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Experimental Study on Delayed Cell Death After Thawing and the Effect of Cryoprotective Base Solutions

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Abstark: Kriopreservasi merupakan teknologi fundamental dalam pengobatan regeneratif, terapi sel, dan biobanking; namun, kematian sel tertunda (delayed cell death, DCD) yang terjadi 24–48 jam pasca-pencairan masih menjadi hambatan kritis dalam mempertahankan sel hidup berkualitas tinggi—bahkan ketika viabilitas segera pasca-pencairan melebihi 95%. Penelitian ini bertujuan untuk menyelidiki terjadinya DCD pada sel setelah pencairan pasca-kriopreservasi, serta mengevaluasi efek regulasi dari berbagai larutan dasar krioprotektif, dengan fokus pada pengembangan protokol kriopreservasi berbasis larutan UW. Enam lini sel (PESUN, Vero, RD, RK13, VeroE6, L20B) digunakan untuk membandingkan dua metode pembekuan (pembekuan cepat vs. pembekuan lambat) dan dua larutan dasar krioprotektif (DMEM vs. larutan UW). Viabilitas sel dideteksi dengan pewarnaan trypan blue, sel apoptosis dengan pewarnaan Giemsa, dan karakteristik biologis sel (proliferasi, kerentanan terhadap virus) dinilai. Hasil penelitian menunjukkan bahwa DCD mencapai puncaknya pada 24 jam pasca-pencairan, dengan angka kematian 42,2% dan angka apoptosis 28,9% pada kelompok berbasis DMEM. Larutan UW (tipe intraseluler, Na^+ rendah/K^+ tinggi) secara signifikan mengurangi DCD: angka kematian 24 jam pasca-pencairan adalah 21,8% (vs. 43,2% pada DMEM) dan angka apoptosis 13,9% (vs. 28,9% pada DMEM, keduanya $P<0,05$). Protokol optimal berbasis UW dikonfirmasi sebagai: 20% dimetil sulfoksida (DMSO), ekuilibrasi 1 menit pada suhu ruang, pembekuan cepat, pencairan dalam penangas air 37°C, glukosa 2,5% dalam medium pengencer, dan pengenceran ≥4 kali lipat. Sel yang diawetkan dengan protokol ini mempertahankan karakteristik pra-kriopreservasi (indeks proliferasi: 3,27 vs. 3,23; waktu penggandaan: 41,8 jam vs. 42,2 jam; Echovirus B1 $\log_{10}$ $TCID_{50}$/mL: 7,50 vs. 7,50). Sebagai kesimpulan, DCD pasca-pencairan berkaitan erat dengan apoptosis. Protokol kriopreservasi berbasis larutan UW secara efektif mengurangi DCD sekaligus mempertahankan fungsi sel, sehingga memberikan strategi yang andal untuk kriopreservasi sel klinis.

Kata kunci: Kriopreservasi; Kematian Sel Tertunda Pasca-Pencairan; Apoptosis; Karakteristik Biologis Sel.

Abstarct : Cryopreservation is a cornerstone technology in regenerative medicine, cell therapy, and biobanking; however, delayed cell death (DCD) occurring 24–48 hours post-thaw remains a critical barrier to maintaining high-quality viable cells—even when immediate post-thaw viability exceeds 95%. This study aims to investigate the occurrence of DCD in cells after thawing following cryopreservation, and to evaluate the regulatory effect of different cryoprotective base

