



Original Article

Cryoprotective enhancing effect of very low concentration of trehalose on the functions of primary rat hepatocytes

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ABSTRACT

Introduction: Cells have various applications in biomedical research. Cryopreservation is a cell-preservation technique that provides cells for such applications. After cryopreservation, sensitive cells, such as primary hepatocytes, suffer from low viability due to the physical damage caused by ice crystals, highlighting the need for better methods of cryopreservation to improve cell viability. Given the importance of effectively suppressing ice crystal formation to protect cellular structure, trehalose has attracted attention as cryoprotectant based on its ability to inhibit ice crystal formation; however, trehalose induces osmotic stress. Therefore, to establish a cell-cryopreservation technique, it is necessary to provide an optimal balance between the protective and damaging effects of trehalose.

Methods: In this study, we evaluated the effects of osmotic stress and ice crystal formation on the viability and function of primary rat hepatocytes at wide range of trehalose concentration.

Results: There was no osmotic stress at very low concentrations (2.6 μ M) of trehalose, and 2.6 μ M trehalose drives the formation of finer ice crystals, which are less damaging to the cell membrane. Furthermore, we found that the number of viable hepatocytes after cryopreservation were 70% higher under the 2.6 μ M trehalose-supplemented conditions than under the dimethyl sulfoxide-supplemented conditions. Moreover, non-cryopreserved cells and cells cryopreserved with trehalose showed comparable intracellular dehydrogenase activity.

Conclusions: We showed that trehalose at very low concentrations (2.6 μ M) improved dramatically viability and liver function of hepatocyte after cryopreservation.

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Abbreviations: DMSO, dimethyl sulfoxide; CMIC, cell-membrane-impermeable cryoprotective agent; DMEM, dulbecco's modified Eagle medium; CCK-8, Cell Counting Kit-8; FBS, fetal bovine serum.

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1. Introduction

Cells have diverse applications in biomedical research. Cells are utilized for the investigation of life processes, serve as hosts for the production of useful physiologically active substances, and provide resources for regenerative medicine. Therefore, it is important to ensure a safe and stable supply of cells. This is achieved with cell-preservation technologies, such as cryopreservation, which is a widely used strategy for storing cells in a physiologically intact form. Cryopreservation reduces the physical damage to cellular structure caused by ice crystals through the use of ice crystal-formation inhibitors, such as dimethyl sulfoxide (DMSO). Highly

sensitive cells, such as primary hepatocytes, exhibit low viability during cryopreservation [1–3], which needs to be improved. Low viability raises concerns of physical damage to cells during ice crystal formation, because the suppressive effects of DMSO for ice crystal formation are inadequate to protect hepatocyte structure. Therefore, it is important to effectively suppress ice crystal formation to protect cellular structure.

On the other hands, the disaccharide trehalose is a key metabolite produced during cryptobiosis, during which all metabolic activities of an organism reach a reversible suspended state [4–7]. The cytoprotective effects of trehalose are well known [8–10] and have attracted increased attention in recent years for suppression of ice crystal formation. Trehalose has been used to cryopreserve both erythrocytes as established cell lines and stem cells [11–14]; however, trehalose might not be able to suppress ice crystal formation inside of cells due to its inability to permeate the cell membrane. Therefore, protecting cells both inside and outside might necessitate the use of trehalose combined with a cell-membrane-permeable cryoprotective agent, such as DMSO. Previous studies indicate that this type of combined treatment improves cell viability following cryopreservation [13,15].

Cell-membrane-impermeable cryoprotective agents (CMICs) function in two different ways: modulation of osmotic stress and suppression of ice crystal formation. However, there are few reports examining both of these mechanisms, and the concentration range of trehalose used in these studies (0.1–0.3 M) is considerably narrow [16]. Additionally, the optimal trehalose concentration (0.05–0.6 M) proposed in previous studies was evaluated based on only viability and function of cells [3,17–19]. Therefore, to establish a cell-cryopreservation technique that provides an optimal balance between the protective and damaging effects of trehalose, evaluation of a wider concentration range is necessary.

In this study, we used primary hepatocytes, because their viability and function are severely affected during freeze and thawing process. To investigate the effects of trehalose on these cells, we evaluated the effects of osmotic stress and ice crystal formation on hepatocytes at wide concentration range of trehalose. Moreover, we assessed ice crystal morphology in cryopreservation media and correlated the findings with those of cell viability and function.

