



Microplate-Based Cryopreservation of Adherent-Cultured Human Cell Lines Using Amino Acids and Proteins

Morita, Kenta ; Yashiro, Tomoko ; Aoi, Takashi ; Imamura, Ryutaro ; Ohtake, Tomoyuki ; Yoshizaki, Norihiro ; Maruyama, Tatsuo

(Citation)

ACS Biomaterials Science & Engineering, 10(4):2442-2450

(Issue Date)

2024-04-08

(Resource Type)

journal article

(Version)

Accepted Manuscript

(Rights)

This document is the Accepted Manuscript version of a Published Work that appeared in final form in ACS Biomaterials Science & Engineering, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see <https://doi.org/10.1021/acsbmaterials.3c01834>

(URL)

<https://hdl.handle.net/20.500.14094/0100488553>



Microplate-based cryopreservation of adherent cultured human cell lines using amino acids and proteins

Kenta Morita^{†, #}, Tomoko Yashiro[†], Takashi Aoi[‡], Tatsuo Maruyama^{†, #}*

[†]Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe 657-8501, Japan

[#]Research Center for Membrane and Film Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

[‡]Graduate School of Medicine, Kobe University, 7-5-2 Kusunoki-cho, Chuou-ku, Kobe 650-0017, Japan

*Corresponding author

E-mail: tmarutem@crystal.kobe-u.ac.jp

Abstract

With the progression of regenerative medicine and cell therapy, the importance of cryopreservation techniques for cultured cells continues to rise. Traditional cryoprotectants, such as dimethyl sulfoxide and glycerol, are effective to cryopreserve suspended cells, but do not demonstrate sufficient efficacy for two-dimensional (2D)-cultured cells. In the past decade, small molecules and polymers have been studied as cryoprotectants. Some L-amino acids have been reported to be natural and biocompatible cryoprotectants. However, the cryoprotective effects of D-amino acids have not been investigated for such organized cells. In the present study, the cryoprotective effects of D- and L-amino acids, and previously reported cryoprotectants were assessed using HepG2 cells cultured on a microplate without suspending the cells. D-proline had the highest cryoprotective effect on 2D-cultured cells. The composition of the cell-freezing solution and freezing conditions were then optimized. The D-proline-containing cell-freezing solution also effectively worked for other cell lines. To minimize the amount of animal-derived components, fetal bovine serum in the cell freezing solution was substituted with bovine serum albumin and StemFit (a commercial supplement for stem cell induction). Further investigations on the mechanism of the cryopreservation suggested that D-proline protected enzymes essential for cell survival from freeze-induced damage. In conclusion, an effective and xeno-free cell-freezing solution was produced using D-proline combined with dimethyl sulfoxide and StemFit for 2D-cultured cells.

KEYWORDS

D-amino acids, adherent cells, cryopreservation, cryoprotective agents, xeno-free, tissue engineering

1. Introduction

Cryopreservation of cells is a vital technique in various fields, including regenerative medicine,^{1,2} supplying transfusion,^{3,4} cellular therapy,^{5,6} biobanking within both plants and animals.⁷⁻⁹ Cryopreservation allows long-term storage and transportation of cells, while maintaining their viability and functionality. Therefore, cryoprotectants to preserve cultured cells through freezing have been the subject of extensive research.

Traditionally, dimethyl sulfoxide (DMSO) and glycerol have been considered the gold standard for cell preservation.^{10,11} DMSO protects cells from the damage induced by osmotic pressure and ice crystal formation.¹² During the cell freezing process without DMSO, the concentration of solutes in the unfrozen extracellular solution increases. Cells are dehydrated and damaged by the hypertonic environment. Cells are also damaged by ice crystal formation. In the presence of DMSO in the cell freezing, DMSO penetrates a cell membrane and helps to balance the osmotic pressure during freezing. DMSO can disrupt an order of the ice lattice and reduce/delay the formation of ice crystals, leading to the improved viability of cryopreserved cells.^{13,14} Immortalized cell lines and cancer cell lines have been stored in 10% DMSO in a suspended state. Adherent cells must be detached from the substrate to prepare a cell suspension for freezing, followed by centrifugation, which is often a time-consuming and laborious process. This process is a major bottleneck to automate cryopreservation of cultured cells. In the past decade, cryopreservation of two-dimensional (2D)-cultured cells has emerged as a critical challenge in the field of regenerative medicine. DMSO has a limited cryoprotective effect on 2D-

cultured cells. Because cell sheets cultured in microplates can be applied to medical assay,^{15,16} the development of advanced cryoprotectants is desired for direct cryopreservation of 2D-cultured cells.

To develop effective cryoprotectants, several factors need to be considered. A good cryoprotectant should ensure high cell survival rates after thawing, exert minimal toxicity, and maintain cell functionality. Animal sera^{17–19} and protein^{20,21} have demonstrated high cryoprotective effects on cells. However, for medical applications, minimizing or eliminating animal-derived components in the cryoprotectant formulation is desirable to mitigate the risk of immunogenic and pathogenic contamination, which is referred to as “xeno free”. Amphiphilic small molecules and polymers¹⁶ as xeno-free cryoprotectants have shown promising results in improving cell viability and reducing cryoinjury, namely amino acids,^{22,23} block copolymers with 2-methacryloyloxyethyl phosphorylcholine (MPC) and di(ethylene glycol) methyl ether methacrylate (DEGMA),²⁴ poly(vinyl alcohol),²⁵ tertiary sulfonium,²⁶ and hydroxyethyl starch (HES).^{25,27} These compounds stabilize cellular structures and minimize osmotic stress, leading to improved post-thaw cell survival and functionality. Considering our background in the development of peptide-based biomaterials, we began to focus on amino acids.^{28,29} L-proline and trehalose are natural cryoprotectants that accumulate in organisms as a defense mechanism against freezing in low temperature.^{14,15,30} L-proline has been studied as a cryoprotectant for various biological samples, including red blood cells,³ spermatozoa,^{22,31,32} oocytes,³³ and reproductive cells.³⁴ Recently, the mechanisms by which L-proline aids in cellular cryopreservation have been postulated. L-proline acts as a biological compound that modulates cellular metabolism rather than acts as an ice nucleator.^{35,36} However, the potential of other L- and D-amino acids to be cryoprotectants remains unexplored. Amino acids are a cost-effective

option because they can be readily mass produced unlike compounds and polymers that require chemical synthesis. Considering their high biocompatibility, amino acids are promising xeno-free cryoprotectants for organized cells.

In this study, we investigated the cryoprotective potential of L- and D-amino acids, focusing on their application in direct cryopreservation of 2D-cultured cells on microplates. We evaluated cell recovery by measuring the total ATP content after thawing using various amino acid combinations as cryoprotectants. Moreover, we attempted to reduce or replace the use of fetal bovine serum (FBS), a commonly employed cryoprotectant, with xeno-free components to enhance the safety and compatibility of the cryopreservation process.

2. Materials and Methods

2.1. Materials

Dulbecco's phosphate buffered saline (PBS), a 2.5 g/L trypsin and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) solution, penicillin-streptomycin mixed solution, Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium with L-glutamine, DMSO, D-leucine, L-serine, L-alanine, L-methionine, L-cysteine, L-isoleucine, D-phenylalanine, glycine, L-tyrosine, D-tryptophan, and L-tryptophan were purchased from Nacalai Tesque (Kyoto, Japan). D-alanine and D-isoleucine were purchased from Watanabe Chemical Industries (Hiroshima, Japan). Albumin from bovine serum, fraction V pH 7.0, disodium hydrogenphosphate, sodium dihydrogenphosphate, D-glutamic acid, D-valine, L-valine, D-aspartic acid, L-lysine, L-phenylalanine, dextran 200,000, and L-lactate dehydrogenase (recombinant, rabbit muscle) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Triblock copolymers with 2-methacryloyloxyethyl phosphorylcholine (MPC) and di(ethylene glycol)

methyl ether methacrylate (DEGMA),²⁴ and tertiary sulfonium/methacrylate copolymer³⁷ were obtained from NOF Corp. (Tokyo, Japan). Poly(vinyl alcohol) (MW = 10,000) and hydroxyethyl starch (HES) were purchased from Sigma Aldrich (St Louis, MO). Other chemicals were purchased from Tokyo Chemical Industry (Tokyo, Japan). Water was purified using the Milli-Q Water Purification system (Merck Millipore, Burlington, MA).

2.2. Preparation of cell-freezing solutions

Cryoprotectants were mixed and dissolved in water to prepare a cell-freezing solution. Typically, 50% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4, 25 mM) were mixed to prepare the cell-freezing solution. Polymers with low water solubility were dissolved by vortex mixing and ultrasonication on ice. The conductivity of a cell-freezing solution was measured using a LAQUAtwin EC-33B (HORIBA, Tokyo, Japan).

