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CRYOPRESERVATION OF HEP-G2 CELLS ATTACHED TO SUBSTRATES: THE BENEFIT OF SUCROSE AND TREHALOSE IN COMBINATION WITH DIMETHYL SULFOXIDE

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ABSTRACT

BACKGROUND: Cryopreservation of mammalian cells is mainly done in cryovials as free cell suspension in 5 to10% (v/v) dimethyl sulfoxide (DMSO). Relatively little attention has been paid to cryopreservation of adherent cell monolayers. **OBJECTIVE:** To investigate the appropriate cryoprotectant (CPA) formulations for the cryopreservation on HEP-G2 human tumor cells attached to the polystyrene plate and plastic surfaces. **MATERIALS AND METHODS:** Five CPA formulations were evaluated for the cryopreservation of HEP-G2 cells attached to polystyrene plates and and plastic coverslips, using post-thaw cell viability as a performance indicator. **RESULTS AND CONCLUSION:** Hep-G2 cells attached to the plastic coverslips and polystyrene plate surfaces were successfully cryopreserved in 10% DMSO with sucrose and trehalose. The addition of saccharides enabled the reduction of DMSO concentration, replaced serum, and improved the functional capacity of post-thaw Hep-G2 cells. Cells attached to the plastic coverslips show significantly better results than those attached to the polystyrene plate surfaces after cryopreservation.

Keywords: attached HEP-G2 cells; cryopreservation; sucrose; trehalose.

INTRODUCTION

Cell cryopreservation is a well-established tool. Ice crystallization during freezing can significantly damage the cells and result in the loss of viability (1, 2, 3, 4). Liver cell preservation protocols typically use 5 to 20% (v/v) dimethyl sulfoxide (DMSO) supplemented with fetal bovine serum (FBS) (5). DMSO in the freezing medium prevents excessive dehydration of cells upon freezing and also inhibits intracellular ice formation (IIF) (6).

There are a few studies on cryopreservation of cells adhering to substrates. Corsini et al. (7) cryopreserved several mammalian cells attached to a flask with 10% DMSO in phosphate buffered saline (PBS), and observed that most cells detached after thaw. Hornung et al. (8) showed that, by adding FBS into the freezing solution, post-thaw cells were still attached to the glass and proliferated well. Coating with cell-adhesive molecules to the plastic plate (9) or more percise control of the cooling rates (10, 11) also allowed successful cryopreservation of cells in the attached state. Another approach to improve the cryopreservation of the attached cells is the incorporation of other cryoprotective compounds into the DMSO solution, such as alginate, poly-L-lysine, sucrose, trehalose and/or high K^+ concentration (12, 13, 14, 15, 16).

For successful cryopreservation of Hep-G2 human tumor cells in the attached state, the required DMSO concentration is high, with 20% v/v being the most appropriate. The concentration

is much higher when compared to the DMSO concentrations used for cells in the suspended state. In the present study, we investigated sucrose and trehalose in combination with DMSO for the cryopreservation of Hep-G2 in the attached state. We stored the cultured and attached cells in a -80°C freezer, a method that is more practical and simpler for regularly daily use in comparison to cryopreservation in liquid nitrogen.

MATERIALS AND METHODS

Materials and reagents

The HEP-G2 human hepatoma cell line was purchased from Chinese Academy of Sciences. Thermanox® plastic coverslips were purchased from ThermoFisher Sientific (Waltham, MA, USA). Costar® polystyrene culture plates (6- and 12-well) were purchased from Corning Inc. (Corning, New York, USA). HyClone fetal bovine serum was purchase from ThermoFisher Biochemical (Beijing, China). Dulbecco's modified eagle medium (DMEM) was secured from GIBCO Co (Beijing, China), dimethyl sulfoxide from APPLICHEM (Darmstadt, Germany), and sucrose and trehalose from China Pharmaceutical Group (Shanghai, China).

Cell culture

To seed cells on the surface of plastic coverslips, Hep-G2 cell suspension containing 2×104 cell/mL was pipetted on the surface of coverslips that were placed at the bottom of 12-well plates. To seed the 6-well polystyrene plates, Hep-G2 cell suspension containing 8×104 cell/mL were seeded directly. Cells were cultivated for 2 days in the incubator at 37°C and with 5% CO_2 atmosphere before cryopreservation. The culture medium was changed daily.

Freezing solutions

Five freezing solutions were ued as listed in Table 1. The prepared solutions were sterile-filtered with a 0.22 μm filter, and kept at 4°C before use.