2. Materials and methods

2.1. Hepatocyte isolation

Primary hepatocytes were isolated from male Sprague–Dawley rats (6- to 8-week-old; Japan SLC, Inc., Hamamatsu, Japan) and prepared using the two-step collagenase perfusion method [20]. Cell viability was ~90% according to Trypan Blue dye-exclusion assays. For experiments involving hepatocytes, we used serum-free medium supplemented with growth factors [Dulbecco's modified Eagle medium (DMEM; Funakoshi Co., Ltd., Tokyo, Japan) supplemented with 0.05 mg/L Epidermal Growth Factor (Funakoshi Co., Ltd., Tokyo, Japan) 10 mg/L insulin obtained from bovine pancreas (Sigma, Tokyo, Japan), 7.5 mg/L hydrocortisone (Sigma), and 60 mg/L L-proline (Sigma); referred to as DHDM] [21,22]. This protocol was reviewed and approved by the Ethics Committee on Animal Experiments of Kyushu University (A29-413-1; 29 Jun 2018).

2.2. Measurement of cell volume and cellular activity

Hepatocytes were suspended at a density of 1×10^6 viable cells/mL in DHDM supplemented with various concentrations of trehalose (0, 26 μ M, 26 mM, 260 mM, 520 mM, 1 M; Wako, Osaka, Japan).

Cells were incubated for 60 min at 4 °C, and the suspension was placed on a hemocytometer and photographed to allow measurement of the projection area using ImageJ software (National Institutes of Health, Bethesda, MD, USA), as described previously [23]. The sphere-equivalent volume of cells was calculated from the projection area. To determine cellular activity, hepatocytes exposed to trehalose were resuspended in DHDM and seeded in collagen I-C-coated 96-well plates at a density of 2.5×10^4 cells/cm² and incubated at 37 °C under 5% CO₂, with the medium replaced once at 4-h post-inoculation. Intracellular dehydrogenase activity was evaluated at 24-h post-inoculation using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan).

2.3. Evaluation of ice crystal morphology

We prepared DHDM supplemented with up to 26 mM trehalose and 10% DMSO (Wako) and transferred it to sandwich-glass cells with a cell gap of 50 μ m (EHC. Co. Ltd, Tokyo, Japan). The optical texture of the sample was observed while lowering the temperature from 20 °C at a rate of 5 °C/min using a polarized light microscope equipped with a cross Nicol prism (POM; ECLIPSE E600 PDL; Nikon, Tokyo, Japan). The temperature of the sample was precisely controlled by a hot stage (LTS E350; Linkam, Fukuoka, Japan) using a temperature controller (10013L; Linkam) at an accuracy of ± 0.1 °C.

2.4. Hepatocyte cryopreservation and cultivation

Cryopreservation solutions were prepared by adding up to 260 mM trehalose and 10% DMSO to DHDM, and hepatocytes were suspended in the cryopreservation solutions at a density of 1×10^6 cells/mL. The suspended hepatocytes were dispensed at 1×10^6 cells/vial, and the temperature was lowered to –80 °C at a rate of 1 °C/min by BICELL (NIHON FREEZER CO., LTD., Tokyo, Japan).

After 5 days of cryopreservation, the cryopreserved hepatocytes were thawed in a water bath at 37 °C, and the cryopreservation solution was removed by dilution with 5 mL of DMEM. The cells were centrifuged at 50 \times g for 90 s and resuspended in DHDM supplemented with 10% fetal bovine serum (FBS). Cell viability was evaluated using the Trypan Blue dye-exclusion test.

Hepatocytes were cryopreserved using DHDM containing 2.6 μ M trehalose and 10% DMSO. The cryopreserved hepatocytes were thawed using the method described in this section and inoculated in 96-well plates coated with collagen film (Cellmatrix type I-C: 3 mg/mL; Nitta Gelatin, Osaka, Japan), [24]. The plates were incubated under standard conditions (37 °C, 5% CO₂, and 95% air) at a seeding density of 1.25×10^5 cells/cm² (based on cell number before cryopreservation) at monolayer state. The seeding density was determined in consideration of the cell recovery efficiency after cryopreservation. The culture medium (DHDM supplemented with 10% FBS) was changed at 2 h, 1- and 3-days post-inoculation. Additionally, freshly isolated hepatocytes were cultivated under similar conditions at a seeding density of 2.5×10^4 cells/cm².