2.3. Cell culture conditions

A431 human epidermoid carcinoma cells, HepG2 human hepatocyte carcinoma cells, and NCI-H1975-Luc human lung adenocarcinoma cells stably expressing luciferase were purchased from the JCRB Cell Bank (Ibaraki, Osaka, Japan). A431 and HepG2 cells were cultured in DMEM. NCI-H1975-Luc cells were cultured in RPMI 1640 medium. The media were supplemented with 10% FBS (Nichirei Biosciences, Tokyo, Japan) and 1% penicillin-streptomycin mixed solution. All cells were cultured at 37 °C in a humidified incubator with 5% CO₂.

2.4. Cryopreservation and recovery of suspended cells

Cells were seeded at 1×10^4 cells/100 μ L/well into a white 96-well plate (Thermo Fisher Scientific, Waltham, MA), followed by incubation for 3 days. After removing the medium, the cells were washed with PBS (-) and detached using a trypsin-EDTA solution. Immediately after,

100 μ L cell freezing solution was added to each of six wells, and cells were carefully suspended by pipetting. The plate was covered with a PP Sticking Plate Seal (WATSON, Tokyo, Japan), wrapped with aluminum foil, and stored flat on a shelf in a freezer at -80°C .

The plate was gently retrieved from the freezer, and the seal was checked for integrity. The plate was floated in a 37°C water bath for rapid thawing in <3 minutes. The cell suspension was transferred to a centrifuge tube and centrifuged ($300 \times g$, 5 min) to remove the supernatant and then resuspended in 100 μ L fresh medium. The cells were transferred to a 96-well plate and incubated at 37°C . Cell recovery was assessed by total ATP content using CellTiter-Glo 2.0 (Promega, Madison, WI) before cryopreservation and at 24 and 48 h of incubation after thawing.

2.5. Cryopreservation and recovery of 2D-cultured cells

Similar to suspended cells, cells were seeded into a 96-well plate and incubated for 3 days. The total ATP content of the cells was assayed prior to cryopreservation. The medium was exchanged with a cell-freezing solution, and the cells were frozen on the plate without detachment. After thawing in a water bath at 37°C , the supernatant was exchanged with fresh medium, followed by incubation at 37°C . Cell recovery was then assessed after 24 and 48 h.

2.6. Total ATP measurement of cells

The total ATP content of suspended and 2D-cultured cells was assessed using CellTiter-Glo 2.0. To each well of a white-walled 96-well plate containing cells in 100 μ L medium, an equal volume of CellTiter-Glo 2.0 was added, followed by mixing for 2 min. Luminescence intensity was measured with a multiwell plate reader (SH-9000Lab; Hitachi High-Tech, Tokyo, Japan) after incubation for 10 min. The luminescence intensity of sample well was subtracted by the averaged intensity of three control wells (contains medium and detection reagent only). Cell

recovery was calculated as the ratio of the total ATP content to the averaged value of it measured prior to cell cryopreservation.

2.7. Effect of cryopreservation on the hepatic function of HepG2 cells

HepG2 cells were seeded at 1×10^4 cells/100 μ L/well into a white 96-well plate. After incubation for 1 day, the cell culturing medium was replaced with the medium containing 25 μ M rifampicin, followed by the additional incubation for 2 days. The total ATP content and CYP3A4 activity were assayed prior to cryopreservation using CellTiter-Glo 2.0 and P450-Glo™ CYP3A4 Assay (Luciferin-IPA), respectively. The culturing medium was replaced with 50 μ L medium containing 6 μ M luciferin-IPA, followed by incubation for 30 min. To measure the CYP3A4 activity, 25 μ L of the medium was transferred to another 96-well plate and mixed with 25 μ L detection reagent. Luminescence intensity was measured using a multiwell plate reader after incubation for 10 min. The cell recovery was also determined by measuring the total ATP content of the rest 25 μ L medium containing cells after adding 25 μ L CellTiter-Glo 2.0.

The cell culturing medium was exchanged with a cell-freezing solution consisted of 40% FBS, 10% DMSO, 10% DMEM, 30% phosphate buffer, and 2% (w/v) D-proline. The cells were frozen at -80 °C for 3 days. After thawing in a water bath at 37 °C, the supernatant was exchanged with fresh medium, followed by incubation at 37 °C for 1 day. The cells were incubated for another 2 days in fresh medium containing 25 μ M rifampicin. Cell recovery and CYP3A4 activity was then assessed again.

2.8. Cryopreservation of L-lactate dehydrogenase (LDH) in solution

LDH was dissolved to a final concentration of 0.05 U/mL. The LDH solution was transferred into a 1.5 mL tube and cryopreserved in a -80 °C freezer for 3 days. Upon removal from the freezer, the LDH solution was immediately thawed in a 37 °C water bath. Enzymatic activity was

then measured using a Cytotoxicity LDH Assay Kit-WST (Dojindo, Kumamoto, Japan). The activity of LDH cryopreserved in the control solution without BSA or proline was considered to be 100%, from which the relative activity of LDH cryopreserved in the sample solution was calculated.

2.9. Cooling rate measurement for cryopreservation of 2D-cultured cells

For rapid freezing, a plate was wrapped with aluminum foil and placed on a shelf in a freezer at -80°C . For slow freezing, a plate was wrapped with a paper towel, placed in a polystyrene foam box, and then stored in the freezer. A temperature logger (KT-155F/EX, Fujita Electric Works, Nakagun, Kanagawa, Japan) was affixed to the plate to measure the cooling rate.

2.10. Statistical analysis

Data represented as mean $\pm$ standard deviation (SD) from at least three wells in a 96-well plate. Student's two-tailed t -test was performed using Microsoft Excel 2016 to assess statistical significance. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

3. Results and Discussion

3.1. Cryopreservation of suspended and 2D-cultured cells

HepG2, A431, and NCI-H1975-Luc cells were employed for cryopreservation tests in suspended and 2D-cultured states. Medium containing 10% DMSO was used as a conventional cell-freezing solution. CELLBANKER 1 plus (Zenogen Pharma, Fukushima, Japan) was also used as an established commercial cell-freezing solution. After the exchange of medium with the cell freezing solution, cells in 96-well plate were frozen in a deep freezer at -80°C . The procedures were intended to be simple and ordinary, assuming an automated cell culture system. The recovery of post-cryopreserved cells was assessed using total ATP content measured by CellTiter-Glo 2.0, which were able to avoid false-positive results (data not shown). Cell recovery

was calculated by comparing the total ATP contents before and after cryopreservation. The total ATP content before freezing was set as 100%. Cells in a suspended state recovered from cryopreservation to a high degree in 10% DMSO-containing medium and CELLBANKER 1 plus. Using medium containing 10% DMSO for suspended cells, the viabilities of HepG2, A431, and NCI-H1975-Luc cells were 89%, 73%, and 60%, respectively, at 48 h after thawing (Fig. 1). These viabilities were higher than those obtained at 24 h after thawing. When CELLBANKER 1 plus was used, the viabilities of the suspended cells were 78%, 69%, and 70%, respectively, at 48 h after thawing. The viabilities at 48 h were similar to those obtained with the 10% DMSO solution. Only the recovery of NCI-H1975-Luc cells was decreased significantly from 24 to 48 h after thawing.

Conversely, adhered cells (2D-cultured cells) did not recover to a high degree. The viabilities of 2D-cultured HepG2, A431, and NCI-H1975-Luc cells in the 10% DMSO solution were 12%, 0%, and 14%, respectively. When CELLBANKER 1 plus was used, HepG2 and NCI-H1975-Luc cells showed viabilities of 60% and 56%, respectively, after 48 h, but A431 cells showed very low recovery of 5% after 48 h. Using CELLBANKER 1 plus, the recovery of 2D-cultured cells at 48 h after thawing was lower than that of suspended cells. Therefore, we attempted to establish a universal cryoprotectant combination for 2D-cultured cells in the following experiments.