Cell cryopreservation

After 2-day cultivation, the culture medium was removed from the 12-well plates (with plastic coverslip) and 6-well polystyrene plates, and replaced by freezing solution. Plates were settled in a Styrofoam box (the container) and placed into a low-temperature freezer at -80°C for 1 d. Then, the frozen samples were taken out from the freezer, and thawed at 37℃ in a water bath for 5 min.

Table 1. Freezing solutions for Hep-G2 cells attached to the substrates. Solutions were prepared in DMEM with 20% FBS added.

No.	Cryoprotectant(s)
1	10%DMSO
2	10% DMSO + 0.3 M sucrose
3	10% DMSO + 0.4 M sucrose
4	10% DMSO + 0.3 M trehalose
5	10% DMSO + 0.4 M trehalose

Freezing container and cooling rate

A Styrofoam container was handmade to hold culture plates for freezing (Fig. 1). For most

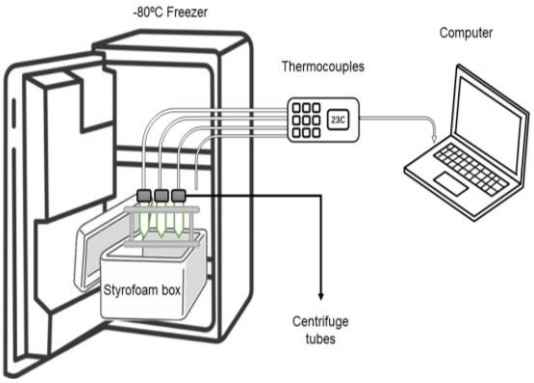


Figure 1. Schematic of the freezing container and cooling rate measurement.

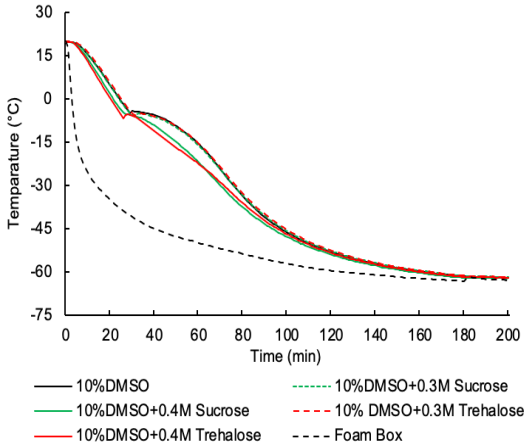


Figure 2. Cooling curves of freezing solutions in the Styrofoam freezing container for culture plates.

cells, the slow cooling rate at ~1°C/min was reported to obtain the best result. To measure the cooling rate, a Cu-constantan thermocouple was inserted into the freezing solution, and additional thermocouple to the container (Fig. 1). The cooling rate was measured in the -80°C freezer with the arrangement described. Freezing solution (3 mL) was added to each of three 15-mL cornical tubes with thermocouples inside. The Styrofoam container was tightly closed. The temperature of the freezing solution was recorded every 1 min. The average cooling rate was 1.2°C/min in the freezing solution and 4.5°C/min inside the foam container (Fig. 2).

Cell adhesion rate

Cell adhesion rates (CAR) on the plastic coverslips and polystyrene plates were evaluated under an inverted microscope, and calculated by:

$$\text{CAR}\,(\%) = \frac{\text{No.of attached cells after freezing}}{\text{No.of attached cells before freezing}} \times 100\% \quad [1]$$

Cell morphology and post-thaw viability

Changes in cell morphology before and after cryopreservation was monitored. The viability of the attached cells was estimated by staining with acridine orange (AO) (2.0 mg/mL) and propidium iodide (PI) (2.0 mg/mL). AO is an intercalating dye, can permeate into both alive and dead cells, and will stain all nucleated cells to engender green fluorescence. PI can only enter dead cells, so it stains all dead nucleated cells to engender a red fluorescence. Nucleated cells that are alive stain green. Cell viability (CV) is calculated by:

$$\text{CV}(\%) = \frac{\text{AO stained cells}}{\text{AO+PI}-\text{ stained cells}} \times 100 \quad [2]$$

Cell proliferation assays

For cells attached to the plastic coverslips, the freezing solution was removed after thawing. An aliquot of 0.4 mL fresh medium was added to each well. Cells were incubated at 37°C for 4 h after the addition of 80 µL MTT solution. The plastic coverslips were then placed into a new 12-well plate, where an aliquot of 600 µL DMSO was added to each well and mixed by shaking for 10 min. The absorbance at 490 nm for the solution was measured using the microplate reader.

