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Comparative Efficacy of the Dimethyl Sulfoxide, Glycerol and Methanol on the Post-Thaw Cell Viability of HeLa Cells

Zeyno NUHOĞLU ÖZTÜRK[1,*] Orhan TOKUR[1] Abdurrahman AKSOY[1]

[1]*Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Veterinary Pharmacology and Toxicology, 55200, Samsun, Türkiye*

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ABSTRACT Cryoprotectants are used to protect cells during freezing. The concentration, type, and freeze-thaw conditions of these substances vary depending on the type of cell to be used. It is very important to determine the appropriate cryopreservation method for the particular cell. This study aims to provide insights into the optimal cryopreservation method for HeLa cells by comparing the performance of different cryoprotectants and evaluating their effectiveness under various freezing and storage conditions. Cell suspensions were frozen with a freezing media composed of cryoprotectant + fetal bovine serum + medium at a ratio of 5:10:85 (v:v:v) and stored under the following conditions: 3 months (-20 °C), 1 month (-80 °C), and 6 months (-80 °C). Cell viability and recovery rates were analyzed immediately post-thaw and after 48 h using the trypan blue dye exclusion assay. In 3 months (-20 °C), viability and recovery rates were higher in the methanol group. Glycerol showed better performance in 1 month (-80 °C). DMSO was the most efficient in 6 months (-80 °C). Methanol failed at -80 °C storage temperature. This study demonstrates the effect of these cryoprotectants in HeLa cells on cell viability and cell recovery rates immediately after thawing and after 48 hours of cultivation.

Keywords: *Cell culture techniques, Cell survival, Cryopreservation, Freezing, HeLa cells.*

ÖZ

Dimetil Sülfoksit, Gliserol ve Metanol'ün HeLa Hücrelerinin Çözdürme Sonrası Hücre Canlılığı Üzerindeki Karşılaştırmalı Etkinliği

Kriyoprotektanlar, hücrelerin dondurma işlemi sırasında korunması için kullanılır. Bu maddelerin, konsantrasyonu, tipi ve dondurma-çözdürme koşulları kullanılacak hücre tipine göre değişir. Uygun kriyoprezervasyon yönteminin hücreye özel olarak belirlenmesi oldukça önemlidir. Bu çalışma, farklı kriyoprotektanların performansını karşılaştırarak ve bunların çeşitli dondurma ve saklama koşulları altında etkinliğini değerlendirerek, HeLa hücreleri için en uygun kriyoprezervasyon yöntemine ilişkin bilgiler sağlanması amaçlanmaktadır. Hücre süspansiyonları 5:10:85 (v:v:v) oranında kriyoprotektan + fetal sığır serumu + medyumdan oluşan bir dondurucu besiyerinde donduruldu ve 3 ay (-20 °C), 1 ay (-80 °C) ve 6 ay (-80 °C) koşullarında saklandı. Hücre canlılığı ve geri kazanım oranları, çözülmeden hemen sonra ve çözülmeyi takiben 48 saat sonra, tripan mavisi kullanılarak analiz edildi. Canlılık ve geri kazanım oranları 3 ay -20 °C'de, metanol grubunda daha yüksekti. Gliserol grubunda ise canlılık ve geri kazanım oranları 1 ay -80 °C'de daha iyi performans gösterdi. DMSO grubunda ise bu oranlar, 6 ay -80 °C'de en yüksekti. Metanol grubu -80 °C'deki depolama koşullarında başarısız oldu. Bu çalışma, HeLa hücrelerindeki bu kriyoprotektanların, çözdürme işleminden hemen sonra ve 48 saatlik kültivasyondan sonra hücre canlılığı ve hücre geri kazanım oranları üzerindeki etkisini göstermektedir.

Anahtar Kelimeler: *Dondurarak saklama, Donma, HeLa hücreleri, Hücre kültürü teknikleri, Hücre yaşamı.*

INTRODUCTION

Cryopreservation is preserving cells at low temperatures (-80 °C or -196 °C) for future use. This process should ensure post-thaw cell viability (Gupta et al. 2017), which could be affected by many variables, including cryoprotectant type and concentration, media and reagents' quality, freezing/thawing speed, storage length, and practitioner's experience (Baust et al. 2017). Among these, cryoprotectants are considered indispensable for maintaining cell survival.