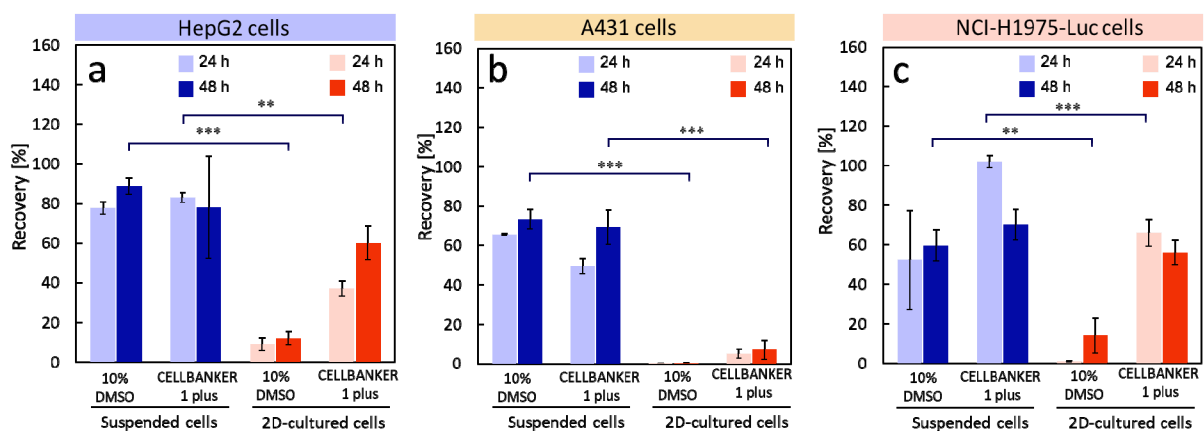


Figure 1. Cryoprotective effects of DMSO and CELLBANKER 1 plus on (a) HepG2 cells, (b) A431 cells, and (c) NCI-H1975-Luc cells. Cell recovery at 24 and 48 h after thawing was determined by total ATP content. 10% DMSO represents DMEM containing 10% DMSO. The data values represent the mean $\pm$ SD of at least three wells in a 96-well plate. ** $p < 0.01$, *** $p < 0.005$.

3.2. Effects of amino acids and polymers on 2D-cultured cell cryopreservation

As mentioned above, DMSO has been the gold standard as a cryoprotectant. FBS has also been studied for its cryoprotective properties, as it contains the abundance of components beneficial for cell growth.^{41–43} Therefore, 2D-cultured cells were first cryopreserved in the cell-freezing solution consisting of 50% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4). We then examined the effect of the electrical conductivity of the cell-freezing solution on HepG2 cell recovery. Two-dimensional cultured HepG2 cells showed the highest recovery when frozen and thawed in a cell-freezing solution with 7.2 mS/cm, which corresponded to 7.5 mM phosphate (Fig. S1). Therefore, 50% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4, 25 mM) were employed as the basic cell-freezing solution in the following experiments.

Several D- and L-amino acids (1% (w/v)) were added to the basic cell-freezing solution to evaluate them as cryoprotectants for 2D-cultured HepG2 cells, except for “No amino acid” group as a control. Both enantiomers of proline, glutamic acid, valine, and arginine, and L-threonine exhibited significantly enhanced cryoprotective effects on 2D-cultured cells compared with the controls without amino acids (Fig. 2a). For proline, threonine, aspartic acid, cysteine, and tryptophan, significant differences in cryoprotective effects were observed between their enantiomers. These differences were potentially attributed to physiological influences exerted on the cells, considering that D-amino acids have been reported to be physiologically active.³⁸

Among the amino acids tested, D-proline had the highest efficacy for cryoprotection. The recovery of cells cryopreserved in the freezing solution containing D-proline at 48 h after thawing was 87%, which was higher than that of cells cryopreserved in CELLBANKER 1 plus. The cryoprotective effect of L-proline has been reported in previous studies.^{3,22,31–34} The present results showed that D-proline had a superior effect on cryopreservation of 2D-cultured cells.

A low-volume cryopreservation in a 96-well plate might possibly vary in its outcome due to supercooling.³⁹ To validate the reproducibility of the data, additional experiments conducted three times using different microplates that contained 6 wells per sample. D-proline consistently showed a significant cryoprotective effect compared to L-proline (Fig. S2).

Polymer- and saccharide-type cryoprotectants were also tested. Although all the substances have been reported as cryoprotectants, only poly(DEGMA/MPC) marked a higher cryoprotective effect than CELLBANKER 1 plus did (Fig. 2b). However, the difference between poly(DEGMA/MPC) and CELLBANKER 1 plus was not significant. These results indicated that D-proline was the most promising cryoprotectant for adhered cells among the additives tested.

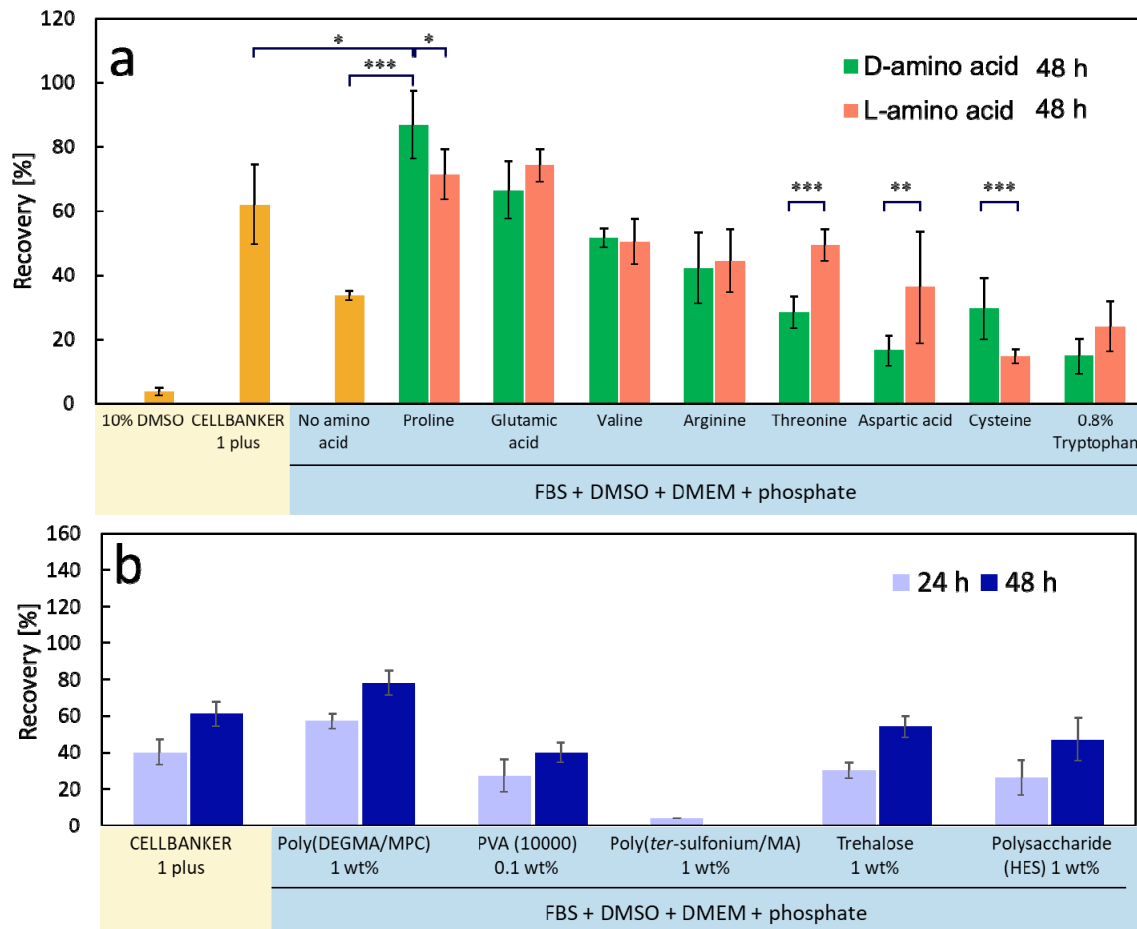


Figure 2. (a) Cryoprotective effects of D- and L-amino acids on 2D-cultured HepG2 cells. Cell recovery at 48 h after thawing was calculated from total ATP content. (b) Cryoprotective effects of polymers and saccharides on 2D-cultured cells. Cell-freezing solutions consisted of 1% (w/v) cryoprotectants (unless stated otherwise), 50% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4, 25 mM). 10% DMSO represents DMEM containing 10% DMSO. The data values represent the mean $\pm$ SD of at least three wells in a 96-well plate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3.3. Effects of D- and L-proline on 2D-cultured cell cryopreservation

The cryoprotective effects of various concentrations of D- and L-proline were examined using 2D-cultured HepG2 cells. Cell recovery increased with the proline concentration (Fig. 3). When D-proline was used, the cells obviously grew between 24 and 48 h after thawing, indicating healthy growth of the cells after thawing.