2.5. Evaluation of intracellular dehydrogenase activity and liver-specific functions about hepatocytes after cryopreservation

Intracellular dehydrogenase activity of hepatocyte after cryopreservation was evaluated at 1-, 3-, and 5-days post-inoculation using the Cell Counting Kit-8. In addition, albumin synthesis was evaluated in order to evaluate the liver-specific function of hepatocyte after cryopreservation. Albumin concentration in culture medium was measured using an enzyme-linked immunosorbent assay involving the horseradish peroxidase/3, 3', 5, 5'-

tetramethylbezidine system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) [25]. The rat albumin standard and anti-rat albumin antibody were purchased from ICN Pharmaceuticals (Aurora, OH, USA). Meanwhile, Ethoxyresorufin-O-deethylase (EROD) activity, an indicator of CYP1A1 (Cytochrome P450 family 1 subfamily A member 1) activity, was also evaluated at 3-days post-inoculation in order to evaluate the liver-specific function of hepatocyte after cryopreservation. EROD activity was estimated by measuring the intensity of resorufin fluorescence in the medium. In this assay, CYP1A1 was induced by 3-methylcholanthrene (Sigma). Briefly, the medium was substituted by 2 μM 3-methylcholanthrene (3MC, Sigma)-supplemented D-HDM and incubated for 24 h. After that, the medium was replaced with 10 μM ethoxyresorufin (Wako)-supplemented D-HDM and incubated for 1 h. The intensity of resorufin fluorescence in the medium was measured using a fluorescence plate reader. Furthermore, the ammonia metabolism was estimated at 1-day post-inoculation by measuring ammonia concentration in the medium. Briefly, the medium was substituted with 1 mM ammonium chloride (Wako Pure Chemical Industries, Osaka, Japan)-supplemented D-HDM and incubated for 4 h. Ammonia metabolism was measured using the ammonia test (Wako Pure Chemical Industries). EROD activity and ammonia metabolism activity were normalized to intracellular dehydrogenase activity indicates these activities per viable cell.

2.6. Statistical analysis

Results are presented as the mean \pm standard deviation (SD), and statistical analysis was performed using a one-sided unpaired Student's *t* test. A $p < 0.05$ and 0.01 was considered statistically significant.

3. Results

3.1. Effect of trehalose on cell volume and intracellular dehydrogenase activity

First, we investigated the effect of trehalose osmotic pressure on cell volume and intracellular dehydrogenase activity. In this experiment, intracellular dehydrogenase activity was taken as an index of hepatocytes viability. We found that as trehalose concentration in the medium increased, the cell volume decreased (Fig. 1A), and we noted a dramatic decrease in cell volume along with a 1000-fold change in trehalose concentration from 26 μM to 26 mM. However, as trehalose concentration reached 1 M, cell volume began to increase. Additionally, hepatocytes exposed to trehalose at a concentration ≥ 26 mM showed decreased intracellular dehydrogenase activity (Fig. 1B). Moreover, cell volume was significantly altered in these cells (Fig. 1A), and intracellular dehydrogenase activity was significantly lower (almost 0) in cells treated with trehalose at a concentration of ≥ 1 M (Fig. 1B). At a trehalose concentration of 26 μM , no change in cell volume was observed (Fig. 1A), and intracellular dehydrogenase activity was similar to that of cells not exposed to trehalose (Fig. 1B).

3.2. Ice crystal morphology and the viability of cryopreserved hepatocytes

We analyzed the shape of ice crystals and the number of viable cells after cryopreservation with various trehalose concentrations in order to establish a correlation between the two. Ice crystals of DHDM containing 10% DMSO showed a linear orientation (Fig. 2A-a), and their shape was similar to that of ice crystals of DHDM containing 10% DMSO and 260 nM trehalose (Fig. 2A-b). By contrast, a linear orientation disappeared at DHDM supplemented

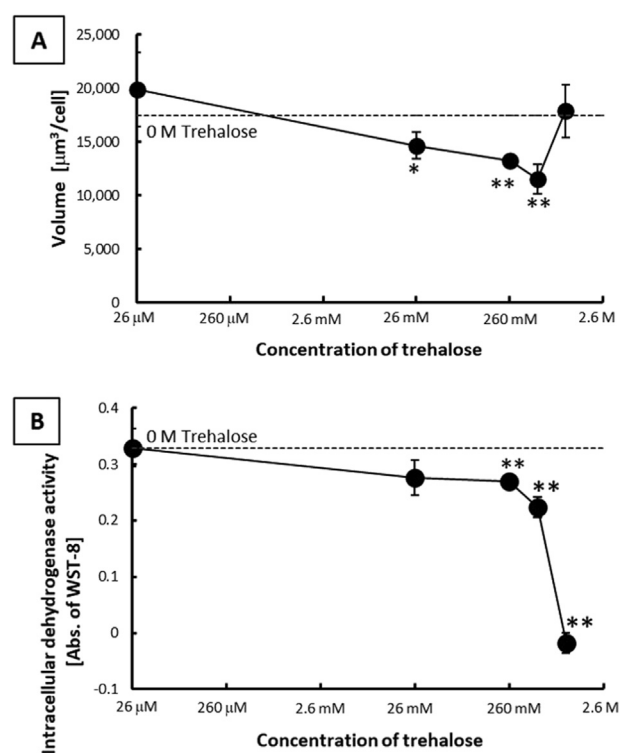


Fig. 1. Osmotic effect of trehalose on hepatocytes. (A) The sphere-equivalent volume and (B) intracellular dehydrogenase activity of hepatocytes after being exposed to various concentrations of trehalose (0, 26 μM , 26 mM, 260 mM, 520 mM, 1 M). Bars represent the S.D. ($n = 3$). ** $p < 0.01$, * $p < 0.05$ vs. 0 M trehalose.

