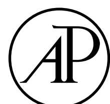
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Modulation of the cryopreservation cap: elevated survival with reduced dimethyl sulfoxide concentration☆,☆☆

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Abstract

The development of cryopreservation (CP) strategies has traditionally focused on the cellular chemo-osmometric characteristics attendant to the freeze-thaw process. This approach coupled with a limited understanding of cellular physiological and biochemical responses to the CP process often yields sub-optimal cell survival. Recently, we have reported on the benefits of the utilization of an intracellular-like preservation solution, HypoThermosol (HTS), as well as incorporating a molecular approach to improving CP outcome [In Vitro Cell. Dev. Biol. Anim. 36(4) (2000) 262]. We now report on the elucidation of a cryoprotective agent (CPA)-dependent survival limit (cap) associated with standard CP methodologies. We further demonstrate an elevation and shift in the CP cap through the utilization of HTS coupled with a reduction in CPA levels necessary to achieve "successful" cell preservation.
Methods. Human fibroblasts, keratinocytes, hepatic, and renal cells were cryopreserved in a standard fashion ($\sim 1\,^{\circ}C\,min^{-1}$ cooling and storage in LN_2) in culture media (serum-free) or HTS with varying levels of dimethyl sulfoxide (Me_2SO). Samples were allowed to recover for 24-h prior to survival assessment. Survival was assessed using alamarBlue (metabolic activity indicator) and calcien-AM (membrane integrity stain) in comparison with non-frozen controls.
Results. (1) A limit in cell survival was identified following CP in media-based CP solutions yielding a cell-type specific CPA-dependent survival limit, (2) peak cell survival resulted in the identification of "optimal" Me_2SO concentrations for CP of each cell type, (3) incorporation of HTS as the carrier medium at typical Me_2SO concentrations substantially elevated survival, and (4) utilization of HTS allowed for the successful preservation of all systems examined at significantly reduced Me_2SO levels.
Conclusion. The data presented in this study illustrate that the utilization of HTS as the carrier medium during CP facilitated a significant improvement in efficacy at reduced Me_2SO levels. Further, the utilization

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of HTS offers the potential for successful Me_2SO-free CP. These findings may prove significant to the advancement in the development of cell-based clinical therapies by providing an improved biocompatible CP methodology.

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The success of cryopreservation (CP) has been due, in part, to the development of protocols that limit intracellular ice formation, cellular volume excursions, and cryoprotectant toxicity while providing optimal cooling rates. In 1949, Polge et al. [30] reported on the successful CP (−79 °C) of spermatozoa using 5–20% glycerol. Later, Lovelock and Bishop [21] identified the beneficial effects of dimethyl sulfoxide (Me_2SO) in CP. In the following years, methodological improvements led to the wide spread expansion of the CP sciences. Studies into the effects of cooling rate (slow vs rapid), cryoprotective agent (CPA) type and concentration (i.e., glycerol, dimethyl sulfoxide, ethylene glycol, etc.), CPA formulations (single vs multiple agents), serum concentration (0–>50%), warming rate, dilution procedure (single-step vs serial), etc. were undertaken in attempts to improve post-thaw survival [10,11,17,22–25,29, 32,33,41]. In 1985, Rall and Fahy [31] further extended CP methodologies by describing the advantage of ice-free cell storage. They described the utilization of high molar concentrations of a mixture of cryoprotectants coupled with rapid cooling to induce vitrification in both the sample and its medium. As a result of these studies, significant improvements in CP outcome were achieved. Despite these advances, however, a significant number of cells die during the freeze-thaw process resulting in the level of CP success being limited (capped) at sub-optimal levels [1,7,12]. This cell loss can be substantial in homogeneous cell suspensions and increases as the system undergoing preservation becomes more complex (i.e., tissues and organs) [2,15,28].

One possible reason for this limitation may be traced to the development of CP strategies focusing on cellular volumetric behavior and the avoidance of ice (intra- and extracellular) formation during CP. Accordingly, models have defined the cell as a passive osmometer during the freezing process. Until recently, consideration of cellular molecular and biochemical responses to the CP process has not played a role in the development of application-based CP strategies. With the goal of the CP sciences being that of initiating temperature dependent suppression of biological activity, an understanding of the cellular responses to the freeze-thaw excursion may provide clues important to elevating the survival cap. A review of the literature reveals that post-thaw survival estimates, even for a single-cell type, range between 20% and 90% based typically on quantitative assays such as dye exclusion, nuclear counts, etc. performed within 1-h of thawing [12,19,26,28]. Accordingly, short-term post-thaw assessment of CP outcome tends to yield overestimates due to sequential apoptotic and necrotic processes contributing to cell death not evident immediately subsequent to thawing [1,3,4,12]. We now describe an elevation in cell survival through the reduction in the extent of cryopreservation-induced delayed-onset cell death (*CIDOCD*), as well as a shift in the survival cap with lower CPA concentrations through the incorporation of HypoThermosol (HTS) [37,38], a preservation solution designed to alleviate cellular stress (ionic, osmotic, biochemical, etc.) under conditions of extended hypothermia (intracellular-like preservation solution) [8,34–36].