Cell-freezing solutions containing D- and L-proline were tested in four kinds of cell lines (Fig. 4). D-proline was effective to cryopreserve all cell lines tested except A431 cells compared with L-proline. In particular, D-proline showed a remarkable cryoprotective effect on MCF-7 cells, which exceeded the cryoprotective effect of CELL BANKER 1 plus (Fig. 4a and 4d). D-proline was moderately effective for NCI-H1975-Luc and HEK293 cells (Fig. 4c and 4e), and not at all for A431 cells (Fig. 4b). Microscopy observation revealed that A431 and NCI-H1975-Luc cells had formed tight intercellular adhesions prior to cryopreservation (Fig. S3a and S3b). Conversely, HepG2 cells did not fully establish contact with each other and exhibited isolated growth (Fig. S3c). Ice crystal formation propagates through the cell–cell adhesion interface in an organized cell system, which might lead to freeze-induced damage. Strong intercellular adhesion would account for the weak effect of cryoprotectants on the recovery of these cells.⁴⁰

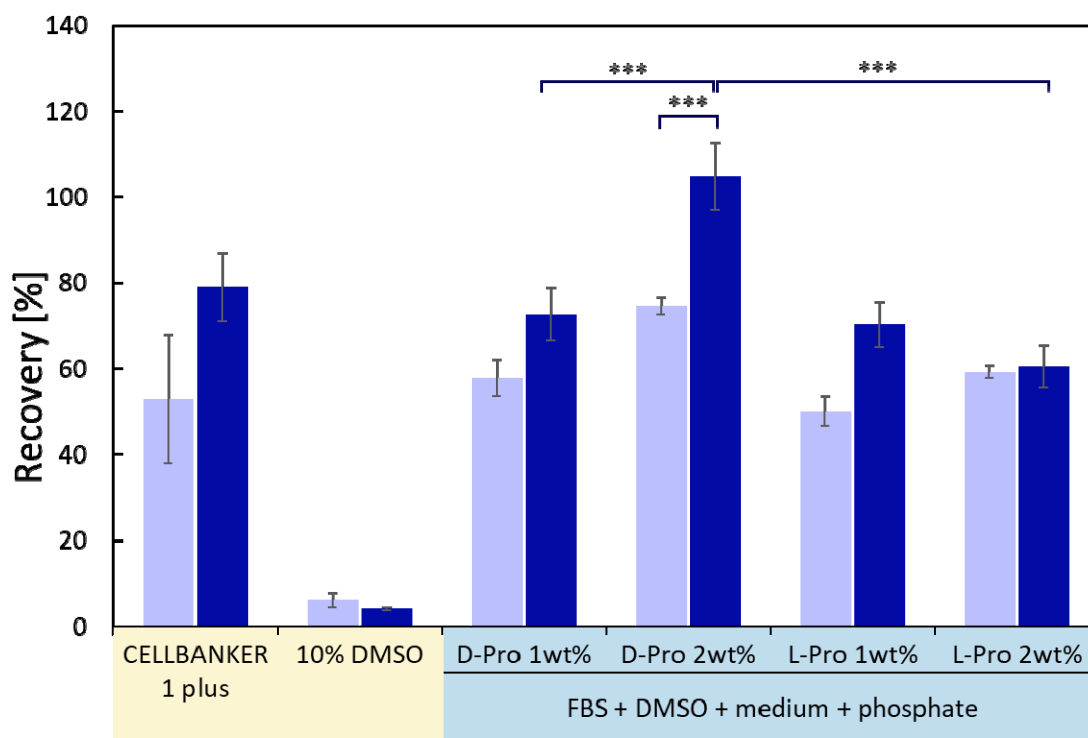


Figure 3. Cryoprotective effects of D- and L-proline on 2D-cultured HepG2 cells. Cell recovery at 24 and 48 h after thawing was calculated from total ATP content. Cell-freezing solutions consisted of 50% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4, 25 mM). The 10% DMSO represents DMEM containing 10% DMSO. The data values represent the mean $\pm$ SD of at least three wells in a 96-well plate. *** $p < 0.005$.

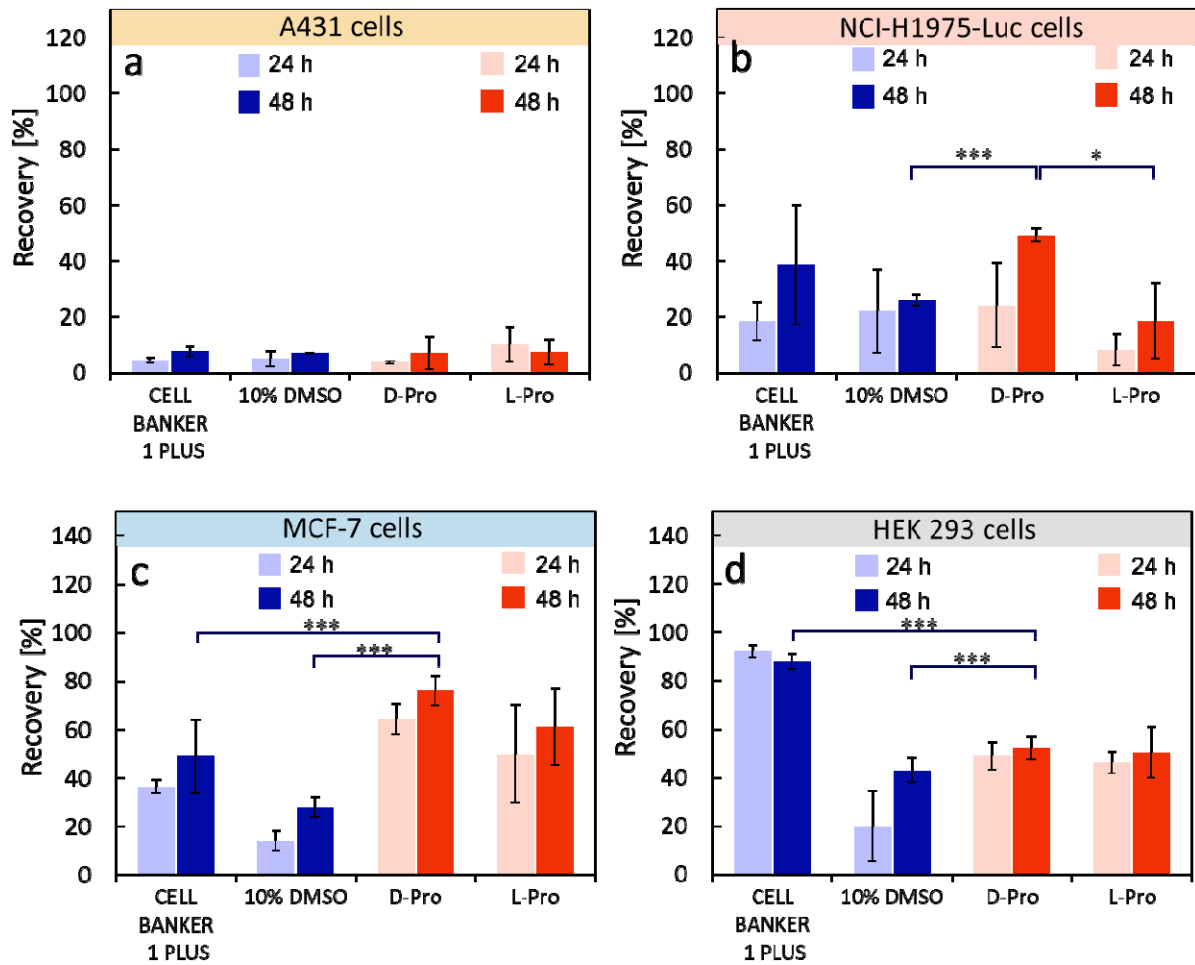


Figure 4. Cryoprotective effects of D- and L-proline on 2D-cultured cell lines. (a) A431 cells, (b) NCI-H1975-Luc cells, (c) MCF-7 cells, and (d) HEK293 cells. Cell recovery at 24 and 48 h after thawing was calculated from total ATP content. Cell-freezing solutions consisted of 2% (w/v) amino acids, 50% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4, 25 mM). The 10% DMSO represents DMEM containing 10% DMSO. The data values represent the mean $\pm$ SD of at least three wells in a 96-well plate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3.4. Cryopreservation conditions

The effect of the cooling rate on 2D-cultured cell cryopreservation were investigated. Two-dimensional cultured HepG2 cells were cryopreserved in cell-freezing solutions consisting of D-proline, 50% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4, 25 mM). While the cooling rates were tested at -2.2 and -0.75 K/min (Fig. S4), the difference in cooling rate did not affect cell recovery in any cryoprotective solution (Fig. S5). These results were unexpected because the rate at which ice crystals form is considered to affect cells.⁴¹

We next examined the effect of the cryopreservation period on cell recovery. The cell-freezing solution consisting of 90% DMEM and 10% DMSO resulted in no viable 2D-cultured HepG2 cells after cryopreservation for 2 months (Fig. 5). Cryopreservation with CELLBANKER 1 plus and the cell-freezing solution containing D-proline resulted in cell viabilities of 58% and 72%, respectively, at 24 h after cryopreservation for 2 months. Cell recovery at 24 h after thawing did not change whether the cells were cryopreserved for 1 day, 1 month, or 2 months. However, cell recovery after 48 hours decreased as the duration of cryopreservation increased, indicating slight cell damage induced by prolonged cryopreservation.