For cells attached to the polystyrene plates, aliquots of 0.8 mL fresh medium and 160 µL MTT solution were added to each well after removing the freezing solution. After 4-h incubation at 37°C, 1200 µL DMSO was added to each well. The absorbance at 490 nm was measured using the microplate reader. Cell recovery rate (CRR) is calculated according to the following formula:

$$\text{CRR}(\%) = \frac{\text{Post}-\text{thaw cell absorbance}}{\text{Fresh cell absorbance}} \times 100\%. \quad [3]$$

Evaluation of 24 h adherent rate

After thawing, 1- and 2-mL fresh culture medium were added respectively to the 12-well plates with the plastic coverslips and to the 6-well polystyrene plates. The culture medium was collected after 24-h culture incubation at 37°C with 5% CO_2 and 70% humidity. The culture was washed twice with D-Hanks solution (2 mL). The collected supernatant was used to determine the number of non-adherent cells. For determining the number of adherent cells, trypsin was added to free the attached cells, which was collected after 1500 rpm centrifugation for 5 min. The adherent rate is calculated by:

$$AR(\%) = \frac{\text{Adherent survival cells}}{\text{Adherent+ non}-\text{adherent cells}} \times 100\%. \quad [4]$$

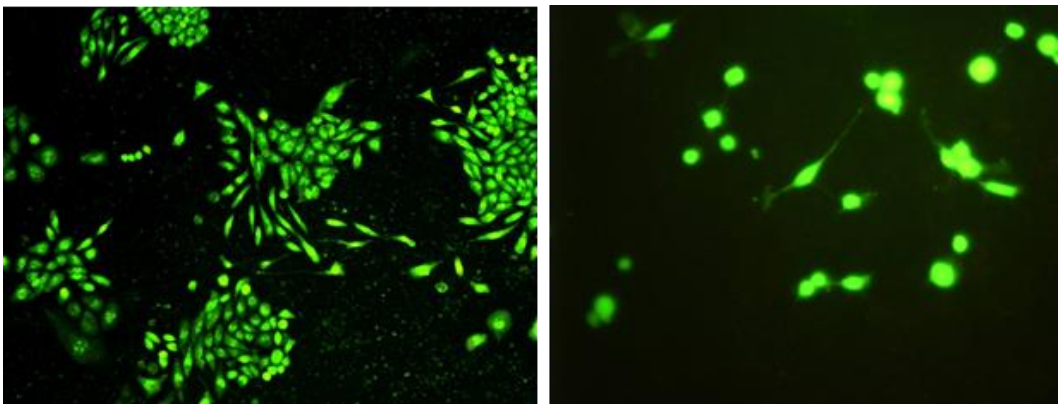
Statistical analysis

For each experiment, coverslips or plates (N = 4 or 5) were used to evaluate the cell attachment or viability. Four images were taken of each sample at random positions. The average cell number of the four images was used for data analysis. By calculating the area of each image, plastic coverslips, and polystyrene plate, the number of cells attached to the surface were estimated. One-way ANOVA was used for statistical significance analysis.

RESULTS

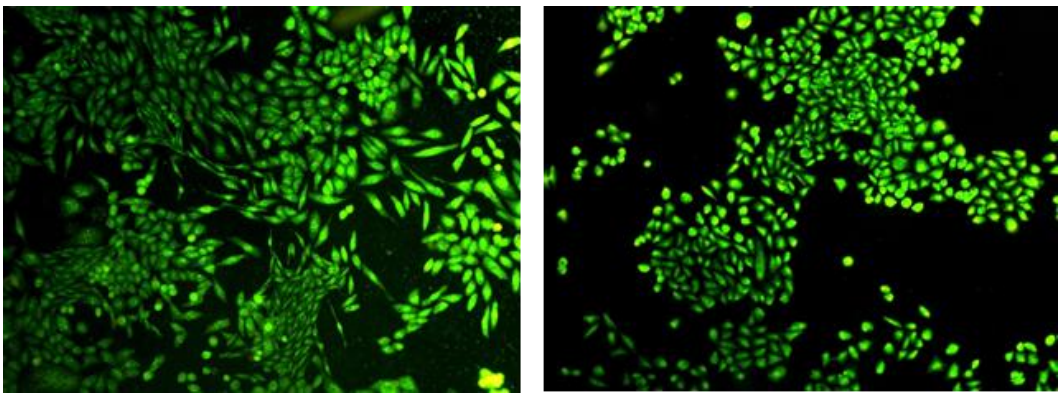
Cell morphology

A noticeable difference in cell morphology can be observed on two substrate surfaces, plastic coverslip and polystyrene plate. Figure 3 shows micrographs of elongated cells and round cells, respectively. After 2-d culture in the adherent state, about 92 to 97% Hep-G2 cells were elongated, and the remaining 3 to 8% cells were round. Plastic coverslips had 1 to 2% less elongated cells in comparison to the polystyrene surface. Despite that the majority of the adhered cells on both substrates were elongated, the Hep-G2 cells on the polystyrene surface were spindle-shaped, having more attached areas whereas Hep-G2 cells on the plastic coverslip were stretched and flat (Figure 4).