Cryoprotectants reduce ice crystal formation, which can puncture cell membranes and damage internal structures. They also help maintain cells' structural integrity and prevent cellular dehydration during freezing (Baust et al. 2011). Some cryoprotectant agents are dimethyl sulfoxide, propylene glycol, ethylene glycol, methanol, amino acids and oligosaccharides, amides, albumin, and

✉ *Corresponding author: zeyno.nuhoglu@omu.edu.tr

polyvinylpyrrolidone (Elliott et al. 2017). Each has unique features, advantages and disadvantages. These are typically added to freezing media at concentrations between 5-20%, with the remaining usually composed of medium and fetal bovine serum (FBS) mixture (Baust et al. 2017). Although specific freezing/thawing protocols exist for most cell lines, the optimal cryoprotectant concentration that maximizes cell viability is still in debate. Moreover, external factors, such as storage temperature and length, impact cryoprotectants' activity (Gao et al. 2020). Therefore, investigations are needed to understand their efficiency under different conditions.

Herein, DMSO, glycerol and methanol were tested for their cryoprotective capabilities. HeLa cells were frozen with each agent and kept at –80 °C for 1 and 6 months. Another set of HeLa cells was kept at –20 °C for 3 months. Finally, viability/recovery scores were compared at the 0^{th} and 48^{th} hours post-thaw. The purpose of this study is to investigate and compare the cryoprotection efficacy of three different cryoprotectants (dimethyl sulfoxide, glycerol, and methanol) on the HeLa cell line. Additionally, the study aims to investigate and identify the most efficient cryopreservation methods tailored to maintain the viability and functionality of HeLa cells under cryogenic conditions.

MATERIAL AND METHODS

Reagents and culturing conditions

Cervical cancer cell line, HeLa, and Eagle's Minimal Essential Medium (EMEM, with l-glutamine) were from the American Type Culture Collection (ATCC), USA. Heat-inactivated FBS was obtained from Gibco, USA. Trypsin-EDTA (0.05%), trypan blue (0.5%), sodium pyruvate, penicillin-streptomycin, and non-essential amino acids were from Biological Industries, USA. Analytical grade DMSO, methanol, and glycerol were from Sigma-Aldrich, Germany. Cells were cultured with EMEM media (10% FBS, 1% penicillin-streptomycin, standard media) at 37 °C, 5% CO_2 in a humidified incubator (EC 160, Nüve, Türkiye).

Freezing

ATCC procedures were employed for the freezing and thawing of HeLa cells (ATCC 2022). Cells were rinsed with PBS and detached by trypsin-EDTA solution. After centrifuging (1000 rpm, 10 mins), supernatant was discarded, and cells were counted using a hemocytometer. Finally, $2.5x10^5$ live cells/mL in freezing media (Table 1) were portioned into cryovials. Then cells were equilibrated with freezing media for 15 mins. Cryovials were placed in a pre-cooled cryorack half-filled with ethanol. Cryorack was transferred into a polystyrene box to allow slow cooling. Finally, cells were either kept at –80 °C for 1 and 6 months in a deep freezer (DF 490, Nüve, Türkiye) or –20 °C for 3 months in a freezer (Bosch, Germany). Some cryovials were lost due to operational errors, and "N" numbers are shown in Table 2.

Table 1: Constituents of freezing media and culture

Cryoprotectant	Freezing media
DMSO	85% MEM + 10% FBS + 5% DMSO
Glycerol	85% MEM + 10% FBS + 5% Glycerin
Methanol	85% MEM + 10% FBS + 5% Methanol

Thawing

Cryovials were immediately placed into a water bath (NB20, Nüve, Türkiye) at 37 °C for 2 mins. Cells were transferred into tubes containing 9 mL pre-warmed media and centrifuged (125 g, 10 mins). The supernatant was removed, and cells were resuspended in media. Cell viability and recovery rates were determined post-thaw 0^{th} and 48^{th} hours.

Trypan blue assay and viability/recovery calculation

Trypan blue assay was performed according to the method described by Murray and Gibson (2020). Briefly, 50 µL cell suspension and 50 µL trypan blue (0.5%) were mixed and vortexed. Then, 10 µL of the mixture was transferred to a hemocytometer, and dead/alive cells were counted with a light microscope (PrimoStar, Zeiss, Germany). The recovery rate and cell viability were calculated using Eq. 1.