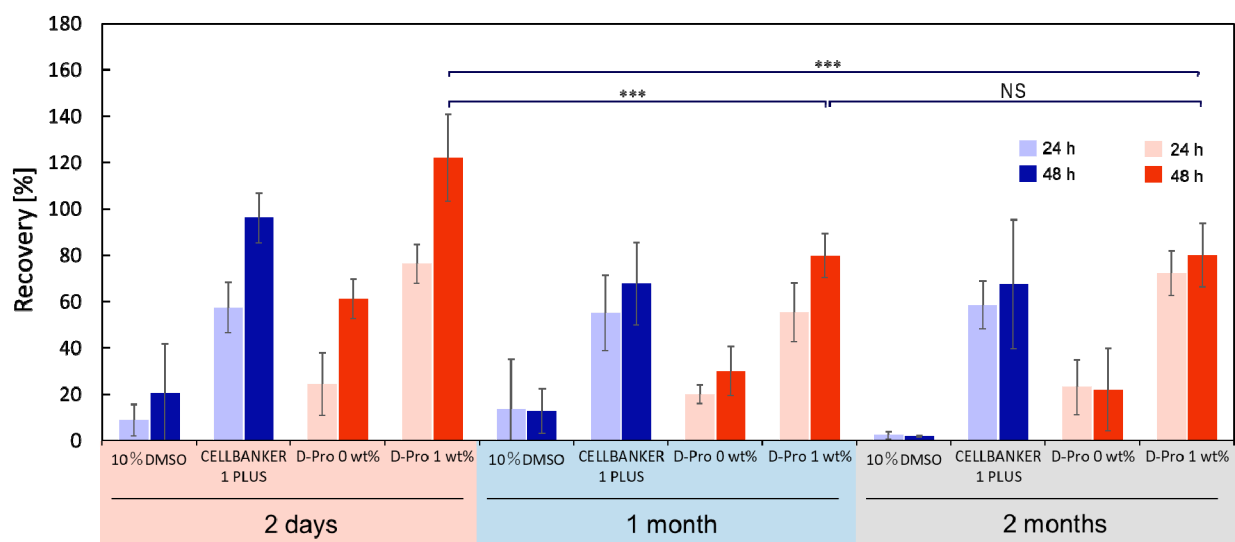


Figure 5. Long-term cryopreservation of 2D-cultured HepG2 cells. Cell recovery at 24 and 48 h after thawing was calculated from total ATP content. Cell-freezing solutions consisted of 50% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4, 25 mM). The 10% DMSO represents DMEM containing 10% DMSO. The data values represent the mean $\pm$ SD of at least three wells in a 96-well plate. *** $p < 0.005$, NS: no significance.

3.5. Reduction and replacement of FBS

FBS contains abundant growth factors and proteins. It is commonly employed for cell cryopreservation solution.^{42–44} However, animal-derived components are unfavorable for medical use. Animal-derived components include a risk of immunogenic contamination. Hence, we attempted to reduce or replace FBS in the cell-freezing solution containing D-proline.

We reduced the FBS concentration in the cell-freezing solution and evaluated the cryopreservation effect. The cell freezing solution consisted of 2% (w/v) D-proline, X% FBS, 10% DMSO, 10% DMEM, and 80–X% phosphate buffer (pH 7.4, 7.5 mM, final). Cell recovery was remarkably low at FBS concentrations less than 20% (Fig. S6). This result indicated that FBS was necessary to cryopreserve 2D-cultured cells even when D-proline was supplemented. We hypothesized that the abundant proteins in FBS played an important role in cryopreservation of 2D-cultured cells. Next, FBS in the cell-freezing solution was replaced with BSA. When the cell-freezing solution contained 5% FBS and 2% (w/v) BSA, cell recovery at 48 h after thawing the cells was 103%, which was superior to that when 40% FBS was used (Fig. 6a). Even in the absence of FBS, cell recovery was 88% with 3% (w/v) BSA in the cell-freezing solution. These results suggested that FBS could be replaced with BSA in the presence of D-proline.

BSA is an animal-derived material. Thus, we employed StemFit for Differentiation (Ajinomoto Healthy Supply, Tokyo, Japan) (StemFit). StemFit is a chemically defined and animal origin-free supplement for ES and iPS cells (the composition is not disclosed). FBS in the cell-freezing solution was replaced with StemFit in a stepwise manner, and the cryopreservation effect was examined using 2D-cultured HepG2 cells. Cell recovery was only slightly reduced by replacing FBS with StemFit in the cell-freezing solution (Fig. 6b). Consequently, StemFit resulted in a xeno-free 2D-cultured cell-freezing solution without any animal-derived proteins. The xeno-free composition of the cell-freezing solution was 2% (w/v) D-proline, 40% StemFit, 10% DMSO, 10% DMEM, and 40% phosphate buffer (pH 7.4, 18.75 mM).

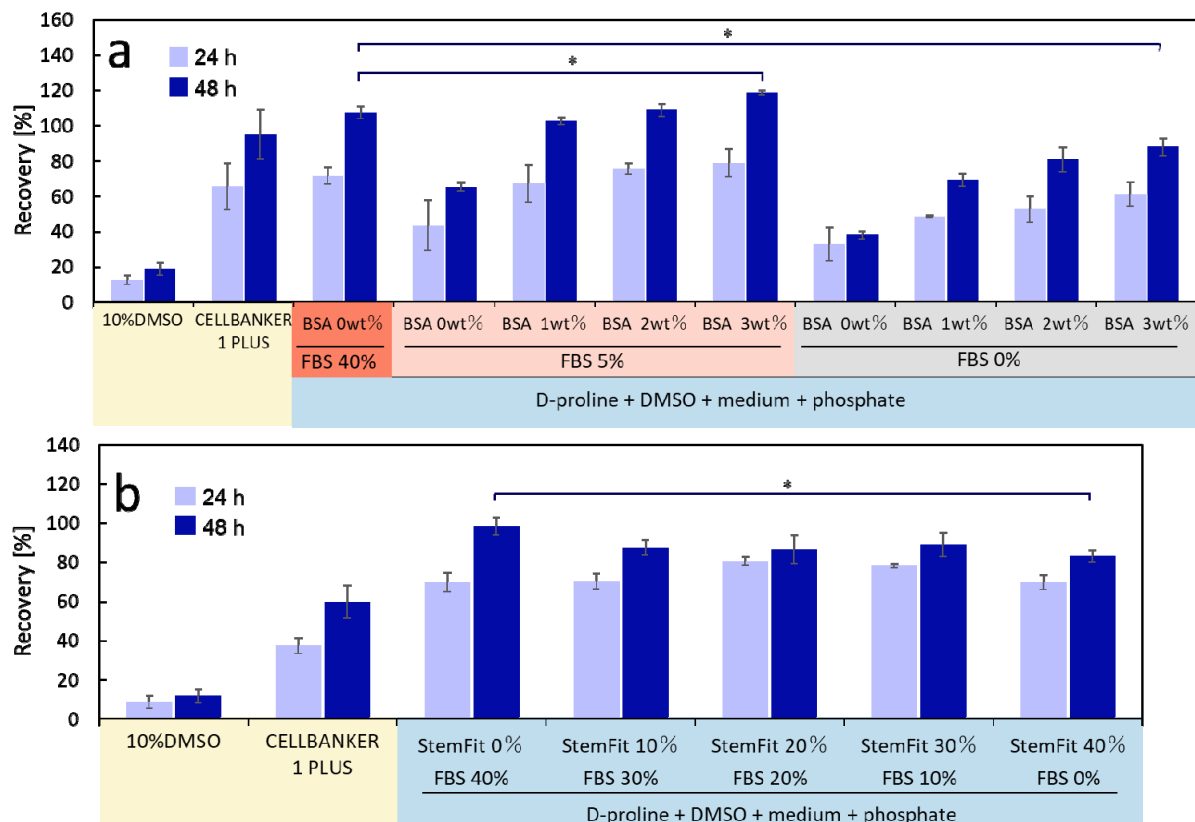


Figure 6. Cryoprotective effects of (a) BSA and (b) StemFit on 2D-cultured HepG2 cells in the presence of D-proline. FBS was replaced with BSA and StemFit. Cell recovery at 24 and 48 h after thawing was calculated from total ATP content. The composition of the cell-freezing solution containing BSA was 2% (w/v) D-proline, 10% DMSO, 10% DMEM, X% FBS, and 80–X% phosphate buffer (pH 7.4, 7.5 mM, final), and that of the cell-freezing solution containing StemFit was 2% (w/v) D-proline, 10% DMSO, 10% DMEM, X% FBS, 40–X% StemFit, and 40% phosphate buffer (pH 7.4, 18.75 mM). The 10% DMSO represents DMEM containing 10 vol% DMSO. The data values represent the mean $\pm$ SD of at least three wells in a 96-well plate. $^*p < 0.05$.