Elongated cells (10x) Round cells (20x)

Figure 3. Observed cell morphology. Elongated cells (left) and round cells (right).



Polystyrene plates (10x) Plastic Coverslips (10x)

Figure 4. Cell morphology on the polystyrene plates (left) and plastic coverslips (right).

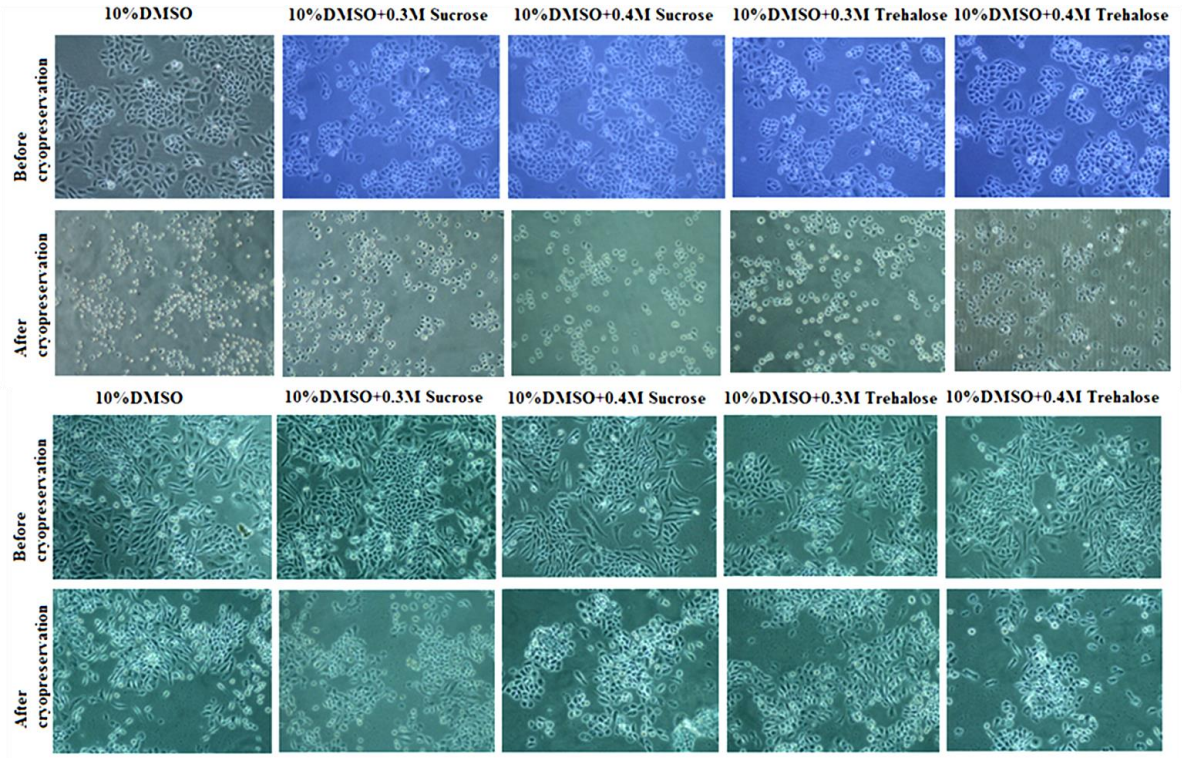


Figure 5. Micrographs of Hep-G2 cells attached to different surfaces before and after cryopreservation. Plastic coverslips (top) and polystyrene plates (bottom).