with 10% DMSO and 2.6 μM trehalose condition, and formed some branching (Fig. 2A-c). This branching exhibited a trehalose-dominated shape rather than a DMSO-dominated shape, despite the low trehalose concentration. In addition, increases in trehalose concentration also increased branching, and the ice crystals became finer (Fig. 2A-d and e).

The number of viable cells increased from 0.30×10^6 to 0.54×10^6 as the concentration of trehalose increased from 0 μM to 2.6 μM (Fig. 2B). However, at 260 mM, the number of viable cells decreased to 0.1×10^6 . Overall, the viability determined by Trypan Blue exclusion ranged from 65% to 80% at all trehalose concentrations, except 2.6 mM (37%) and 260 mM (17%). The 2.6 μM trehalose condition had the highest viable cell number (0.54×10^6), which was 1.8-fold compared to 0 M trehalose condition.

3.3. Intracellular dehydrogenase activity and the liver-specific functions of hepatocytes cultivated after cryopreservation

DMSO adversely affected to adhesion and extension of cryopreserved cells. On the other hand, the addition of trehalose improved cell adhesion and extension, and the cell state approached to the state of non-cryopreserved cell (Fig. 3A). Hepatocytes were cultured after cryopreservation to evaluate their intracellular dehydrogenase activity and liver function. Compared with non-cryopreserved cells, those cryopreserved with only DMSO showed a considerable reduction in intracellular dehydrogenase activity. Although reductions were also observed under DMSO- and trehalose-supplemented conditions, the extent of reduction was lower than that observed under only DMSO-supplemented conditions. Upon cultivation post-cryopreservation, hepatocytes cryopreserved using combined trehalose and DMSO showed >2.4-fold higher intracellular dehydrogenase activity

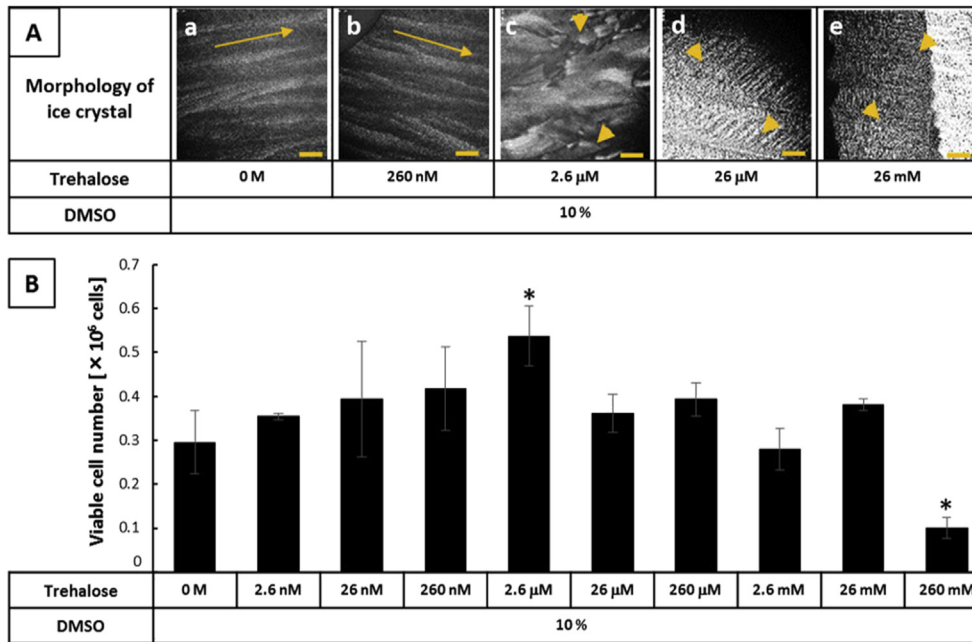


Fig. 2. Cryopreservation effect of trehalose on hepatocytes. (A) Observation of ice crystal morphology of DHDM supplemented with trehalose (0, 26 μM, 26 mM) and DMSO (0, 10%). Arrows represent a linear orientation, and arrow heads represent branching of ice crystal formation. Scale bar = 200 μm. (B) Determination of viable cell number after cryopreservation. 0, 26 μM, 26 mM, 260 mM trehalose and 10% DMSO to DHDM were used as cryopreservation solutions. Bars represent the S.D ($n = 3$). * $p < 0.05$ vs. 0 M trehalose.

relative to those cryopreserved using DMSO alone (Fig. 3B). In particular, the intracellular dehydrogenase activity of cells cryopreserved under trehalose-supplemented conditions increased during cultivation and reached a level reported for non-cryopreserved cells on day 3 post-inoculation.