Eq. 1: Cell viability % and recovery % calculation formulas

Viability (%) = (Viable cell count)/ (Total post thaw cell count) ×100

Recovery (%) = (Viable cell count)/ (Total frozen cell count) ×100

There is no standardized test to evaluate the effects of cryoprotectants on cell viability. However, two parameters, recovery and viability, are usually measured for the assessment (Weinberg et al. 2009). "Recovery" refers to the cell count that successfully grows in a new culture and considers the initial frozen cell count. "Viability", on the other hand, considers only post-thaw cell counts and does not consider any cells that may have been damaged during the freeze/thaw process. Therefore, recovery rates are regarded as a more accurate indicator of overall cell health, especially in evaluating cryoprotectant potency. Indeed, using the viability scores alone can lead to false positives (Murray and Gibson. 2020).

Statistical Analysis

The data were analyzed by GraphPad Prism 9.4 (San Diego, California, USA). Parametric data were compared by one-way ANOVA posthoc Tukey test with an alpha level of 0.05. Non-parametric data were analyzed by Kruskal-Wallis with Post-Hoc Mann-Whitney U tests using a Bonferroni-adjusted alpha level of 0.017 (0.05/3). Results were presented as mean±SEM.

Limitations

This study has some limitations. Cellular morphology and oxidative stress parameters were not investigated. Storage conditions were limited. Only one concentration of the cryoprotectant and FBS was tested. Only one cell line was tested.

RESULTS

This study tested the cryoprotective efficiency of DMSO, glycerol, and methanol on HeLa cells. Experiments were carried out at two different temperatures and three different periods because storage length and temperature are significant factors affecting long-term cell viability (Gaoet al. 2020). Although liquid nitrogen (-196 °C) is considered a well-settled approach for long-term cryopreservation, many laboratories still store their cells in a deep freezer at -80 °C. Therefore, we tested –80 °C storage temperature at 1 and 6 months. We also investigated whether cells may be viable after storing at -20 °C at an ordinary freezer temperature (-20 °C). Descriptive parameters are given in Table 2. Statistical comparisons of viability and recovery values are shown in Fig 1-3.

Table 2: Descriptive parameters of the DMSO, glycerol, and methanol groups.

		Group	N	Post-thaw	Mean	S.E.M.	Min.	Max.	Post-thaw	Mean	S.E.M.	Min.	Max.
1 month –80 °C	Viability	DMSO	14	0th	73.75	2.98	50	87.5	48th	90.99	3.14	66.67	100
		Glycerol	14		70.74		12.5	100		76.66	8.49	0	100
		Methanol	14		75.81	3.12	50	88.89		40.96	5.3	0	83.33
	Recovery	DMSO	14		54.85	7.63	8	120		52	7.62	16	128
		Glycerol	14		32.57	4.55	8	72		171.73	28.99	0	336
		Methanol	14		36.57	4.64	8	64		17.6	2.83	0	40
6 months –80 °C	Viability	DMSO	9	0th	81.55	3,83	61.11	100	48th	66.15	1.94	57.97	73.02
		Glycerol	14		85.9	1.55	75	95		60.81	3.54	31.58	82.76
		Methanol	15		42.89	5.99	11.11	100		37.82	3.55	20	57.14
	Recovery	DMSO	9		90.66	6.79	64	120		294.22	34.12	128	416
		Glycerol	14		108	12.68	48	232		124.57	18.18	40	280
		Methanol	15		23.46	2.86	8	48		19.33	2.5	8	32
3 months –20 °C	Viability	DMSO	13	0th	80.2	2.86	50	94.74	48th	47.58	3.99	25	66.67
		Glycerol	14		73.11	4.13	33.33	88.89		62.25	4.18	33.33	85.71
		Methanol	12		81.86	1.71	71.43	90.91		81.7	2.21	70	91.67
	Recovery	DMSO	13		90.67	11.47	16	160		33.85	2.59	16	48
		Glycerol	14		38.29	4.83	8	80		54.29	7.17	16	96
		Methanol	12		95.33	8.44	48	160		76	4.35	56	96