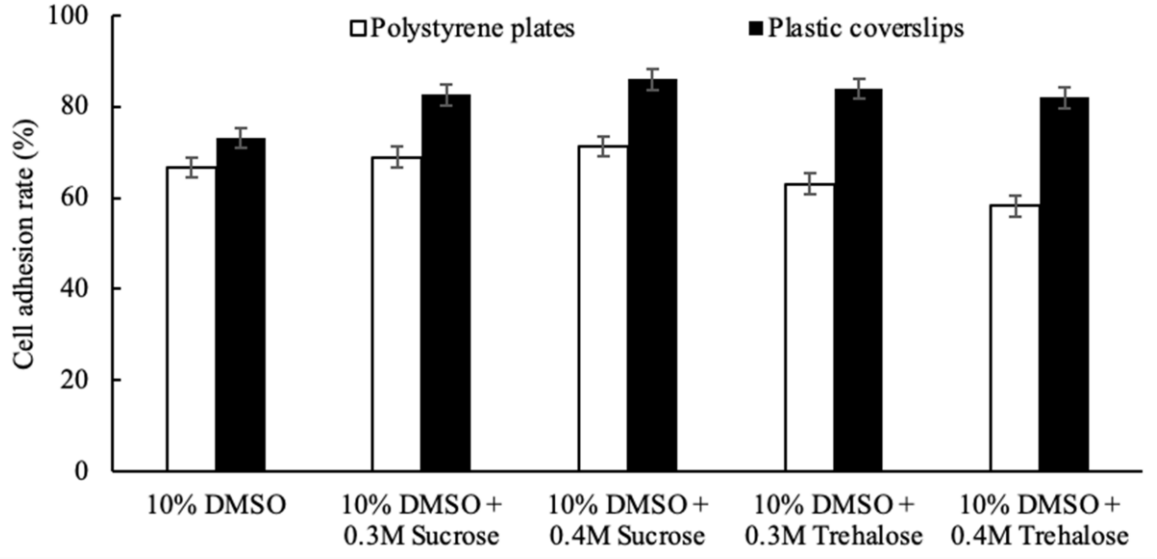


Figure 6. Cell adhesion rate after cryopreservation. Bars: standard deviation (*N*=3).

