopreservation using DEPAK and Proton freezers kept most of the spheres alive. At the same time, the two different slow-freezing methods resulted in poor sphere viability, probably due to freeze-thaw damage (Fig. 5B). Similarly, the DEPAK freezing method showed the highest cryopreservation efficiency for 1231A3-derived neurospheres (Fig. 5C).

To further assess the differentiation potential of the frozen-thawed neurospheres, we calculated the area of neurite outgrowth by subtracting the nuclear area from the TUBB3 immunostained area. As shown in Fig. 5D, cryopreservation using the DEPAK freezer tended to exhibit a larger neurite area among the four freezing methods in 201B7-Ff-derived neurospheres; however, this was not statistically significant because only a few neurospheres were alive when cryopreserved using slow freezing methods (shown in Fig. 5B). In contrast, we obtained sufficient data on neurite area in 1231A3-derived neurospheres, which showed moderate viability even with the slow-freezing methods (Fig. 5C), and observed that DEPAK-frozen neurospheres exhibited a significant increase in neurite outgrowth compared with those of the slow-freezing methods (Fig. 5E). Collectively, these results suggest that DEPAK freezing is suitable for spheroid cryopreservation because of its high differentiation potential.

4. Discussion

4.1. Advantages of the DEPAK freezing method in cryopreservation of biological materials

In this study, we attempted to improve the cryopreservation efficiency of cells and spheroids by diverting the freezing technology used in the food industry. Compared with that in the