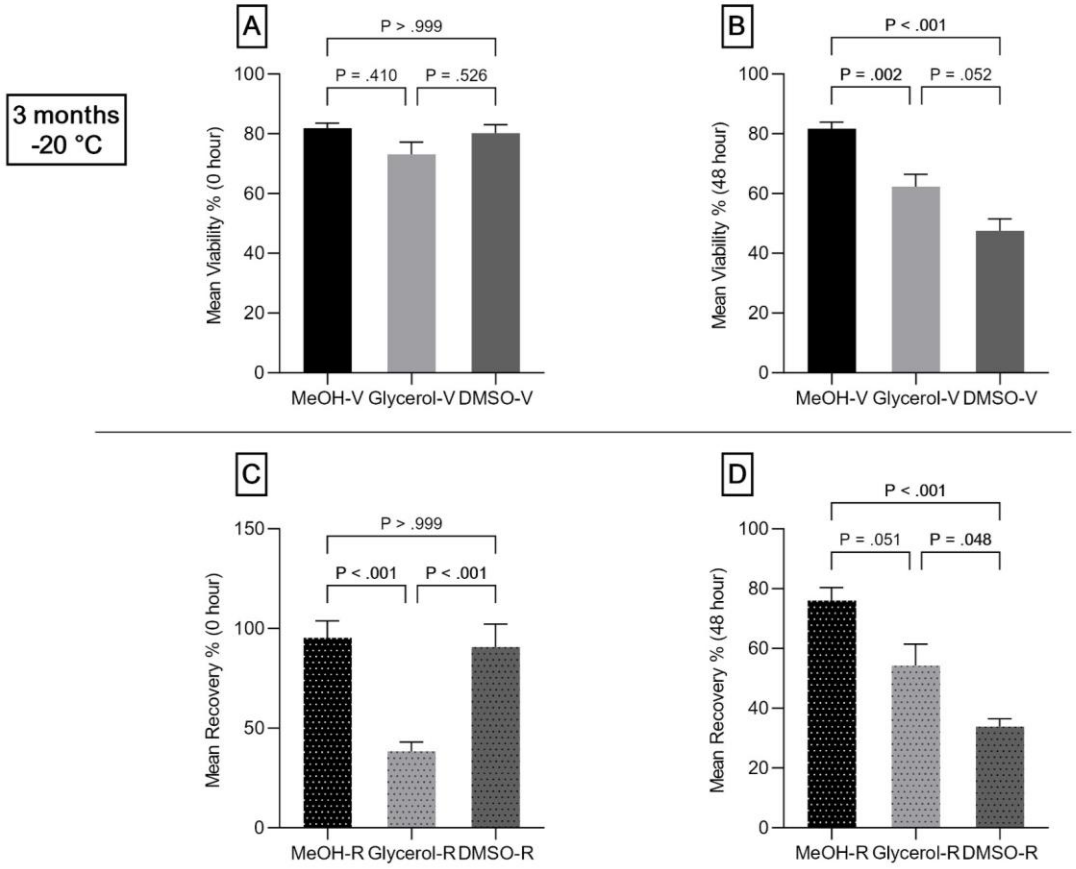

Figure 1: Comparison of DMSO, glycerol, and methanol groups stored at -20 °C for 3 months, A, B: Viability "V" (0, 48 h) C, D: Recovery "R" (0, 48 h).