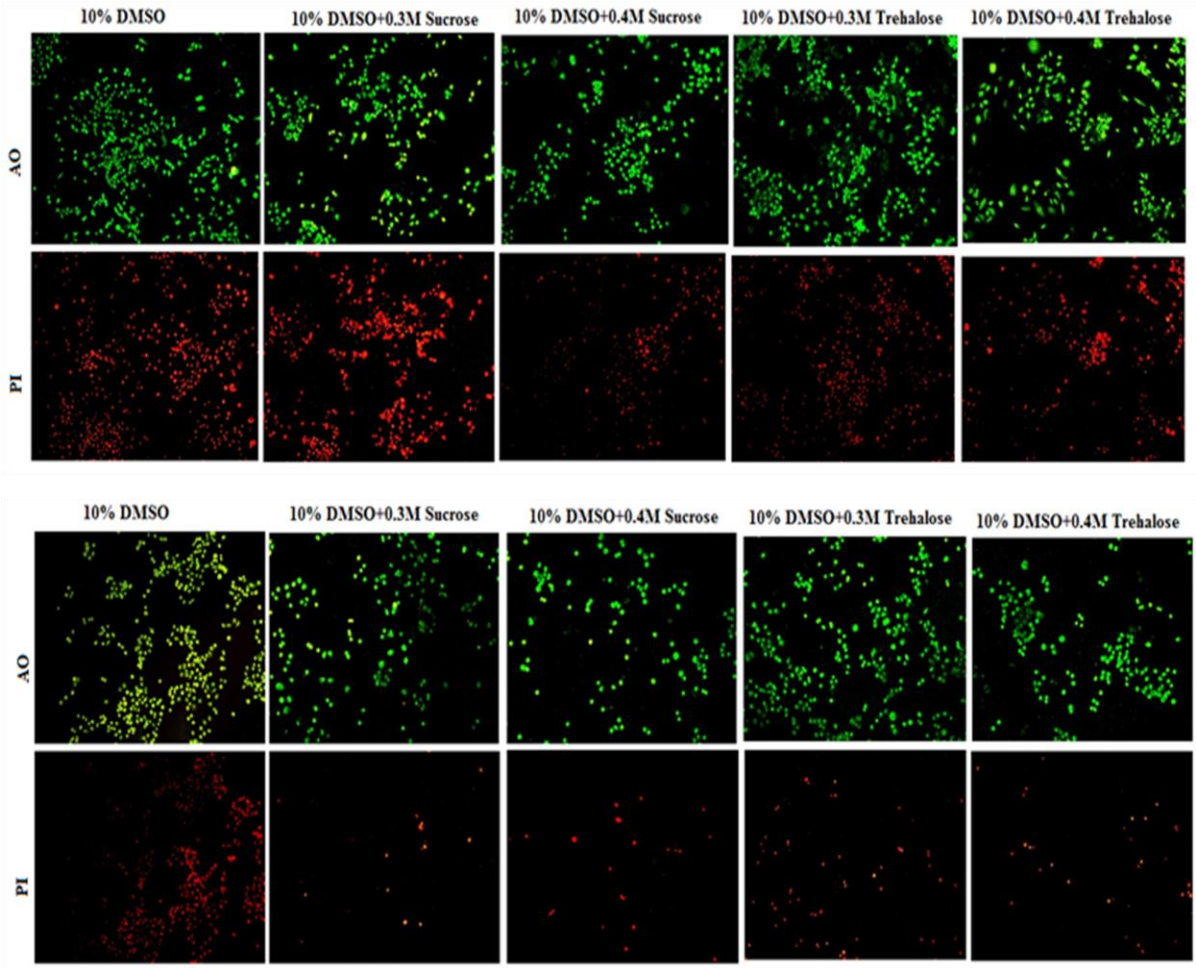


Figure 7. Viable or dead Hep-G2 cells after cryopreservation in the attached state. Cells attached to polysryrene plate surfaces (top) and plastic coverslips (bottom) were stained with AO and PI after cryopreservation.

Cell adhesion

In an attempt to understand the effects of sucrose and trehalose, the state of Hep-G2 cell adhesion before and after cryopreservation was investigated on the polystyrene plates and plastic coverslips (Figure 5). Hep-G2 cells (2×104) were seeded in the ϕ=13 mm circular plastic coverslips in the 12-well plates for 2 d (Figure 5 top), and Hep-G2 cells (8×104) were seeded in 6-well polystyrene plates for 2 d (Figure 5 Bottom). Before cryopreservation, cells extended well on the polystyrene surfaces, but after cryopreservation many cells were detached. Cells attached to the plastic coverslips were retained better and cell loss was insignificant. Figure 6 shows the rate of cell adhesion, the fraction of cells remaining attached after cryopreservation when compared to the total number of cells attached before cryopreservation. In the plastic coverslips, the cell adhesion rate increased significantly by adding sucrose and trehalose in the freezing solution. But sucrose did not improve and trehalose decreased the cell adhesion rate on the polystyrene plate surfaces.

Post-thaw cell viability and proliferation

Figures 7 and 8 show the viability of cells attached to the plastic coverslips and polystyrene plates after cryproservation. Viable cells stain green, and dead cells stain red. The addition of sucrose and trehalose in addition to 10% DMSO increased cell viability after cryopreservation. The highest cell viability was observed in 10% DMSO + 0.4 M sucrose on both plastic coverslips (93.5 ± 2.0%) and polystyrene plate surfaces (91.2 ± 2.0%). The viability of Hep-G2 cells was significantly higher on plastic coverslip than on the polystyrene plate surfaces.

MTT assay assessed the metabolic activity of the attached cells with different freezing solutions 4 h after thawing. The metabolic activity of attached cells after cryopreservation showed a similar trend as cell viability (Figure 8). Attched cells were further cultured for 24 h in the 6-well or 12-well culture plates at 37°C. Again the percentage of surviving cells after 24-h culture showed a similar trend as cell viability.

Cell viability, metabolic activity and 24 h survival rate were all significantly higher with 10% DMSO + 0.4 M sucrose. Cells attached to the plastic coverslips survived better than cells attached to the polystyrene plate surfaces.

DISCUSSION

The formation of ice crystallization, cellular dehydration, the toxicity of cryoprotectants all impact the outcome of cell cryopreservation. Their harmful effects can be reduced by adjusting the conditions such as cooling rate, ramps, warming rate and cryoprotective solution (17, 18). Among cryoprotectants, DMSO is the most commonly used and has several advantages (19). Its lower molecular weight allows it to permeate into mammalian cells and protect intracellularly.

Saccharides, such as trehalose and sucrose, also provide cryoprotection (20, 21, 22). They stabilize cell membranes (phospholipids and proteins) via direct interaction between sugar molecules and the polar groups of membranes during freezing and dehyudration (21). The combination of different protectants is known to have a synergistic effect. A number of studies have confirmed that sucrose as an additive in the freezing medium improves cryopreservation of rat hepatocytes (23, 24, 25). Furthermore, sucrose is used in various vitrification studies as an extracellular material (26, 27, 28). In the present study, therefore, we chose sucrose and trehalose in combination with DMSO for the cryopreservation of Hep-G2 in the attached state. We stored the cultured and attached cells in a -80°C freezer, a method that is more practical and simpler for regularly daily use in comparison to cryopreservation in liquid nitrogen.