We then measured albumin concentration in the medium and calculated the albumin-secretion rate. The albumin-secretion rate of hepatocytes cryopreserved using a mixture of trehalose, DMSO was 3.6-fold higher on days 2 and 3, and 4.2-fold higher on days 4 and 5 relative to that of cells cryopreserved using only DMSO (Fig. 3C). Additionally, the albumin-secretion rate increased during the cultivation period and approached the level of non-cryopreserved condition. Total albumin secretion depends on overall cell number, and albumin secretion normalized to intracellular dehydrogenase activity indicates albumin secretion per viable cell. In the results, determination of the albumin-secretion rate according to intracellular dehydrogenase activity (Fig. 3D) revealed a similar trend to total albumin secretion (Fig. 3C).

In the absence of 3MC, EROD activity was hardly observed in non-cryopreserved condition. However, EROD activity of hepatocytes cryopreserved with DMSO only was induced 109-fold higher than that of non-cryopreserved cells (Fig. 3E). In contrast, trehalose suppressed the increase of EROD activity (17-fold higher than that of non-cryopreserved cells). Furthermore, EROD activity was more induced in the presence of 3MC (Fig. 3E). Hepatocytes cryopreserved with only DMSO had 2.2-fold higher EROD activity relative to that of non-cryopreserved cells, which was far from the value in the non-cryopreserved cells. On the other hand, the extent of increase (1.5-fold higher) was suppressed under DMSO- and trehalose-supplemented conditions.

Meanwhile, hepatocytes cryopreserved under trehalose-supplemented conditions had 1.5-fold higher ammonia metabolic rate (Fig. 3F) and 1.7-fold higher ammonia metabolic rate per intracellular dehydrogenase activity (Fig. 3G) relative to that under only DMSO supplemented condition. On the other hands, the ammonia metabolic rate of non-cryopreserved cells was 6.1 ± 0.6 [nmol/h/well], and the ammonia metabolic rate per intracellular

dehydrogenase activity was 16.9 ± 1.6 [nmol/h/well/Abs. of WST-8]. Although, the ammonia metabolic rate per intracellular dehydrogenase activity was higher in cryopreserved conditions than in non-cryopreserved condition, it was difficult to directly compare cryopreserved and non-cryopreserved conditions that had different intracellular dehydrogenase activities since the ammonia metabolism activity of hepatocytes depends on oxygen-tension and substrate concentration [26]. In any case, this result showed that trehalose improved ammonia metabolism ability after cryopreservation at least.

4. Discussion

4.1. Effects of CMICs on hepatocyte cryopreservation

Trehalose is a CMIC that has attracted increased attention due to its ability to prevent ice crystal formation and exert cryoprotective effects [8–10]. CMICs can produce finer ice crystals and induce osmotic stress, and previous studies report that combined treatment with trehalose and DMSO increases cell viability [11–13]. In a previous report, combine use of trehalose and DMSO decreased hepatocyte viability [3]; therefore, cell-cryopreservation technology using trehalose remains to be effectively established.

Hepatocytes are large in size and exhibit various specific functions [27]. Cells suspended in a solution are susceptible to differences in the osmotic pressure between the inside and outside of the cell, which changes in pressure likely to induce movement of the solution until isotonic conditions are achieved. This movement induces cell contraction or expansion, with differences in osmotic pressure increasing the amount of solution movement per cell-membrane area along with increases in cell diameter. This suggests that a larger cell volume invokes a greater burden on the cell membrane. For hepatocytes, which are large in size, a difference in osmotic pressure can be a serious issue due to their involvement in various functions associated with key metabolic processes. Specifically, the cell membrane plays an important role hepatocyte function [28]; therefore, to maintain liver-specific functions