Methods and materials

Cell culture

Normal Human Dermal Fibroblast (NHDF), Normal Human Epidermal Keratinocytes (NHEK), and Renal Proximal Tubule Epithelial (RPTEC) cells were obtained from Clonetics (San Diego, CA.). Madin Darby Canine Kidney [MDCK (NBL-2)] and Human Hepatocarsinoma [C3A(HepG2/C3A)] cells were obtained from American Type Culture Collection (Rockville, MD). Cultures were maintained at 37 °C, 5% CO_2 – 95% air in their respective growth medium in Falcon 75 cm^2 T-flasks. NHDF, NHEK, and RPTEC cell culture media (FBM, KBM, ReBM, respectively) were purchased (Clonetics), formu-

lated and used in accordance to the Clonetics normal human cell systems instructions. MDCK cells were cultured in media consisting of Dulbecco's version of Eagles Minimal Essential Medium (DME) (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Atlanta Biological, Atlanta, GA) and 1% Penicillin/Streptomycin (Life Technologies). C3A cells were cultured in medium consisting of Eagles Minimal Essential Medium (EMEM) (Life Technologies) fortified with 10% fetal calf serum (Atlanta Biological), and 1% Penicillin/Streptomycin (Life Technologies). Cells were sub-cultured every 5 days (~95% culture confluence) and replenished with new growth medium every 3 days. All experiments were preformed using NHDF, NHEK, and RPTEC cells between passage 4 and 9 and MDCK and C3A cells between passage 60 and 68.

Cryopreservation protocol–serum free

Twenty-four hours prior to freezing, the cell culture medium was replaced with 12.0 ml of fresh growth medium. The adherent monolayer of cells (~95% confluence) was detached from the bottom of the T-flask with the addition of 4.0 ml of 1% Trypsin (Sigma, St. Louis, MO) and 5 mM EDTA (Sigma) for 5 min at 37 °C. After the 5 min incubation period, 14.0 ml of growth medium were added to the cell suspension to inhibit trypsinization. The cell suspension was divided equally, $\sim 1 \times 10^6$ cells per treatment, and the cells were pelleted at 500*g* for 6 min. The trypsin/EDTA/medium supernate was decanted, and the cell pellet was re-suspended in 1.0 ml of the appropriate CP solution at 10 °C. Cells were allowed to equilibrate in their respective solutions for 10 min. The CP solutions consisted of various derivatives of serum-free culture media (FBM, KBM, ReBM, DME, EMEM), HTS (BioLife Solutions, Binghamton, NY) containing 0–20% Me_2SO (Sigma). Me_2SO containing HTS was purchased from BioLife Solutions (Binghamton, NY) under the trade name CryoStor. All CP solutions were serum free. The components of HTS have been reported by Taylor et al. [37,38] and are reproduced in Table 1.

Cells suspended in respective CP solutions (1.0 ml) were placed into cryovials (1.0×10^6 cells/ml) and frozen in a standard non-linear, two-step process in which the cell suspensions were exposed to −20 °C for 1.25 h and then transferred to −80 °C for a subsequent 1.25-h period. Cryovial temperatures were monitored each minute by surface mounted thermocouples, yielding a cooling rate of 0.8 °C min^{-1} from −2 to −20 °C. Once samples reached −20 ° C, a 10 min hold at −20 °C was implemented to ensure that all solutions reach osmotic equilibrium with the freeze-concentrated media. Following the hold, cryovials were cooled at ~0.8° C min^{-1} to −80 °C and then quenched and stored in liquid nitrogen. Following storage, cells were rapidly thawed in a 37 °C water bath for 4 min while gently agitated to achieve uniform thawing. A one-step dilution (1:12 and 1:24) in growth medium was performed and the resulting cell suspension was divided into 150 μl aliquots, placed in separate wells of Falcon 96-well plates, and incubated at 37 °C, 5% CO_2 – 95% air. Growth medium was replenished in the cultures at 24-h intervals.