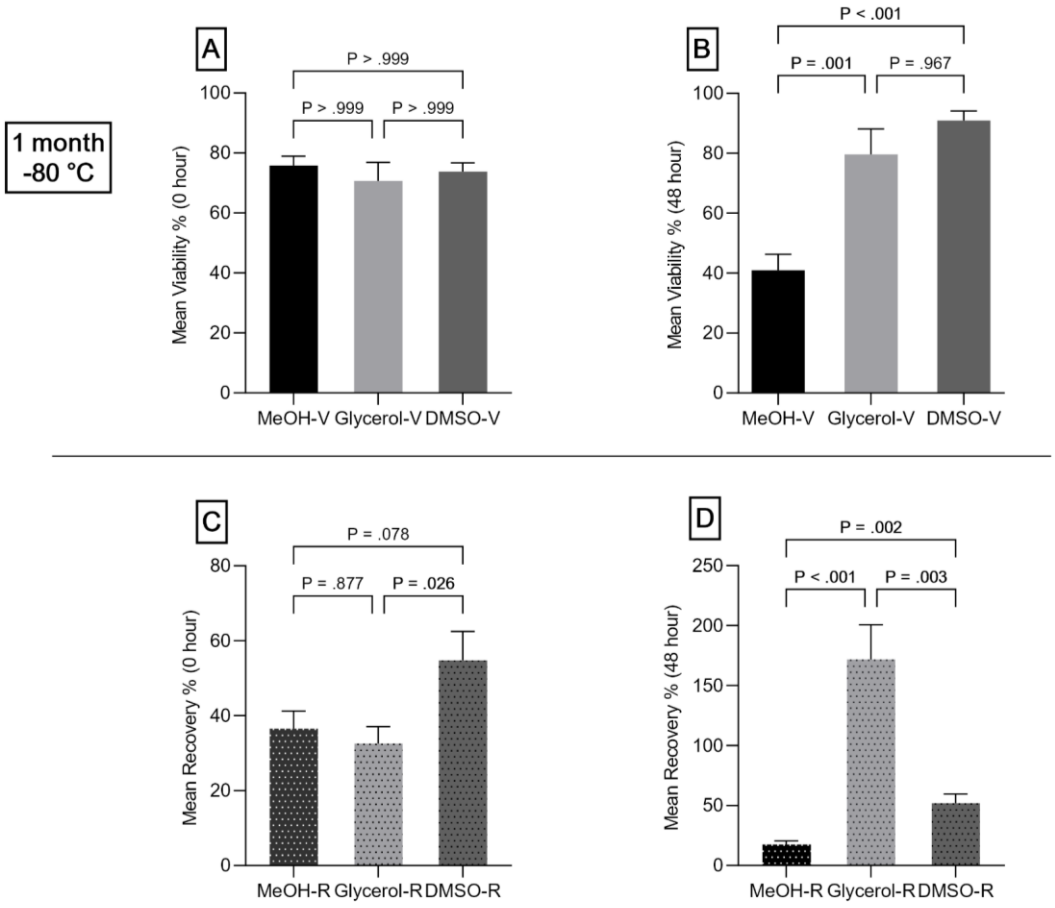

Figure 2: Comparison of DMSO, glycerol, and methanol groups stored at -80 °C for 1 month, A, B: Viability "V" (0, 48 h) C, D: Recovery "R" (0, 48 h).

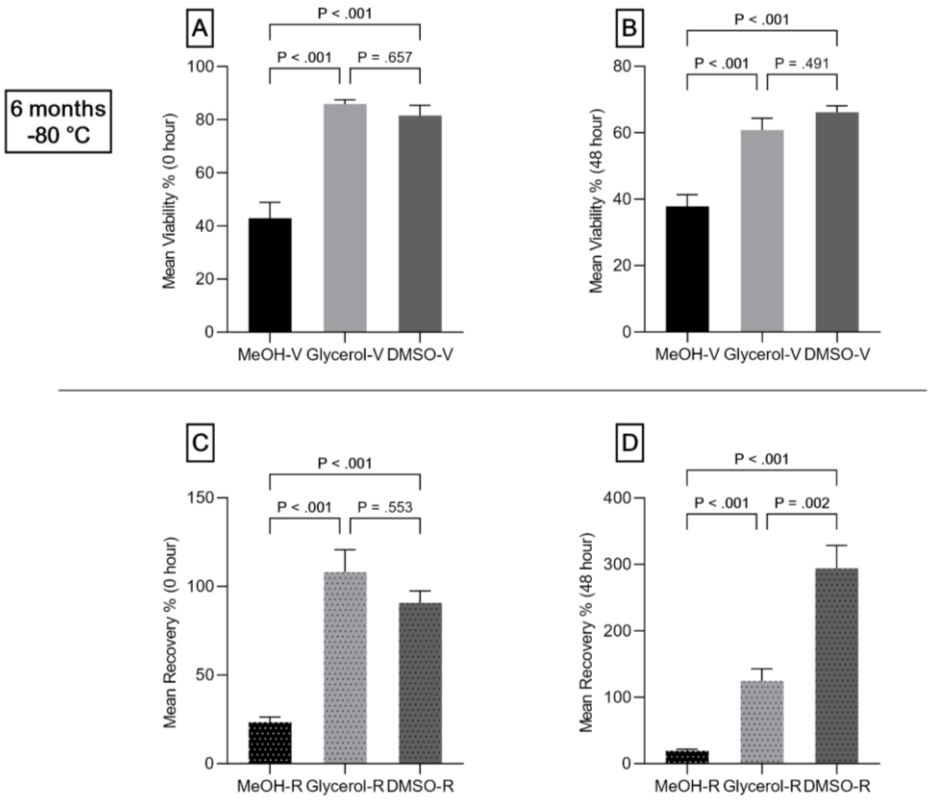

Figure 3: Comparison of DMSO, glycerol, and methanol groups stored at -80 °C for 6 months, A, B: Viability "V" (0, 48 h) C, D: Recovery "R" (0, 48 h).