3.6. Role of D-proline as a cryoprotectant

Cells recovered from cryopreservation needs to express their original traits. CYP3A4 is a typical detoxifying enzyme produced by hepatocytes under an induction by rifampicin. We then tested the CYP3A4 activity of HepG2 induced by rifampicin after 2D-cultured cell cryopreservation, comparing to that before cryopreservation. When a cell-freezing solution containing 40% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4, 25 mM), with 2% (w/v) D-proline, was used, the cells' relative CYP3A4 activity was 53% (Fig. 7a), with cell recovery at 89% (Fig. 7b). In contrast, cryopreservation with CELLBANKER 1 PLUS resulted in a relative CYP3A4 activity of only 14.3%, while cell recovery remained at 83%. While there was no significant difference in cell recovery between the D-proline-containing cell-freezing solution and CELLBANKER 1 PLUS, there was a notable difference in CYP3A4 activity, suggesting that the cryopreservation with CELLBANKER 1 PLUS might temporarily impair the hepatic function of HepG2 cells. The cryopreservation with a medium containing 10% DMSO without D-proline and with a cell-freezing solution gave very low CYP3A4 activity due

to the reduced number of living cells recovered from cryopreservation. D-proline proved effective in maintaining cellular function as well as in cryopreserving cells.

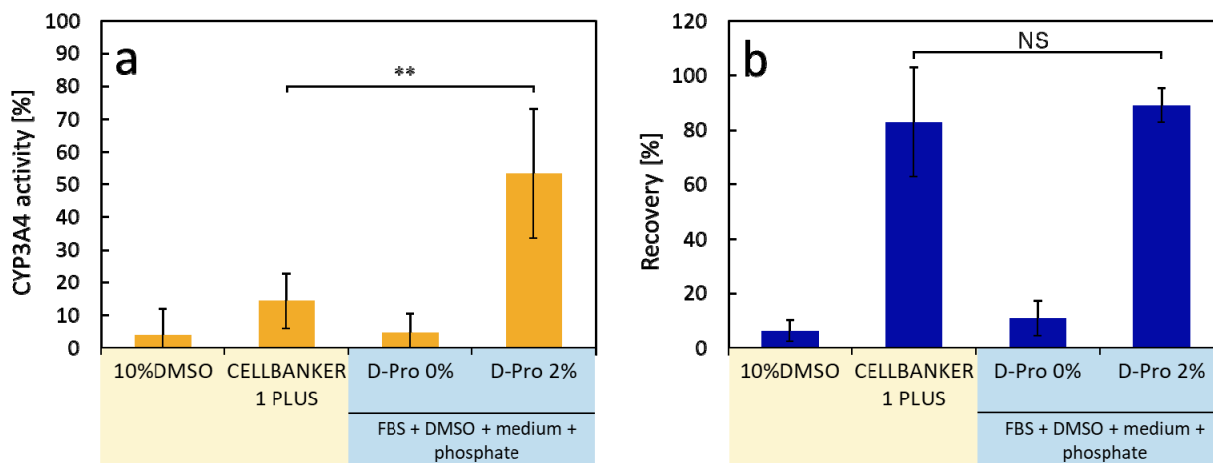


Figure 7. Effect of 2D-cultured cell cryopreservation on the hepatic function of HepG2 cells. (a) CYP3A4 activity and (b) cell recovery at 72 h after thawing the cryopreserved cells was determined by total ATP content. The CYP3A4 activation was induced by 25 μ M rifampicin for 48 h. Cell-freezing solutions (D-Pro 0% and D-pro 2%) consisted of 40% FBS, 10% DMSO, 10% DMEM, and 30% phosphate buffer (pH 7.4, 25 mM). The 10% DMSO represents DMEM containing 10% DMSO. The data values represent the mean $\pm$ SD of at least three wells in a 96-well plate. ** p < 0.01, NS: no significance.

L-proline is a natural cryoprotectant that accumulates in organisms.³⁰ Recently, the mechanisms by which L-proline protects cells from cryoinjury have been postulated. L-proline acts as a signaling compound that modulates cellular metabolism rather than acts as an ice nucleator.^{35,36} Thus, we investigated the possible biochemical role of D-proline as a cryoprotectant. L-Lactate dehydrogenase (LDH) was dissolved and frozen in a cell-freezing solution consisting of 10% DMSO, 10% DMEM, and 80% phosphate buffer (pH 7.4, 9.38 mM)

containing BSA, D-proline, or L-proline as cryoprotectants. Enzymatic activity in each cryopreservation solution was assayed before and after freezing, and the activity was expressed as a ratio to that before freezing without cryoprotectants. Without cryoprotectants, the enzymatic activity of LDH was reduced to 13% after cryopreservation. However, when 6% (w/v) BSA, 4% (w/v) D-proline, and 4% (w/v) L-proline were used, the relative enzymatic activities after cryopreservation were 106%, 73%, and 127%, respectively (Fig. 8). We already suggested that these three substances effectively acted as cryoprotectants for 2D-cultured cells (Figs. 2a and 6a). The cellular cryoprotective effect of BSA and D- and L-proline might be derived from protecting intracellular enzymes from cryoinjury. D-proline demonstrated a superior cryoprotective effect on 2D-cultured cells, and L-proline showed a stronger cryoprotective effect on LDH. This discrepancy suggested that D-proline influenced not only LDH but also other enzymes involved in cell recovery. While previous studies proved the biochemical effect of L-proline on cellular metabolism,^{35,36} our results suggested the new biophysical effects of D- and L-proline on intracellular proteins, both of which led to cryoprotection of the cells. The enzyme stabilization by D-proline might attribute to maintaining HepG2 function during the cryopreservation.

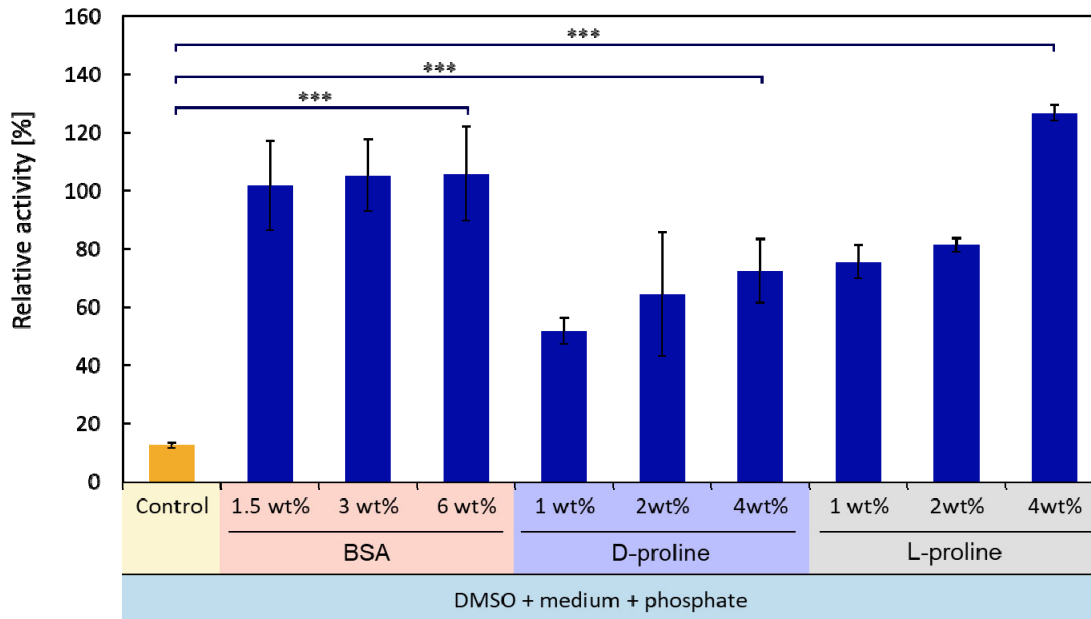


Figure 8. Cryoprotective effects of BSA, and D- and L-proline on lactate dehydrogenase (LDH). Enzyme solutions were frozen at -80°C for 2 days. Cell-freezing solutions consisted of 10% DMSO, 10% DMEM, 80% phosphate buffer (pH 7.4, 9.38 mM), and a cryoprotectant. The data values represent the mean $\pm$ SD of at least three wells in a 96-well plate. *** $p < 0.005$.

4. Conclusions

Microplate-based cryopreservation of human cells would greatly simplify the maintenance of organized cells used in regenerative medicine and cell therapies. However, the existing cryoprotectants for suspended cells have not been effective for 2D-cultured cells. In this study, we demonstrated the high efficacy of D-proline as a cryoprotectant for 2D-cultured HepG2 cells. Among eight types of D- and L- amino acids and five types of polymers, D-proline showed the highest cryoprotective effect on cells. The efficacy of the cell-freezing solution containing D-proline even surpassed that of a commercially available cell-freezing solution. D-proline was also effective to cryopreserve other 2D-cultured cell lines without tight intracellular adhesion.

The cryoprotective effect was maintained when FBS was replaced with BSA or StemFit to minimize or eliminate animal-derived components. Upon investigating the mechanism of the cryoprotective effect, we found that D-proline stabilized an enzyme vital for cell survival. The protection of enzymes from cryoinjury would allow HepG2 cells to maintain their original function as hepatocytes even after cryopreservation. The present study demonstrates the strong potential of D-proline for use in the cryopreservation of 2D-cultured cells as a xeno-free cryoprotectant.