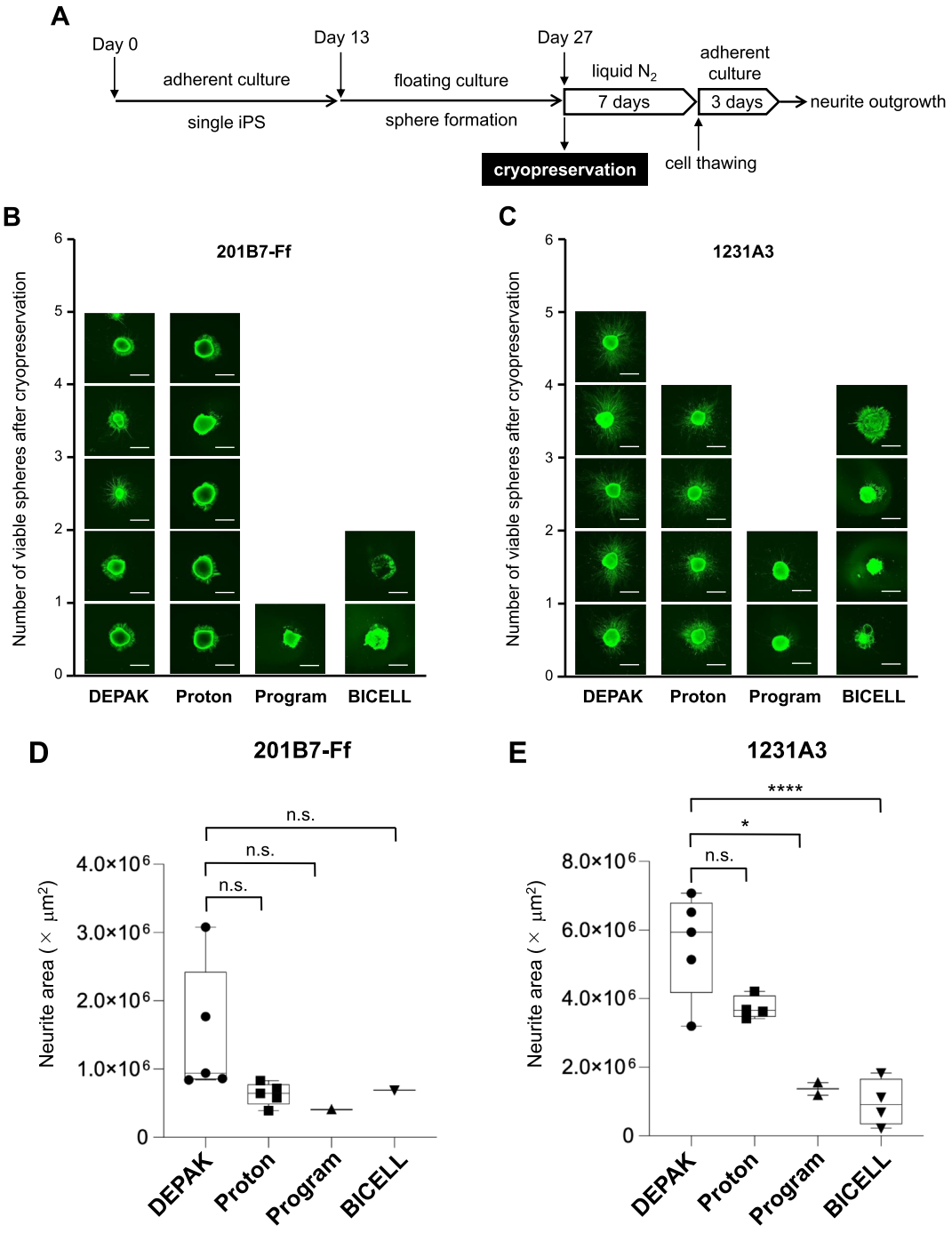

Fig. 5. Effects of four freezing methods on neurite outgrowth capacity of the two iPSC-derived neurospheres. (A) Schematic overview of the protocol for testing the effects of four freezing methods on the neurite outgrowth capacity of iPSC-derived neurospheres. iPSC-derived neurospheres in floating culture were cryopreserved on day 27 and stored in the vapor phase in liquid nitrogen for seven days. After thawing, neurospheres were grown in adherent culture for three days, and then neurite outgrowth was observed under a fluorescent microscope. (B and C) The fluorescent images of neurite outgrowth using immunostaining with an anti-TUBB3 antibody. Neurospheres derived from 201B7-Ff (B) and 1231A3 (C) were cryopreserved using the four freezing methods as indicated. The number of viable spheres out of six in a single cryotube after thawing was shown as a stack of image panels. Scale bars, 1 mm (B and C). The area of neurite outgrowth of 201B7-Ff (D) and 1231A3 (E) was defined by the exclusion of the DAPI staining area from the TUBB3 staining area with the BZ-H3M measurement module. Data are shown as means ± SD, *$p < 0.05$, ****$p < 0.0001$. n.s.; not significant.

conventional slow-freezing methods, cryopreservation using the DEPAK freezer resulted in a significant increase in the viability and proliferation of the four cancer cell lines that were particularly susceptible to freeze-thaw damage (Fig. 2). In addition, the DEPAK freezing method yielded higher cell viability and sphere formation capacity in undifferentiated and neural differentiated human iPS cells, respectively (Figs. 3 and 4). The growth rate of cancer cell lines and undifferentiated iPS cells was temporarily reduced by freezing and thawing, but no effect of the freezing method on the growth rate was observed. Furthermore, this method sustained the differentiation potential of iPSC-derived neurospheres (Fig. 5), allowing for its use in drug discovery and regenerative medicine. What makes the DEPAK freezing method even more noteworthy is that, unlike a programmed freezer or deep freezer, there is no need for a

slow-freezing step (−1 °C/min) because this method rapidly quenches cells at −35 °C immediately followed by preservation in liquid nitrogen. Thus, this simple, quick, and efficient DEPAK freezing method could be an alternative to conventional slow-freezing methods.

4.2. What is the mechanism underlying higher cryopreservation efficiency of the DEPAK freezing method

We evaluated the potential utility of DEPAK and Proton freezers, originally used to preserve fresh foodstuffs during the cryopreservation of biological materials. The DEPAK freezer displayed a higher cryopreservation efficiency in cancer cell lines than the Proton freezer (Fig. 2E–G). In contrast, their effects on spheroid cryopreservation were comparably higher than those of the slow-freezing methods (Fig. 5). What mechanisms are responsible for these results? The Proton freezer combines a weak static magnetic field (1–200 mT), an alternating electric field in the radio waves (0.2–1 MHz), and a cold airflow [17]. It has been assumed that a static magnetic field increases the hydrogen bonds between water molecules and generates a more stable water cluster, promoting ice nucleation and suppressing supercooling. Considering a previous report demonstrating that a Proton freezer is suitable for the cryopreservation of iPSC-derived dopaminergic neurons, a combination of a static magnetic field and an alternating electric field appears to minimize ice crystals, thereby improving the cell viability of iPSC-derived neurospheres [19]. In contrast, the DEPAK freezer freezes samples under a minute alternating current (several dozen μA) generated by high-voltage electrostatic induction and consequently suppresses the quality deterioration of foodstuffs [18]. Although the precise mechanisms underlying the higher cryopreservation efficiency of the DEPAK freezing method for biological materials have not been completely elucidated, we predict this technology will prevent ice crystals formation during freezing.

4.3. Perspectives for further development of the DEPAK freezing method in cryopreservation

Our findings suggest that the DEPAK freezing method can be applied to the cryopreservation of not only spheroids, organoids, tissues and organs. To achieve this objective, further improvements are required in DEPAK freezing methods. First, because the equipment settings of the DEPAK freezer in this study were the same as those for food preservation, we still had the opportunity to adjust the parameters to obtain the highest efficiency for the cryopreservation of biological materials. Second, given that DEPAK technology was originally dedicated to food defrosting, as mentioned in the Introduction, using the DEPAK freezer in the cell thawing process would be worthwhile. Third, considering the importance of cell-freezing media in cryopreservation, optimizing its chemical composition, including cryoprotectants, may be an alternative strategy for making the most of DEPAK freezing technology. Thus, these examinations using the DEPAK freezer will lead to the establishment of cryopreservation techniques for various biological materials and contribute to basic biology, drug discovery, and regenerative medicine.

5. Conclusions

Our study attempted to divert the innovative food-freezing technology called “DEPAK” to the cryopreservation of biological materials. In the cryopreservation of freeze-thaw damage-sensitive cell lines and undifferentiated human iPSCs, DEPAK freezing resulted in significantly higher cell proliferation than conventional slow-freezing methods. Importantly, freezing with DEPAK did not impair the sphere formation capacity of neurally differentiated human iPSCs. Furthermore, DEPAK-frozen neurospheres exhibited higher viability and neurite outgrowth after thawing than slow-freezing neurospheres. It may be possible that the DEPAK freezing method can overcome the difficulties associated with the cryopreservation of three-dimensional cell aggregates, including organoids, tissues, and organs.

Author contributions

Conceptualization, K.B. and A.K.; methodology, K.B.; investigation, K.B. and M.O.; supervision, K.B., H.D, and A.K.; original draft preparation, K.B. and H.D.; writing-review and editing, H.D. and A.K.

Declaration of competing interest

The authors declare no conflict of interest.

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