Viability is the percentage of viable cells in a culture. The post-thaw 0th-hour viability was measured immediately after thawing. All cryoprotectants presented similar post-thaw 0th-hour viability. However, the damaged cells may lose their viability later in the culture; hence post-thaw 48th hour viability provides a better understanding of cryopreservation success. The impacts of the tested agents were significant at post-thaw 48th. For 1 and 6 months (at -80 °C), DMSO and glycerol provided better viability (Fig. 2-3), whereas for 3 months period, methanol was better (Fig. 1).

Recovery is the ratio of viable cell count to the initial frozen cell count. Therefore, it is considered a more accurate representation of overall cell health. Recovery results differed in storage conditions and measuring time (0-48 hours). Some principal findings considering post-thaw 48th-hour recoveries were as follows: For 1 month period (at –80 °C), glycerol was better (Fig. 2); for 6 months period (at –80 °C), DMSO was better (Fig. 3); for 3 months period (at –20 °C) glycerol and methanol were better (Fig. 1).

DISCUSSION AND CONCLUSION

As the temperature decreases during freezing, water separates purely, and the remaining solutes cause osmotic disturbance inside cells. Therefore, water passes through the plasma membrane to the outside of the cells in order to establish equilibrium (Baust et al. 2017). Free water molecules inside/outside the cells attach via hydrogen bonding and form ice crystals. When thawing, on the other hand, the leaked water molecules (as well as ice crystals) will rush into cells for the same reason. In both scenarios, osmotic imbalance (shrinking and swelling) and ice crystals cause considerable physical damage to the cells. Therefore, cells primarily die through necrosis when cryoprotectants are not used. Cryoprotectants inhibit ice crystal forming by binding/surrounding free water molecules and blocking their attachment. However, because these agents are solutes that can enter and out the cells, they may become another source of osmotic imbalance. When cryoprotectants are used, particularly at elevated levels, cells usually die through apoptosis triggered by osmotic stress (Baust et al. 2000). For this reason, the optimum cryoprotectant concentrations should be determined.

Various cryoprotectant agents were proposed, such as proline (Bryant et al. 2022), recombinant wheat proteins (Chow-Shi-Yee et al. 2020), and dextran-based hydrogels (Pereira et al. 2019); however, DMSO and glycerol are still widely preferred owing to well-established protocols and accessibility (Freshney 2015; Murray and Gibson 2022). These agents could protect non-sensitive cells such as HeLa without needing other supplies, but neuron-like sensitive cells require supplements such as FBS, albumin, sericin, and maltose (Gonzalez Porto et al. 2018; Yamatoya et al. 2022). FBS is a requirement in most cryopreservation protocols, yet it does not provide enough protection alone, as FBS at 100% concentration fails to protect cells from freezing damage (Fujisawa et al. 2019). On the other hand, the combination of FBS with cryoprotectant agents yields excellent viability (Reuther et al. 2006; Shinde et al. 2019). Cells preserved up to 95% FBS (with 5-10% DMSO) presented higher viability rates and maintained their cellular function (e.g., colony formation, differentiation) better than lower FBS levels (Stevenson et al. 2004; Fujisawa et al. 2019; Sevim and Arat 2021). Therefore, most laboratories utilize FBS in freezing media, usually at 10% concentration (Gomez-Lechon et al. 2006, Myagmarjav and Liu 2022), although some others avoid using FBS because of health concerns (Yamatoya et al. 2022). In this study, FBS concentration in freezing media remained constant at 10%.

In one month –80 °C period (Fig. 2), glycerol was superior to DMSO (p=0.003) in terms of recovery (post-thaw 48th), while viability was the same. Due to its lower toxicity, glycerol may allow cells to recover more quickly than DMSO, which can induce oxidative stress and be toxic to cells at concentrations >1% (Bumbat et al. 2020; Chow-Shi-Yee et al. 2020; Tamagawa et al. 2022). In 6 months –80 °C period, on the other hand, DMSO presented better recovery than glycerol (p=0.002) (Fig. 3), while viability was again the same. DMSO has higher intracellular permeability than glycerol (Vian and Higgins 2014; Myagmarjav and Liu 2022), and thus it may have protected cells more efficiently in long-term preservation. Although the glycerine and proline mixture was as effective as DMSO on four different cell lines (Bryant et al. 2022), cryopreservation with DMSO usually yields better outcomes. DMSO was effective in as low as 2% concentration when cryopreserving adult stem cells, even without FBS (Thirumala et al. 2010), while glycerol requires further concentrations. Glycerol successfully cryopreserved red blood cells at 15% (Poisson et al. 2019), although it caused toxicity on granulocytes at 30% concentration (Moss and Higgins 2016).