We found that the addition of sucrose and trehalose to the 10% DMSO freezing solution reduced the loss of Hep-G2 cells attached to the plastic coverslips and polystyrene plate surfaces upon cryopreservation. Cell adhesion and viability was significantly increased after adding sucrose in the freezing solution (Figures 5 to 8). Nagahara et al. reported that 20% DMSO was the most appropriate for the cryopreservation of Hep-G2 cells in the attached state (29). Stokich et al. found that the use of 0.1 M trehalose provided some protection to frozen cells, but even the protected cells lost their viability if they were frozen as a monolayer (30). Katenz et al. found that the total protein level in attached cells of human hepatocytes significantly increased when using an additional 0.2 M trehalose after cryopreservation (31).

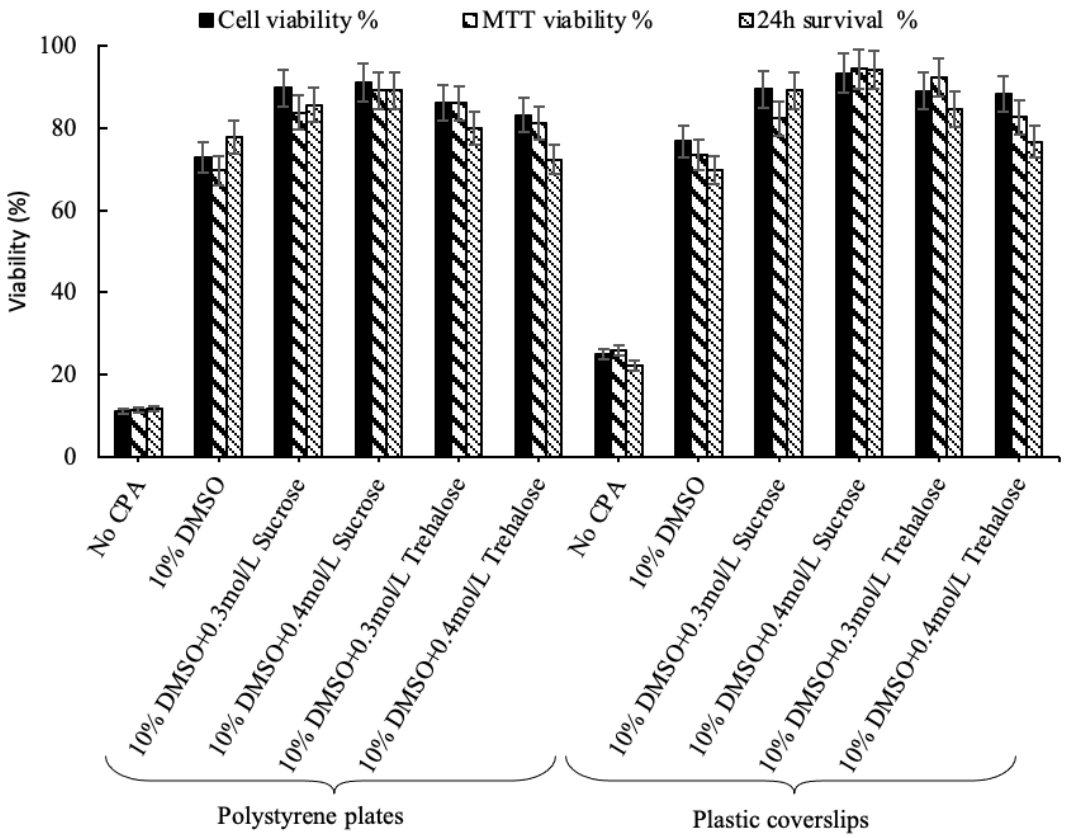


Figure 8. The effect of sucrose and trehalose on cell viability parameters after one day storage at -80°C (*N*=3).

The present study added 0.3 M and 0.4 M trehalose into the freezing solution and significantly reduced Hep-G2 cell loss (31). However, with further increases in trehalose concentration cell viability decreased. This effect was probably due to the cytotoxicity or high extracellular osmolarity that inevitably causes osmotic stress to the cells. Petrenko et al. achieved a higher recovery rate when using the addition of 0.3 M sucrose rather than 2% DMSO+10% FCS mixture (32). Their results were consistent with the data presented by other studies showing the increase in the protective effect during the cryopreservation of mouse sperm. The viability of Hep-G2 cells was the highest with 10% DMSO and 0.4 M sucrose. Although the recovery rate of 5% DMSO with 0.3 M sucrose mixture was 20% higher than that without sucrose, there is no significant difference compared to 10% DMSO alone. The positive effect of sucrose and trehalose may be attributed to the protective mechanisms such as cell dehydration and/or the reduction of external ice formation. Proper cell dehydration could decrease the chance of cell damage during freezing, and the presence of saccharides minimizes the critical size of external ice crystals (33).