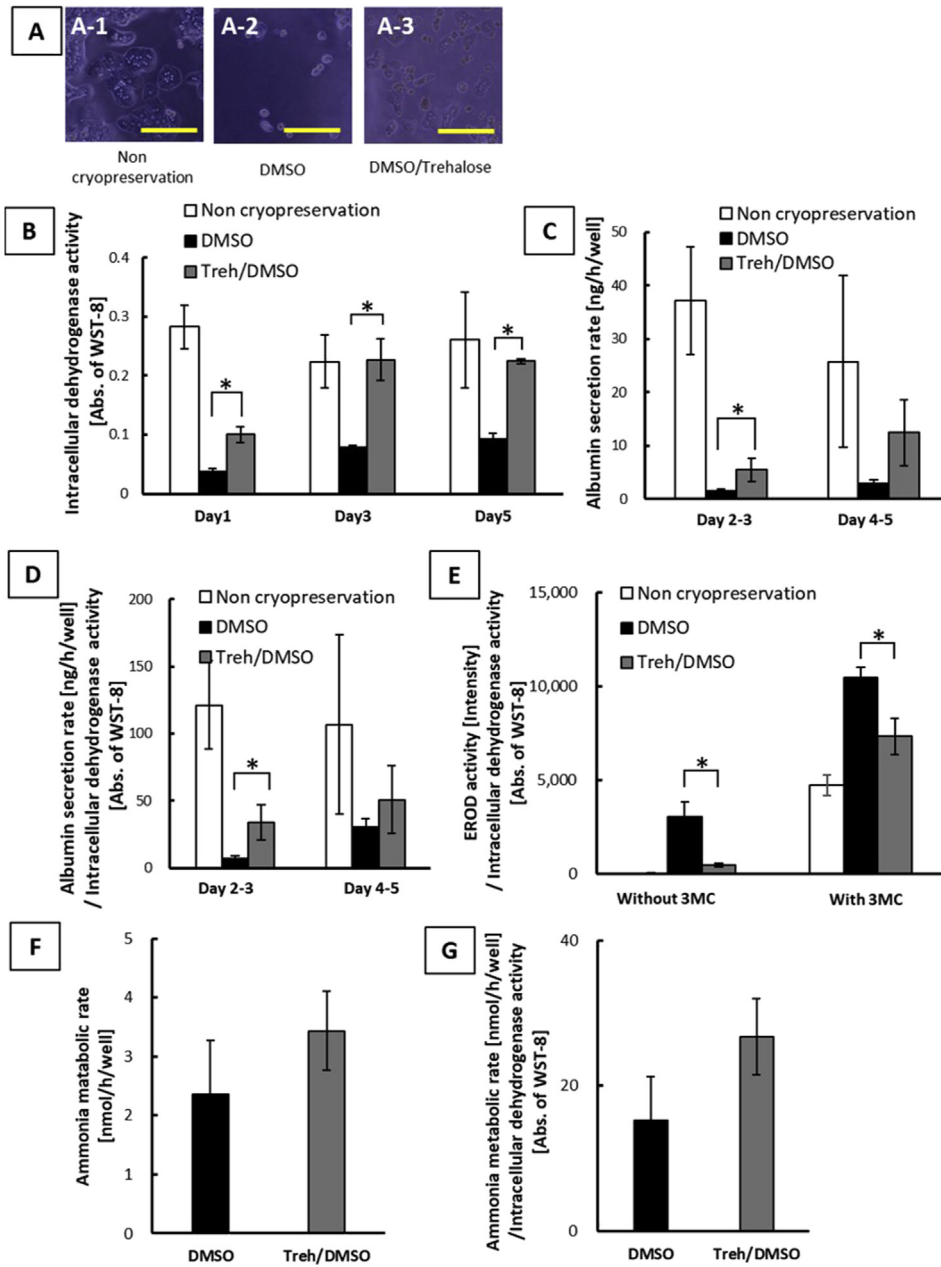


Fig. 3. The function of hepatocytes cultivated after cryopreservation. DHDM containing 2.6 μ M trehalose (Treh) and 10% DMSO were used as cryopreservation solution. The seeding viable cell density was as follows, freshly isolated hepatocytes (non cryopreserved): 2.5×10^4 cells/cm², hepatocytes cryopreserved using only 10% DMSO (DMSO): 4.4×10^4 cells/cm², hepatocytes cryopreserved using 10% DMSO and 2.6 μ M trehalose (Treh/DMSO): 5.0×10^4 cells/cm². (A) Morphology of hepatocytes at 1-days post-inoculation (A-1: non cryopreserved, A-2: DMSO, A-3: Treh/DMSO). Scale bar = 200 μ m. (B) Assessment of intracellular dehydrogenase activity at 1-, 3-, and 5-days post-inoculation. (C) albumin-secretion rate. (D) albumin-secretion rate according to intracellular dehydrogenase activity at 2–3 and 4–5 days post-inoculation, (E) EROD activity according to intracellular dehydrogenase activity at 3-days post-inoculation. (F) ammonia metabolic rate and (G) ammonia metabolic rate according to intracellular dehydrogenase activity at 1 day post-inoculation. Bars represent the S.D (n = 3, *p < 0.05 vs. 0 M trehalose).

following cryopreservation, protecting the cell membrane of hepatocytes is crucial. Hence, osmotic stress on the cell membrane must be removed for hepatocytes cryopreservation.