solutions, with a focus on establishing a UW solution-based cryopreservation protocol. Six cell lines (PESUN, Vero, RD, RK13, VeroE6, L20B) were used to compare two freezing methods (rapid freezing vs. slow freezing) and two cryoprotective base solutions (DMEM vs. UW solution). Cell viability was detected by trypan blue staining, apoptotic cells by Giemsa staining, and cell biological characteristics (proliferation, viral susceptibility) were assessed. The results showed that DCD peaked at 24 h post-thaw, with mortality rate of 42.2% and apoptotic rate of 28.9% in DMEM-based groups. UW solution (intracellular-type, low Na^+/high K^+) significantly reduced DCD: 24 h post-thaw mortality was 21.8% (vs. 43.2% in DMEM) and apoptotic rate was 13.9% (vs. 28.9% in DMEM, both $P<0.05$). The optimal UW-based protocol was confirmed as: 20% dimethyl sulfoxide (DMSO), 1 min room temperature equilibration, rapid freezing, 37°C water bath thawing, 2.5% glucose in dilution medium, and ≥4-fold dilution. Cells preserved by this protocol maintained pre-cryopreservation properties (proliferation index: 3.27 vs. 3.23; doubling time: 41.8 h vs. 42.2 h; Echovirus B1 $\log_{10}$ $TCID_{50}$/mL: 7.50 vs. 7.50). In conclusion, post-thaw DCD is closely associated with apoptosis. The UW solution-based cryopreservation protocol effectively reduces DCD while preserving cell function, providing a reliable strategy for clinical cell cryopreservation.
Keyword: Cryopreservation; Post-Thaw Delayed Cell Death; Apoptosis; Cell Biological Characteristics.

1. Introduction

Cryopreservation is a critical technology in modern medicine and biology, supporting applications such as cell therapy, regenerative medicine, and biobanking [1]. The ideal cryopreservation protocol should not only maintain cell viability post-thaw but also restore cellular functions to pre-cryopreservation levels [2]. However, conventional cryopreservation methods (first-generation protocols) face a major limitation: delayed cell death (DCD) occurs 24–48 h post-thaw, with mortality ranging from 30% to 70%, even if immediate post-thaw viability exceeds 95% [3], [4]. This DCD is primarily driven by apoptosis induced by cold stress, involving the activation of caspase cascades and mitochondrial dysfunction [5], [6]. Recent studies have further identified ferroptosis and necroptosis as potential synergistic mechanisms of DCD, highlighting the complexity of cold stress-induced cell death [7]. First-generation cryoprotective solutions (e.g., DMEM supplemented with DMSO)

are "extracellular-type" (high Na^+/low K^+), which fail to regulate cellular molecular responses to cold stress, leading to oxidative damage and energy depletion [8]. In contrast, second-generation cryoprotective solutions (e.g., UW solution, HypoThermosol) are designed as "intracellular-type" (low Na^+/high K^+) and include buffers, free radical scavengers, and energy substrates, exhibiting potential for reducing DCD [9], [10]. Recent advances in UW solution modification—such as adding trehalose or antioxidants—have further improved its protective efficacy in stem cell cryopreservation [11]. This study systematically explored the dynamics of DCD post-thaw and optimized a UW solution-based cryopreservation protocol, aiming to provide a clinically applicable strategy to improve cryopreservation outcomes for mammalian cells.

2. Research Method

a. Materials

Cell Lines: PESUN (ATCC CRL-1583), Vero (ATCC CCL-81), RD (ATCC CCL-136), RK13 (ATCC CCL-37), VeroE6 (ATCC CRL-1586), and L20B (ATCC CRL-2052) cells were cultured in DMEM (Gibco, USA) supplemented with 5%–10% fetal bovine serum (FBS, Thermo Fisher, USA) at 37°C in a 5% CO_2 incubator.

Cryoprotective Base Solutions:

DMEM (Gibco, USA): Extracellular-type, high Na^+ (140 mM/L), low K^+ (5 mM/L). UW Solution (self-prepared, based on [12]): Intracellular-type. Preparation steps: Dissolve components in ultrapure water (Millipore, USA), adjust pH to 7.4 with 1 M HCl/NaOH, and filter through a 0.22 μm polyethersulfone membrane (Merck, Germany) before use. The composition is shown in Table 1.

Cryoprotectants (CPAs): Dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA, ≥99.9%), glycerol (GLY, Sigma-Aldrich, USA, ≥99.5%), ethylene glycol (EG, Sigma-Aldrich, USA, ≥99.8%).

Viruses: Echovirus B1 (ATCC VR-197), Rotavirus 95 (ATCC VR-2018), Herpes Simplex Virus 2 (HSV-2, ATCC VR-734), stored at -80°C.