In summary, the study highlights that the choice of cryoprotectant should be based on the specific storage conditions and the intended duration of preservation. Glycerol appears to excel in short-term storage at -80 °C, while DMSO is more suitable for long-term storage at the same temperature. Methanol may have better in preserving cells for a limited time at -20 °C but is less effective in other scenarios. The success of the cryopreservation process is subject to variability, contingent upon numerous factors encompassing the conditions of cell culture, the composition of the growth media, the concentration and duration of trypsin application, the period during which cells are exposed to the external environment, and the selection and concentration of cryoprotective agents (Liu et al. 2021). Furthermore, the specific cell type being preserved constitutes a critical determinant influencing the outcomes of cryopreservation endeavors. In light of these multifaceted considerations, it is imperative that each step of the cryopreservation procedure is executed with meticulous care and precision, while the chosen freezing protocol should be systematically optimized, bearing in mind the distinct requisites associated with individual cell types (Elliott et al. 2017; Murray and Gibson 2020). Researchers may consider these findings when selecting cryoprotectants for their HeLa cell preservation needs, taking into account the desired storage duration and temperature conditions.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Idea / Concept: OT, ZNÖ
Supervision / Consultancy: AA
Data Collection and / or Processing: OT, ZNÖ
Analysis and / or Interpretation: OT, ZNÖ
Writing the Article: AA, OT, ZNÖ
Critical Review: AA

REFERENCES

ATCC (2022). American Type Culture Collection Animal Cell Culture Guide. Date of access: 9 April 2023. Access address: https://www.atcc.org/resources/culture-guides/animal-cell-culture-guide.

Baust JG, Corwin WL, Baust JM (2011). Cell Preservation Technology. Moo-Young M (Ed). Comprehensive Biotechnology (pp. 179-190). Elsevier BV.

Baust JM, Campbell LH, Harbell JW (2017). Best practices for cryopreserving, thawing, recovering, and assessing cells. *In Vitro Cell. Dev- An,* 53 (10), 855-871.

Baust JM, Van B, Baust JG (2000). Cell viability improves following inhibition of cryopreservation-induced apoptosis. *In Vitro Cell. Dev- An,* 36 (4), 262-270.

Bryant SJ, Awad MN, Elbourne A et al. (2022). Deep eutectic solvents as cryoprotective agents for mammalian cells. *J Mater Chem B***,** 10 (24), 4546-4560.

Bumbat M, Wang M, Liang W et al. (2020). Effects of Me(2)SO and Trehalose on the Cell Viability, Proliferation, and Bcl-2 Family Gene (BCL-2, BAX, and BAD) Expression in Cryopreserved Human Breast Cancer Cells. *Biopreserv Biobank*, 18 (1), 33-40.

Chow-Shi-Yee M, Grondin M, Ouellet F, Averill-Bates DA (2020). Control of stress-induced apoptosis by freezing tolerance-associated wheat proteins during cryopreservation of rat hepatocytes. *Cell Stress Chaperones*, 25 (6), 869-886.

Elliott GD, Wang SP, Fuller BJ (2017). Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. *Cryobiology*, 76, 74-91.

Freshney RI. (2015). Cryopreservation. Freshney RI (Ed). Culture of animal cells: a manual of basic technique and specialized applications (pp. 317-334). John Wiley & Sons, Inc.

Fujisawa R, Mizuno M, Katano H et al. (2019). Cryopreservation in 95% serum with 5% DMSO maintains colony formation and chondrogenic abilities in human synovial mesenchymal stem cells. *BMC Musculoskelet Disord,* 20 (1), 316.

Gao S, Ogawa M, Takami A et al. (2020). Practical and Safe Method of Long-term Cryopreservation for Clinical Application of Human Adipose-derived Mesenchymal Stem Cells Without a Programmable Freezer or Serum. *Cryoletters*, 41 (6), 337-343.