4.2. The effect of trehalose on cell volume and intracellular dehydrogenase activity

Hepatocytes treated with trehalose showed smaller volumes than untreated hepatocytes (Fig. 1A), indicating that the cells were dehydrated in response to the hypertonicity of the extracellular fluid containing trehalose. In contrast, hepatocytes treated with 1 M trehalose were larger (Fig. 1A), and the cell structure was

greatly damaged due to large differences in osmotic pressure. There are two possibilities regarding the cellular response to high osmolality, such as that at 1 M trehalose. First, the cell membrane cannot withstand the movement of solution, resulting in destruction of the cell membrane. Second, when osmotic pressure is applied and cells excessively contract, deformation leads to destruction of the cell structure. We observed that intracellular dehydrogenase activity began to decrease at trehalose concentrations ≥ 26 mM as a result of changes in cell volume (Fig. 1B). This resulted in a strong correlation detected between changes in cell volume (Fig. 1A) and decreased intracellular dehydrogenase activity (Fig. 1B), thereby confirming that the difference in osmotic

pressure around the cells induced changes in cell structure that was detrimental to hepatocytes.

Previous studies report the use of 0.2 M trehalose for hepatocyte cryopreservation [3], which reduced hepatocyte activity (Fig. 1B). Changes in cell volume can be analyzed and discussed in terms of mathematical modeling of cumulative osmotic stress formation [29]; however, the effect of changes in cell volume on intracellular dehydrogenase activity can only be shown experimentally. A significant aspect of this study was examining the severity of changes in volume and their effect on hepatocyte function. Cell dehydration and osmotic stress were not induced at trehalose concentrations $\leq 26 \mu\text{M}$; therefore, this suggests that trehalose used as a CMIC should be used at concentrations lower than those previously reported [$\leq 100 \text{ mM}$ [16], $\leq 50 \text{ mM}$ [17], and 200 mM [18]]. The results of the present study suggest that trehalose might be effective at a concentration range that does not induce osmotic stress.

4.3. The suppressive effects of trehalose on ice crystal formation

Ice crystal formation is affected by water-retention potential and the concentration of the dissolvent, such as salts and hydrophilic materials. Moreover, the shape of ice crystals changes depending on the type and concentration of the solute [15]. Trehalose forms a sugar–water network and disrupts the water network [30], thereby, inhibiting the water-crystallization process [31]. Furthermore, as the concentration of trehalose increases, the size of the ice crystals decreases [16,32,33]. In the present study, we observed ice crystal formation with polarized light microscopy by replacing polarization and birefringence characteristics with contrast, thereby allowing visualization of ice crystal structure. The microscopy images revealed that the presence of trehalose changed ice crystal morphology, and that branching increased along with increasing trehalose concentration (Fig. 2A). Additionally, evaluation of ice crystal shape at lower trehalose concentrations ($0.26 \mu\text{M}$ – 0.26 M) relative to previous studies (0.1 – 0.3 M [17]) confirmed the presence of a trehalose-dominant crystal shape at very low trehalose concentration ($2.6 \mu\text{M}$; Fig. 2A-c). This is the first report showing that trehalose suppressed ice crystal formation, even at much lower concentrations (in micromolar range) than those used in previous studies [16–18]. Notably, at this concentration, osmotic stress was undetected.

4.4. Trehalose-mediated cryopreservation improves hepatocyte viability

The viability of hepatocytes increased along with cryopreservation with combined trehalose and DMSO (Fig. 2B). At a trehalose concentration of $2.6 \mu\text{M}$, the number of viable cells after cryopreservation was the highest, with the ice crystals exhibiting a trehalose-dominant shape. Therefore, this suggested a correlation between ice crystal shape and cell viability (Fig. 2). Additionally, this result was consistent with the effect of trehalose on cell volume and intracellular dehydrogenase activity. Moreover, the concentration that had highest viable cell number ($2.6 \mu\text{M}$) was much lower than that used in previous studies (100 mM [16], 200 mM [17] and 200 mM [18]).