Table 1. Composition of UW Solution

Component	Concentration (g/L)	Concentration (mM/L)
$NaH_2PO_4 \cdot H_2O$	4.14	25
Potassium Lactobionate	29.03	100
$MgSO_4 \cdot 7H_2O$	1.23	5
Raffinose $\cdot 5H_2O$	15.13	30
Glutathione	0.85	3
Osmolality	-	320
pH	-	7.4

b. Cryopreservation Protocols

Two freezing methods were compared:

Rapid Freezing: Cells in logarithmic growth phase were resuspended in cryoprotective solution (base solution + CPA) at 1×10^6 cells/mL, equilibrated at room temperature (18–25°C) for 1 min, and directly plunged into liquid nitrogen (-196°C).

Slow Freezing: Cells were resuspended in cryoprotective solution, placed in a foam container filled with cotton (to achieve a cooling rate of ~1°C/min), stored at -70°C for 12 h, and then transferred to liquid nitrogen.

Cryoprotective solutions were formulated as follows:

DMEM-based: DMEM + 40% FBS + 15%–35% CPA (DMSO/GLY/EG).

UW-based: UW solution + 40% FBS + 15%–25% DMSO.

c. Thawing Protocol

Cryopreserved cells were removed from liquid nitrogen and thawed in a 37°C water bath with gentle shaking (100 rpm) until >90% of the ice melted (≈60–90 s). Thawed cells were diluted 2–8-fold with DMEM containing 0%–

7.5% glucose (Sigma-Aldrich, USA), centrifuged at 1000 × g for 5 min, and resuspended in fresh DMEM for subsequent experiments.

d. Detection of Cell Viability and Apoptosis

Cell Mortality: Trypan blue staining was used. Cells were mixed with 0.4% trypan blue (Sigma-Aldrich, USA) at a 1:1 ratio, incubated for 1–2 min at room temperature, and counted under an inverted microscope (Olympus, Japan, CKX41) with a 10× objective. Mortality = (number of stained cells / total cells) × 100%.

Apoptotic Rate: Giemsa staining was used. Cell smears were fixed with 4% paraformaldehyde for 10 min, stained with 1% Giemsa solution (pH 6.8, Sigma-Aldrich, USA) for 20 s, and rinsed with distilled water. A total of 200 cells were counted per smear; apoptotic cells were identified by chromatin condensation or apoptotic bodies. Apoptotic rate = (number of apoptotic cells/total cells) × 100%.

e. Assessment of Cellular Biological Characteristics

Proliferation Assays:

Proliferation Index (PI): Calculated as (cell number at 72 h post-thaw / initial seeded cell number) × 100%.

Doubling Time (DT): Determined during logarithmic growth using the formula:

$$DT = (t - t_0) \times \log_{10} 2 / (\log_{10} Nt - \log_{10} N_0)$$

where $t\text{-}t_0$ = culture duration (h), Nt = cell number at time t, and N_0 = initial cell number.

Saturation Density: Cell number at 144 h post-thaw (stationary phase).

Viral Susceptibility: Cells were cultured to 90% confluence in 96-well plates, infected with 1:1000 diluted viruses (diluted in DMEM without FBS), and incubated at 37°C. At 5 days post-infection, cytopathic effects (CPE) were observed, and the 50% tissue culture infective dose ($\log_{10}$ $TCID_{50}$/mL) was calculated using the Reed-Muench method [14].

f. Statistical Analysis

All experiments were independently repeated 5 times. Data were expressed as mean ± standard error (SE). Differences between two groups were analyzed using Student's t-test; multiple comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant. Statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software, USA).

3. Results and Discussion

a. Occurrence of Delayed Cell Death After Thawing

For PESUN cells cryopreserved with DMEM + 10% DMSO (rapid freezing), immediate post-thaw cell mortality was 5.41% ± 0.1%, and no apoptotic cells were detected. Mortality increased gradually, peaking at 24 h (42.2% ± 0.2%) and decreasing to 18.0% ± 0.3% at 72 h. The apoptotic rate showed a similar trend, reaching 28.9% ± 0.7% at 24 h. Pre-cryopreservation cells entered logarithmic growth at 24 h and reached saturation at 72 h (3.84×10^5 cells/mL). Post-thaw cells had a 24 h lag phase, entered logarithmic growth at 48 h, and only reached 3.16×10^5 cells/mL at 96 h (80% of pre-cryopreservation density) (Table 2). These findings confirm that DCD is a significant post-thaw event, peaking at 24 hours and primarily driven by apoptosis, consistent with previous reports [3], [5].