It is interesting that cell adhesion and viability of HEP-G2 cells attached to the plastic coverslips were higher than those attached to the polystyrene plate surfaces. The reason is unknown and remains to be further investigated.

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REFERENCES

1. Fuller BJ (2004) *CryoLetters* **25**, 375-388.
2. Yeh Y & Feeney RE (1996) *Chem Rev* **96**, 601-618.
3. Wang GM & Haymet ADJ (1998) *Phys Chem B* **102**,5341-5347.
4. Mandumpal JB, Kreck CA & Mancera RL (2011) *Phys Chem Chem Phys* **13**, 3839-3842.
5. Miyamoto Y, Suzuki S, Nomura K & Enosawa S (2006) *Cell Transpiration* **15**, 911-919.

6. Lovelock JE & Bishop MW (1959) *Nature*, **183**, 1394-1395.
7. Corsini J & Mann E (2005) *Biol. Proceed. Online* **7**, 26-30.
8. Hornung J, Muller T & Fuhr G (1996) *Cryobiology* **33**, 260-270.
9. Shoji R, Sakai Y, Sakoda A & Suzuki M (2000) *Cytotechnology* **32**, 147-155.
10. Armitage WJ & Juss BK (1996) *CryoLetters* **17**, 213-218.
11. Pasch J, Schiefer A, Heschel I & Rau G (1999) *Cryobiology* **39**, 158-168.
12. Ji L, Pablo JJ & Palecek SP (2004) *Biotechnology and Bioengineering* **88**, 299-312.
13. Pegg DE (2002) *Cryobiology* **44**, 46-53.
14. Ma W, O'Shaughnessy T & Chang E, (2006) *Neuroscience Letters* **403**, 84-89.
15. Malpique R, Tostoes R, Beier AF, Serra M, Brito C, Schulz JC, Bjorquist P, Zimmermann H & Alves PM (2012) *Biotechnology Progress* **28**, 1079-1087.
16. Lloyd TD, Orr S, Skett P, Berry DP & Dennison AR (2003) *Cell Tissue Bank* **4**, 3-15
17. Li AP, Gorycki PD, Hengstler JG, Kedderis GL, Koebe HG, Rahmani R, et al. (1999) *Chemico-Biological Interactions* **121,** 117-123.
18. Yu ZW & Quinn PJ (1994) *Bioscience Reports* **14,** 259-281.
19. Sasnoor LM, Kale VP & Limaye LS (2003) *Stem Cell Research* **12**, 553-564.
20. Crowe LM, Mouradian R, Crowe JH, Jackson SA & Womersley C (1984) *Biochimica et Biophysica Acta* **769**, 141-150.
21. Patist A & Zoerb H (2005) *Colloid and Surf B Biointerfaces* **40**, 107-113.
22. Kravchenko LP, Petrenko A Yu, Somov A Yu, et al. (2001) *Cryobiology* **42**, 218-221.
23. Kravchenko LP, O.A, Semenchenko & Fuller BJ (2004) *The Ukrainian Biochemical* **76,** 136-138.
24. Miyamoto Y, Suzuki S, Nomura K & Enosawa S (2006) *Cell Transplant* **15**, 911–919.
25. Ali J & Shelton JN (1999) *Journal of Reproduction Infertility* **99**, 471-477.
26. Kuleshova LL, MacFarlane DR, Trounson AO & Shaw JM (1999) *Cryobiology* **38**, 119-130.
27. Reubinoff BE, Pera MF, Vajta G & Trounson AO (2001) *Human Reproduction* **16,** 2187-2194.
28. Anderson CD, Pierce J, Nicoud IB, Belous AE, Jones CM & Chari RS (2007) *Journal of Surgucal Research* **141**, 234-240.
29. Nagahara Y, Sekine H, Otaki M, Hayashi M & Murase N (2016) *Cryobiology* **72**, 53-59
30. Stokich B, Osgood Q, Grimm D, Moorthy , Chakraborty N & Menze MA (2014) *Cryobiology* **69**, 281-290.
31. Katenz E, Vondran FWR, Schwartlander R, et al. (2007) *Liver Transplant* **13**, 38-45
32. Petrenko Yu A, Grischuk VP, Roslyakov AD, et al., (1992) *CryoLetters* **13**, 87-98.
33. Koshimoto C & Mazur P (2002) *Cryobiology* **45,** 80-90.