Supporting Information

Supporting Information is available free of charge on the ACS Publications website.

- Supplemental results include the effects of electrical conductivity on cryopreservation (Fig. S1), confirmation of reproducibility in cryopreservation of 2D-cultured cells (Fig. S2), microscopy images of 2D-cultured cells (Fig. S3), temperature profiles of fast and slow freezing of 2D-cultured cells (Fig. S4), effects of the cooling rate on cryopreservation of 2D-cultured HepG2 cells (Fig. S5), long-term cryopreservation of 2D-cultured HepG2 cells (Fig. S6), and cryoprotective effects of FBS on 2D-cultured HepG2 cells in the presence of D-proline (Fig. S7).

AUTHOR INFORMATION

Corresponding Author

* tmarutcm@crystal.kobe-u.ac.jp

ORCID

Tatsuo Maruyama: 0000-0003-2428-1911

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This research was supported partially by JSPS KAKENHI (Grant No. 19H05458 and 23H01774 for T.M., 21K14471 and 23K13610 for K.M.), Eneos Tonengeneral Research/Development Encouragement & Scholarship Foundation, Nakatani Foundation (2022S230), and Toyota Physical and Chemical Research Institute 2023.

ACKNOWLEDGMENTS

NCI-H1975-Luc cells were kindly distributed from T. Murakami. We thank Mitchell Arico from Edanz (<https://jp.edanz.com/ac>) for editing a draft of this manuscript.

NOTES

The authors declare no competing financial interest.

REFERENCES

- (1) Elliott, G. D.; Wang, S.; Fuller, B. J. Cryoprotectants: A Review of the Actions and Applications of Cryoprotective Solutes That Modulate Cell Recovery from Ultra-Low Temperatures. *Cryobiology*. Elsevier Ltd June 2017, pp 74–91.
<https://doi.org/10.1016/j.cryobiol.2017.04.004>.

- (2) Ma, X.; Liu, Y.; Sun, B.; Fan, W.; Cui, Z. Cryopreservation: Organ Preservation. In *Comprehensive Biotechnology*; Elsevier, 2019; pp 689–708. <https://doi.org/10.1016/B978-0-444-64046-8.00277-9>.
- (3) Dou, M.; Lu, C.; Sun, Z.; Rao, W. Natural Cryoprotectants Combinations of L-Proline and Trehalose for Red Blood Cells Cryopreservation. *Cryobiology* **2019**, *91* (1), 23–29. <https://doi.org/10.1016/j.cryobiol.2019.11.002>.
- (4) Lei, Q.; Sun, Y.; Huang, J.; Liu, W.; Zhan, X.; Yin, W.; Guo, S.; Sinelshchikova, A.; Brinker, C. J.; He, Z.; Guo, J.; Wuttke, S.; Zhu, W. Dimensional Reduction of Metal–Organic Frameworks for Enhanced Cryopreservation of Red Blood Cells. *Angew. Chemie Int. Ed.* **2023**, *62* (22). <https://doi.org/10.1002/anie.202217374>.
- (5) Hunt, C. J. Technical Considerations in the Freezing, Low-Temperature Storage and Thawing of Stem Cells for Cellular Therapies. *Transfus. Med. Hemotherapy* **2019**, *46* (3), 134–150. <https://doi.org/10.1159/000497289>.
- (6) Vasovic, L. V.; Reich-Slotky, R.; Goel, R. Hematopoietic Stem Cell Collections and Cellular Therapies. In *Clinical Principles of Transfusion Medicine*; Elsevier, 2018; pp 151–167. <https://doi.org/10.1016/B978-0-323-54458-0.00013-1>.
- (7) Daly, J.; Zuchowicz, N.; Nuñez Lendo, C. I.; Khosla, K.; Lager, C.; Henley, E. M.; Bischof, J.; Kleinhans, F. W.; Lin, C.; Peters, E. C.; Hagedorn, M. Successful Cryopreservation of Coral Larvae Using Vitrification and Laser Warming. *Sci. Rep.* **2018**, *8* (1), 15714. <https://doi.org/10.1038/s41598-018-34035-0>.

- (8) Day, J. G.; Stacey, G. N. Biobanking. *Mol. Biotechnol.* **2008**, *40* (2), 202–213.
<https://doi.org/10.1007/s12033-008-9099-7>.
- (9) Engelmann, F. Plant Cryopreservation: Progress and Prospects. *Vitr. Cell. Dev. Biol. - Plant* **2004**, *40* (5), 427–433. <https://doi.org/10.1079/IVP2004541>.
- (10) LOVELOCK, J. E.; BISHOP, M. W. H. Prevention of Freezing Damage to Living Cells by Dimethyl Sulphoxide. *Nature* **1959**, *183* (4672), 1394–1395.
<https://doi.org/10.1038/1831394a0>.
- (11) C. Polge; A. U. Smith; A. S. Parkes. Revival of Spermatozoa after Vitriification and Dehydration at Low Temperatures. *Nature*. 1949, p 666.
- (12) Rall, W. F.; Mazur, P.; McGrath, J. J. Depression of the Ice-Nucleation Temperature of Rapidly Cooled Mouse Embryos by Glycerol and Dimethyl Sulfoxide. *Biophys. J.* **1983**, *41* (1), 1–12. [https://doi.org/10.1016/S0006-3495\(83\)84399-9](https://doi.org/10.1016/S0006-3495(83)84399-9).
- (13) Dong, J.; Malsam, J.; Bischof, J. C.; Hubel, A.; Aksan, A. Spatial Distribution of the State of Water in Frozen Mammalian Cells. *Biophys. J.* **2010**, *99* (8), 2453–2459.
<https://doi.org/10.1016/j.bpj.2010.08.035>.
- (14) Kratochvílová, I.; Golan, M.; Pomeisl, K.; Richter, J.; Sedláková, S.; Šebera, J.; Mičová, J.; Falk, M.; Falková, I.; Řeha, D.; Elliott, K. W.; Varga, K.; Follett, S. E.; Šimek, D. Theoretical and Experimental Study of the Antifreeze Protein AFP752, Trehalose and Dimethyl Sulfoxide Cryoprotection Mechanism: Correlation with Cryopreserved Cell Viability. *RSC Adv.* **2017**, *7* (1), 352–360. <https://doi.org/10.1039/C6RA25095E>.

- (15) Stokich, B.; Osgood, Q.; Grimm, D.; Moorthy, S.; Chakraborty, N.; Menze, M. A. Cryopreservation of Hepatocyte (HepG2) Cell Monolayers: Impact of Trehalose. *Cryobiology* **2014**, *69* (2), 281–290. <https://doi.org/10.1016/j.cryobiol.2014.08.001>.
- (16) Tomás, R. M. F.; Bissoyi, A.; Congdon, T. R.; Gibson, M. I. Assay-Ready Cryopreserved Cell Monolayers Enabled by Macromolecular Cryoprotectants. *Biomacromolecules* **2022**, *23* (9), 3948–3959. <https://doi.org/10.1021/acs.biomac.2c00791>.
- (17) Ha, S. Y.; Jee, B. C.; Suh, C. S.; Kim, H. S.; Oh, S. K.; Kim, S. H.; Moon, S. Y. Cryopreservation of Human Embryonic Stem Cells without the Use of a Programmable Freezer. *Hum. Reprod.* **2005**, *20* (7), 1779–1785. <https://doi.org/10.1093/humrep/deh854>.
- (18) Son, J. H.; Kim, K.-H.; Nam, Y.-K.; Park, J.-K.; Kim, S.-K. Optimization of Cryoprotectants for Cryopreservation of Rat Hepatocyte. *Biotechnol. Lett.* **2004**, *26* (10), 829–833. <https://doi.org/10.1023/B:BILE.0000025886.57547.04>.
- (19) Zalomova, L. V.; Reshetnikov, D. A.; Ugraitskaya, S. V.; Mezhevikina, L. M.; Zagainova, A. V.; Makarov, V. V.; Yudin, S. M.; Fesenko, E. E. Fetal Serum in Combination with 5% Dimethyl Sulfoxide Efficiently Protects the Human Gut Microbiota during Cryopreservation in Liquid Nitrogen. *Biophysics (Oxf)*. **2021**, *66* (4), 657–664. <https://doi.org/10.1134/S0006350921040230>.
- (20) Cao, T.-T.; Zhang, Y.-Q. The Potential of Silk Sericin Protein as a Serum Substitute or an Additive in Cell Culture and Cryopreservation. *Amino Acids* **2017**, *49* (6), 1029–1039. <https://doi.org/10.1007/s00726-017-2396-3>.