Table 2. Proliferation of PESUN Cells Pre- and Post-Cryopreservation ($\times10^5$ cells/mL, Mean ± SE)

Group	Initial Seeded Cells	12 h	24 h	48 h	72 h	96 h
Pre-cryo	1.0	1.0±0.2	1.23±0.1	2.63±0.2	3.84±0.7	3.94±0.3
Post-thaw	2.0	2.0±0.3	1.98±0.3	2.32±0.2	2.76±0.1	3.16±0.1

Footnote to Table 2: The higher initial seeded cell number in the post thaw group (2.0×10^5 cells/mL) was intentionally set to compensate for expected cell loss during the first 12–24 h after thawing, allowing a more comparable evaluation of proliferation kinetics between pre and post cryopreservation conditions.

b. Factors Influencing Delayed Cell Death

Freezing/Thawing Methods: No significant difference in 24-h post-thaw mortality was observed between rapid freezing (42.2% ± 1.2%) and slow freezing (44.7% ± 0.6%) ($P > 0.05$). Similarly, thawing at 37°C (47.3% ± 1.7%) or 70°C (49.4% ± 1.4%) did not significantly affect DCD ($P > 0.05$). This suggests that when the base solution is not optimized, the freezing/thawing rate has a minimal impact on DCD, a finding that aligns with recent observations [15].

Cryoprotective Base Solution: UW solution significantly reduced DCD compared to DMEM (Table 3). Immediate post-thaw mortality was 5.8% ± 0.2% (UW) vs. 7.21% ± 0.3% (DMEM) ($P > 0.05$). At 24 h post-thaw, mortality was 21.8% ± 0.5% (UW) vs. 43.2% ± 0.3% (DMEM) ($P < 0.05$); apoptotic rate was 13.9% ± 0.2% (UW) vs. 28.9% ± 0.3% (DMEM) ($P < 0.05$). The superior performance of UW solution is attributed to its intracellular-type composition (low Na^+/high K^+), which minimizes osmotic stress and ion imbalance, and its inclusion of free radical scavengers like glutathione [8], [12], [21]. Recent studies have shown that such solutions can reduce reactive oxygen species production by 40%–50% in cryopreserved cells [22].

Table 3. Comparison of Delayed Cell Death Between DMEM and UW Solution (Mean ± SE, n=5)

Base Solution	Immediate Mortality (%)	24 h Mortality (%)	24 h Apoptotic Rate (%)
DMEM	7.21±0.3	43.2±0.3	28.9±0.3
UW Solution	5.8±0.2	21.8±0.5*	13.9±0.2*

Note: *$P < 0.05$ vs. DMEM

CPA Type and Concentration: Optimal CPA concentrations were identified: for EG, 20% (24 h mortality: 69.7% ± 2.2%); for DMSO, 25% (24 h mortality: 43.4% ± 2.4%); for GLY, 30% (24 h mortality: 49.6% ± 1.6%). DMSO showed the lowest DCD among the three CPAs, consistent with its widespread use in mammalian cell cryopreservation [17]. Higher or lower concentrations

increased DCD, likely due to CPA toxicity or insufficient ice crystal inhibition [16].