Gomez-Lechon MJ, Lahoz A, Jimenez N, Castell JV, Donato MT (2006). Cryopreservation of rat, dog and human hepatocytes: influence of preculture and cryoprotectants on recovery, cytochrome P450 activities and induction upon thawing. *Xenobiotica,* 36 (6), 457-472.

Gonzalez Porto SA, Domenech N, Gonzalez Rodriguez A et al. (2018). The addition of albumin improves Schwann cells viability in nerve cryopreservation. *Cell Tissue Bank***,** 19 (4), 507-517.

Gupta V, Sengupta M, Prakash J, Tripathy BC (2017). Animal cell culture and cryopreservation. Gupta V (Ed). Basic and Applied Aspects of Biotechnology. (pp. 59-75) Singapore, Springer**.**

Moss AC, Higgins AZ (2016). Investigating the potential for cryopreservation of human granulocytes with concentrated glycerol. *Cryobiology*, 72 (3), 290-293.

Murray KA, Gibson MI (2020). Post-Thaw Culture and Measurement of Total Cell Recovery Is Crucial in the Evaluation of New Macromolecular Cryoprotectants. *Biomacromolecules***,** 21 (7), 2864-2873.

Murray KA, Gibson MI (2022). Chemical approaches to cryopreservation. *Nat Rev Chem*, 6 (8), 579-593.

Myagmarjav B, Liu B (2022). Cryopreservation of HEP-G2 cells attached to substrates: the benefit of sucrose and trehalose in combination with dimethyl sulfoxide. *Cryoletters,* 43 (3), 175-182.

Pereira J, Ferraretto X, Patrat C, Meddahi-Pelle A (2019). Dextran-Based Hydrogel as a New Tool for BALB/c 3T3 Cell Cryopreservation Without Dimethyl Sulfoxide. *Biopreserv Biobank,* 17 (1), 2-10.

Poisson JS, Acker JP, Briard JG, Meyer JE, Ben RN (2019). Modulating Intracellular Ice Growth with Cell-Permeating Small-Molecule Ice Recrystallization Inhibitors. *Langmuir*, 35 (23), 7452-7458.

Reuther T, Kettmann C, Scheer M et al. (2006). Cryopreservation of osteoblast-like cells: viability and differentiation with replacement of fetal bovine serum in vitro. *Cells Tissues Organs,* 183 (1), 32-40.

Sevim ET, Arat S (2021). Combining dimethyl sulphoxide (DMSO) with different cryoprotectants ensures better cartilage cell cryopreservation. *Cryoletters,* 42 (4), 220-226.

Shinde P, Khan N, Melinkeri S, Kale V, Limaye L. (2019). Freezing of dendritic cells with trehalose as an additive in the conventional freezing medium results in improved recovery after cryopreservation. *Transfusion*, 59 (2), 686-696.

Stevenson DJ, Morgan C, Goldie E, Connel G (2004). Cryopreservation of viable hepatocyte monolayers in cryoprotectant media with high serum content: metabolism of testosterone and kaempherol post-cryopreservation. *Cryobiology*, 49 (2), 97-113.

Tamagawa S, Sakai D, Schol J et al. (2022). N-acetylcysteine attenuates oxidative stress-mediated cell viability loss induced by dimethyl sulfoxide in cryopreservation of human nucleus pulposus cells: A potential solution for mass production. *JOR spine*, 5 (4), e1223.

Thirumala S, Gimble JM, Devireddy RV (2010). Evaluation of Methylcellulose and Dimethyl Sulfoxide as the Cryoprotectants in a Serum-Free Freezing Media for Cryopreservation of Adipose-Derived Adult Stem Cells. *Stem Cells Dev*, 19 (4), 513-522.

Vian AM, Higgins AZ (2014). Membrane permeability of the human granulocyte to water, dimethyl sulfoxide, glycerol, propylene glycol and ethylene glycol. *Cryobiology*, 68 (1), 35-42.

Weinberg A, Song LY, Wilkening C et al. (2009). Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. *Clin Vaccine Immunol*, 16 (8), 1176-1186.

Liu X, Pan Y, Liu F et al. (2021). A review of the material characteristics, antifreeze mechanisms, and applications of cryoprotectants (CPAs). *J Nanomater*, 1-14.

Yamatoya K, Nagai Y, Teramoto N et al. (2022). Cryopreservation of undifferentiated and differentiated human neuronal cells. *Regen Ther,* 19, 58-68.