- (21) Carpenter, J. F.; Hansen, T. N. Antifreeze Protein Modulates Cell Survival during Cryopreservation: Mediation through Influence on Ice Crystal Growth. *Proc. Natl. Acad. Sci.* **1992**, *89* (19), 8953–8957. <https://doi.org/10.1073/pnas.89.19.8953>.
- (22) Kundu, C. N.; Das, K.; Majumder, G. C. Effect of Amino Acids on Goat Cauda Epididymal Sperm Cryopreservation Using a Chemically Defined Model System. *Cryobiology* **2001**, *42* (1), 21–27. <https://doi.org/10.1006/cryo.2001.2296>.
- (23) Trimeche, A.; Yvon, J.; Vidament, M.; Palmer, E.; Magistrini, M. Effects of Glutamine, Proline, Histidine and Betaine on Post-Thaw Motility of Stallion Spermatozoa. *Theriogenology* **1999**, *52* (1), 181–191. [https://doi.org/10.1016/S0093-691X\(99\)00120-X](https://doi.org/10.1016/S0093-691X(99)00120-X).
- (24) Nagao, M.; Sengupta, J.; Diaz-Dussan, D.; Adam, M.; Wu, M.; Acker, J.; Ben, R.; Ishihara, K.; Zeng, H.; Miura, Y.; Narain, R.; Imamura, R.; Mori, H. Synthesis of Highly Biocompatible and Temperature-Responsive Physical Gels for Cryopreservation and 3D Cell Culture. *ACS Appl. Bio Mater.* **2018**, *1* (2), 356–366. <https://doi.org/10.1021/acsabm.8b00096>.
- (25) Deller, R. C.; Vatish, M.; Mitchell, D. A.; Gibson, M. I. Synthetic Polymers Enable Non-Vitreous Cellular Cryopreservation by Reducing Ice Crystal Growth during Thawing. *Nat. Commun.* **2014**, *5* (1), 3244. <https://doi.org/10.1038/ncomms4244>.
- (26) Cañavate, J. P.; Lubian, L. M. Relationship between Cooling Rates, Cryoprotectant Concentrations and Salinities in the Cryopreservation of Marine Microalgae. *Mar. Biol.* **1995**, *124* (2), 325–334. <https://doi.org/10.1007/BF00347136>.

- (27) Rubinstein, P.; Dobrila, L.; Rosenfield, R. E.; Adamson, J. W.; Migliaccio, G.; Migliaccio, A. R.; Taylor, P. E.; Stevens, C. E. Processing and Cryopreservation of Placental/Umbilical Cord Blood for Unrelated Bone Marrow Reconstitution. *Proc. Natl. Acad. Sci.* **1995**, 92 (22), 10119–10122. <https://doi.org/10.1073/pnas.92.22.10119>.
- (28) Morita, K.; Nishimura, K.; Yamamoto, S.; Shimizu, N.; Yashiro, T.; Kawabata, R.; Aoi, T.; Tamura, A.; Maruyama, T. In Situ Synthesis of an Anticancer Peptide Amphiphile Using Tyrosine Kinase Overexpressed in Cancer Cells. *JACS Au* **2022**, 2 (9), 2023–2028. <https://doi.org/10.1021/jacsau.2c00301>.
- (29) Morita, K.; Nishimura, Y.; Ishii, J.; Maruyama, T. Micelle-like Nanoassemblies of Short Peptides Create Antimicrobial Selectivity in a Conventional Antifungal Drug. *ACS Appl. Nano Mater.* **2023**, 6 (2), 1432–1440. <https://doi.org/10.1021/acsanm.2c05183>.
- (30) Hu, Y.; Liu, X.; Liu, F.; Xie, J.; Zhu, Q.; Tan, S. Trehalose in Biomedical Cryopreservation—Properties, Mechanisms, Delivery Methods, Applications, Benefits, and Problems. *ACS Biomater. Sci. Eng.* **2023**, 9 (3), 1190–1204. <https://doi.org/10.1021/acsbmaterials.2c01225>.
- (31) Zhang, W.; Min, L.; Li, Y.; Lang, Y.; Hoque, S. A. M.; Adetunji, A. O.; Zhu, Z. Beneficial Effect of Proline Supplementation on Goat Spermatozoa Quality during Cryopreservation. *Animals* **2022**, 12 (19), 2626. <https://doi.org/10.3390/ani12192626>.
- (32) Moradi, B.; Faramarzi, A.; Ghasemi-Esmailabad, S.; Aghaz, F.; Hashemian, A. H.; Khazaei, M. L-proline as a Novel Additive to Cryopreservation Media Improved Post-thaw Quality of Human Spermatozoon via Reducing Oxidative Stress. *Andrologia* **2022**, 54 (1), 1–8. <https://doi.org/10.1111/and.14301>.

- (33) Zhang, L.; Xue, X.; Yan, J.; Yan, L.-Y.; Jin, X.-H.; Zhu, X.-H.; He, Z.-Z.; Liu, J.; Li, R.; Qiao, J. Cryobiological Characteristics of L-Proline in Mammalian Oocyte Cryopreservation. *Chin. Med. J. (Engl)*. **2016**, *129* (16), 1963–1968.
<https://doi.org/10.4103/0366-6999.187846>.
- (34) Horcharoensuk, P.; Yang-en, S.; Narkwichean, A.; Rungsiwiwut, R. Proline-Based Solution Maintains Cell Viability and Stemness of Canine Adipose-Derived Mesenchymal Stem Cells after Hypothermic Storage. *PLoS One* **2022**, *17* (3), e0264773.
<https://doi.org/10.1371/journal.pone.0264773>.
- (35) Murray, K. A.; Gao, Y.; Griffiths, C. A.; Kinney, N. L. H.; Guo, Q.; Gibson, M. I.; Whale, T. F. Chemically Induced Extracellular Ice Nucleation Reduces Intracellular Ice Formation Enabling 2D and 3D Cellular Cryopreservation. *JACS Au* **2023**.
<https://doi.org/10.1021/jacsau.3c00056>.
- (36) Bailey, T. L.; Hernandez-Fernaund, J. R.; Gibson, M. I. Proline Pre-Conditioning of Cell Monolayers Increases Post-Thaw Recovery and Viability by Distinct Mechanisms to Other Osmolytes. *RSC Med. Chem.* **2021**, *12* (6), 982–993.
<https://doi.org/10.1039/d1md00078k>.
- (37) Imamura, R.; Mori, H. Synthesis of Zwitterionic Polymers Containing a Tertiary Sulfonium Group for Protein Stabilization. *Biomacromolecules* **2019**, *20* (2), 904–915.
<https://doi.org/10.1021/acs.biomac.8b01542>.
- (38) Genchi, G. An Overview on D-Amino Acids. *Amino Acids* **2017**, *49* (9), 1521–1533.
<https://doi.org/10.1007/s00726-017-2459-5>.

- (39) Daily, M. I.; Whale, T. F.; Partanen, R.; Harrison, A. D.; Kilbride, P.; Lamb, S.; Morris, G. J.; Picton, H. M.; Murray, B. J. Cryopreservation of Primary Cultures of Mammalian Somatic Cells in 96-Well Plates Benefits from Control of Ice Nucleation. *Cryobiology* **2020**, *93* (December 2019), 62–69. <https://doi.org/10.1016/j.cryobiol.2020.02.008>.
- (40) Acker, J. P.; Elliott, J. A. W.; McGann, L. E. Intercellular Ice Propagation: Experimental Evidence for Ice Growth through Membrane Pores. *Biophys. J.* **2001**, *81* (3), 1389–1397. [https://doi.org/10.1016/S0006-3495\(01\)75794-3](https://doi.org/10.1016/S0006-3495(01)75794-3).
- (41) Mazur, P. Cryobiology: The Freezing of Biological Systems. *Science* (80-.). **1970**, *168* (3934), 939–949. <https://doi.org/10.1126/science.168.3934.939>.
- (42) Zhao, J.; Hao, H.; Thomas, R. L.; Lyman, W. D. An Efficient Method for the Cryopreservation of Fetal Human Liver Hematopoietic Progenitor Cells. *Stem Cells* **2001**, *19* (3), 212–218. <https://doi.org/10.1634/stemcells.19-3-212>.
- (43) Ha, S. Y.; Jee, B. C.; Suh, C. S.; Kim, H. S.; Oh, S. K.; Kim, S. H.; Moon, S. Y. Cryopreservation of Human Embryonic Stem Cells without the Use of a Programmable Freezer. *Hum. Reprod.* **2005**, *20* (7), 1779–1785. <https://doi.org/10.1093/humrep/deh854>.
- (44) Terada, S.; Nishimura, T.; Sasaki, M.; Yamada, H.; Miki, M. Sericin, a Protein Derived from Silkworms, Accelerates the Proliferation of Several Mammalian Cell Lines Including a Hybridoma. *Cytotechnology* **2002**, *40* (1–3), 3–12. <https://doi.org/10.1023/A:1023993400608>.

TABLE OF CONTENTS GRAPHIC