Cell Line Specificity: All six cell lines showed similar DCD patterns, with 24-h post-thaw mortality ranging from 46.8% ± 0.1% (Vero) to 52.8% ± 0.06% (RD) in DMEM groups, indicating DCD is a universal phenomenon in cryopreserved mammalian cells [18].

c. Optimization of UW-Based Cryopreservation Protocol

Key parameters for the UW-based protocol were optimized: equilibration time (1–2 min), DMSO concentration (20%–25%), glucose in dilution medium (2.5%), and dilution fold (≥4-fold). These parameters collectively resulted in the lowest post-thaw mortality and apoptosis, balancing CPA penetration, toxicity, and osmotic support [16], [19], [20].

d. Biological Characteristics of Cells Preserved with Optimized UW Protocol

The optimized protocol (UW + 40% FBS + 20% DMSO, 1 min equilibration, rapid freezing, 37°C thawing, 2.5% glucose, 4-fold dilution) maintained cellular function. Post-thaw cells had a proliferation index of 3.27 ± 0.08 (vs. 3.23 ± 0.05 pre-cryopreservation), doubling time of 41.8 ± 0.8 h (vs. 42.2 ± 0.4 h), and saturation density of 31.2 ± 0.4×10^5 cells/mL (vs. 31.6 ± 0.5×10^5 cells/mL) ($P > 0.05$ for all). Viral susceptibility for Echovirus B1, Rotavirus 95, and HSV-2 remained comparable to pre-cryopreservation levels ($P > 0.05$) (Table 4). This confirms that the protocol not only reduces DCD but also preserves critical cell functions, making it suitable for virology research and vaccine production [23].

Justification for 20% DMSO: The selection of 20% DMSO in the UW based protocol was based on preliminary dose response experiments (10–25% DMSO, data not shown), which indicated that concentrations below 15% resulted in insufficient protection against delayed cell death (24 h mortality > 35%), while concentrations above 20% did not further improve viability but increased

osmotic stress. Although 20% DMSO is higher than conventional 10% DMSO used in DMEM based protocols, the intracellular type UW solution with high K^+ and lactobionate mitigates DMSO induced cytotoxicity by stabilizing membrane fluidity and reducing osmotic imbalance.

Clinical Safety Considerations: For clinical translation, the 20% DMSO protocol should be followed by DMSO washout steps (e.g., centrifugation and resuspension) to reduce potential adverse effects. Recent guidelines recommend residual DMSO < 1% for infusion [26].

Comparison of Base Solutions: UW solution significantly reduced DCD compared to DMEM. Optimal conditions were: 20% DMSO, 1 min equilibration, rapid freezing, 37°C water bath thawing, dilution with 2.5% glucose, and ≥4 fold dilution.

Post Thaw Cell Function: Post thaw cells had a proliferation index of 3.27 ± 0.08 (vs. 3.23 ± 0.05 pre cryopreservation), doubling time of 41.8 ± 0.8 h (vs. 42.2 ± 0.4 h), and saturation density of 31.2 ± 0.4 × 10^5 cells/mL (vs. 31.6 ± 0.5 × 10^5 cells/mL) ($P > 0.05$ for all). Viral susceptibility for Echovirus B1, Rotavirus 95, and HSV 2 remained comparable to pre cryopreservation levels ($P > 0.05$) (Table 4). This confirms that the protocol not only reduces DCD but also preserves critical cell functions, making it suitable for virology research and vaccine production [23].

Table 4. Viral Susceptibility of PESUN Cells Pre- and Post-Cryopreservation (log_{10} $TCID_{50}$/mL, Mean ± SE)

Virus	Pre-cryopreservation	5 Passages Post-Thaw	10 Passages Post-Thaw
Echovirus B1	7.50	7.50	7.66
Rotavirus 95	7.33	7.50	7.33
Herpes Simplex Virus 2	6.50	6.66	6.33

4. Conclusion

This study confirmed that post-thaw delayed cell death is a universal challenge in cryopreservation, driven by apoptosis and peaking at 24 h. The "intracellular-type" UW solution significantly reduces DCD compared to the "extracellular-type" DMEM. An optimized cryopreservation protocol using UW solution with 20% DMSO, a 1-minute equilibration, rapid freezing, thawing at 37°C, dilution with 2.5% glucose, and a ≥4-fold dilution effectively minimizes DCD and preserves key cellular functions, including proliferation and viral susceptibility. This protocol offers a clinically applicable strategy for improving cell cryopreservation outcomes in regenerative medicine, biobanking, and virology research.

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