Increases in trehalose concentration are accompanied by increases in osmotic stress; therefore, the ideal trehalose concentration is one that yields finer ice crystals and suppresses osmotic stress. Fig. 4 presents a conceptual diagram of the relationship between trehalose and cells during cryopreservation. The response to inhibition of ice crystal formation and osmotic stress is dependent on cryoprotective agents and cell types. In this study, the optimal trehalose concentration range used for cryopreservation of primary rat hepatocytes was around $2.6 \mu\text{M}$, and detailed

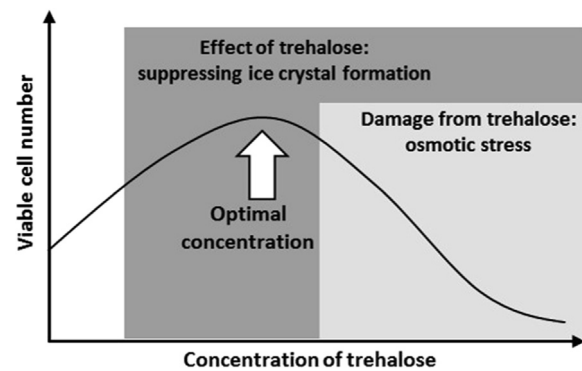


Fig. 4. Conceptual diagram of the relationship between trehalose concentration and viable cell number of hepatocyte after cryopreservation.

verification of the optimum concentration around $2.6 \mu\text{M}$ is necessary in the future. In the case of other cell types, it will be possible to optimize the trehalose concentration using the method described in this study in order and improve the viability of the target cells.

4.5. Intracellular dehydrogenase activity and liver-specific functions of hepatocytes after cryopreservation

We also evaluated the morphology, viability (intracellular dehydrogenase activity) and liver function of hepatocytes after cryopreservation, not only the hepatocytes viability just after thawing. Following hepatocytes cultivation/culture post cryopreservation showed the cytoprotective effect of trehalose significantly. The addition of trehalose improved cell adhesion and extension comparing to only DMSO condition (Fig. 3A). This results showed that trehalose protected cells from damage during freeze-thawing process, and could bring the state of cryopreserved hepatocytes approached to that of non-cryopreserved cells. Intracellular dehydrogenase activity significantly increased in cells cryopreserved using both trehalose and DMSO (Fig. 3B). Because the intracellular dehydrogenase activity of these cells was similar to that of non-cryopreserved cells on days 3 and 5 post-inoculation, cells that were damaged during cryopreservation were likely able to recover during the culture period.

Additionally, hepatocytes cryopreserved with trehalose showed higher albumin-secretion and ammonia metabolism ability than those cryopreserved without trehalose (Fig. 3C, F and G). Moreover, it has already been reported that hepatocytes cultured in the presence of DMSO were markedly increase EROD activity [34]. In our study, the EROD activity of hepatocytes cryopreserved with only DMSO was also higher than non-cryopreserved cells even in the absence of 3MC. The similar increase of the EROD activity of hepatocytes cryopreserved using DMSO was observed in the presence of 3MC. These results suggested that DMSO in cryopreserved solutions affected the markedly increase in EROD activity. On the other hand, there is a previous report that the EROD activity was decreased in the primary rat hepatocytes treated with DMSO-containing solution after cryopreservation [35], in which higher concentrations of 3MC and longer induction time of CYP1A1 than our experiments. Differences in experimental conditions may affect the amount of CYP1A1 induced by DMSO and 3MC and the degree of cell activity. Hence, we considered that the discrepancy of EROD activity between this result and previous studies was observed. However, in our experiments, the addition of trehalose suppressed the excessive increase in EROD activity due to DMSO, and the value approached to that of non-cryopreserved cells (Fig. 3E). Our results suggested that liver-specific functions were

improved after cryopreservation due to the formation of finer crystals, which in turn protected the cellular structure.

Numerous studies have evaluated hepatocyte cryopreservation; however, their findings indicated that hepatocyte functions following cryopreservation deteriorated relative to those of non-cryopreserved cells [36,37]. Interestingly, in the present study, the functions of non-cryopreserved cells and those cryopreserved with trehalose were comparable. These results were mostly a result of the inhibitory effects of trehalose on ice crystal formation, given that hepatocytes are highly sensitive to osmotic stress. Furthermore, it is also expected that trehalose stabilize the cell membrane by the approach from outside the cell to cell membrane and membrane-located proteins [38]. This study noted that trehalose protects cells and improved liver-specific function even if they did not exist in the cells.

5. Conclusion

In this study, we used trehalose as a CMIC and found very low concentration of trehalose had cytoprotective effect by evaluating its effects on osmotic stress and ice crystal formation. We noted that adding trehalose at 2.6 μ M dramatically improved viability and liver function of hepatocyte after cryopreservation. We believe that this result is significant importance to the field of cryopreservation. Furthermore, it is possible that polyol compounds other than trehalose (e.g., sucrose and maltose) and capable of interrupting the hydrogen-bonding network of water might also represent potential cell-cryoprotective agents at concentrations that prevent osmotic stress and allow fine ice crystal formation.

Declaration of interest

None.

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