Table 1
Composition of HypoThermosol (as published by Taylor et al. [37])

Component	Concentration
Ionic	
Na^+	100.00 mM
K^+	42.5 mM
Ca^{2+}	0.05 mM
Mg^{2+}	5.0 mM
Cl^-	17.1 mM
pH Buffers	
$H_2PO_4^-$	10.0 mM
HCO_3	5.0 mM
HEPES	25.0 mM
Impermeants	
Lactobionate	100.00 mM
Sucrose	20.0 mM
Mannitol	20.0 mM
Glucose	5.0 mM
Colloid	
Dextran-40	6.0%
Metabolites	
Adenosine	2.0 mM
Glutathione	3.0 mM
Osmolarity (mOsm/kg)	350
pH	7.6
$[K^+]$ $[Cl^-]$	727

Viability assessment

Post-thaw cell viability was determined both through qualitative and quantitative assessment assays. Qualitative assessment was performed by light microscopy. Primary, quantitative assessment was accomplished using alamarBlue, a non-

toxic, fluorescent metabolic activity indicator (Accumed International, Westlake, OH), diluted 1:20 in Hanks Balanced Salt Solution (Life Technologies) without phenol red (HBSS). Secondary quantitative viability assessment was performed using 0.2% Calcein-AM (Molecular Probes, Eugene, OR), a fluorescent membrane integrity dye, in HBSS.

After a 24-h recovery period, the cultures were removed from the incubator, the medium decanted, and 100 μl of the prepared alamarBlue solution was added to each well. Following the alamarBlue addition, plates were incubated for 1 h at 37 °C in the dark. After the 1-h incubation period (±1.0 min), cell viability was assessed quantitatively using a fluorescent plate reader (Cytoflor 2350, Perseptive Biosystems, Cambridge, MA) with a 530 nm excitation and 590 nm emission filter set. Following each assessment, the alamarBlue was removed from the wells, replaced with 150 μl of growth medium, and plates were returned to the incubator to allow for continued recovery.

Calcein-AM assessment was performed at 1,12, 24, and 48 h post-thaw through the removal of culture medium and the addition of 100 μl of Calcein-AM solution to each well. Working Calcien-AM solution was prepared from a stock Calcien-AM solution and diluted to 0.2% in HBSS. Cultures were incubated at 37 °C for a 1-h period and assessed. Cells stained with Calcein-AM were assessed through manual cell counts via fluorescent microscopy and using a fluorescent plate reader (Cytoflor 2350) with a 485 nm excitation and 590 nm emission filter set.

Data analysis

Fluorescence units were converted to percent survival based upon non-cryopreserved experimental controls (37 °C). Calculations of standard error measurement (SEM) were performed and statistical significance was determined using single factor ANOVA analysis. SEM (±) and ANOVA-based P-values are reported in the text. Each experimental condition represents an $N \geqslant 32$ experimental replicates (minimum of 4 experimental repeats with 8 sample replicates per experiment). Error bars presented for all conditions (controls and experimentals) represent the population variability of each respective sample across all replicates. Control SEM values represent inherent intra- and interexperimental cell culture variability.

Results

Cryopreservation cap traditional preservation medium

Cryopreservation of NHDF and Canine Renal Cells (MDCK) was performed under a standard protocol (serum-free culture media with 5% Me_2SO), and assessment over 48-h post-thaw was performed (Table 2). Assessment of both NHDF and MDCK cells revealed a decline in cell number over the first 24-h post-thaw followed by a increase in cell number by 48-h post-thaw there-by establishing the 24-h post-thaw time point as the nadir in cell survival in both the NHDF and MDCK systems.

Cryopreservation of NHDF was performed under a standard protocol with varying CPA concentrations (serum-free culture media with 0–10% Me_2SO) to determine system survival response (Fig. 1). NHDF preserved in media (FBM) with Me_2SO concentrations ranging from 0% to 1.25% yielded very low survival of 2–6% (±0.27) in comparison with non-frozen controls (100%) ($P < 0.001$). Addition of 2.5% and 5% Me_2SO to the culture medium resulted in a significant improvement in cell survival (24-h post-thaw) over that of media with 0–1.25% Me_2SO ($P < 0.001$), to 24% (±2.41) and 29% (±1.57), respectively. Further increases in CPA concentration (10%) resulted in a decrease in cell survival from both that of 2.5% and 5% Me_2SO to 16% (±1.06) (24-h post-thaw) ($P < 0.001$). These data yielded a peak in NHDF cell survival in media + 5% Me_2SO representing a survival cap.

The identification of a survival cap with peak survival obtained at 5% Me_2SO in the NHDF system prompted analysis of additional cellular systems to determine the universality of the peak. To this extent, NHEK were preserved in serum-free media with Me_2SO concentrations ranging from 0% to 15% (Fig. 2). Attempted preservation of the NHEK cells in media (KBM) with Me_2SO concentrations ranging from 0% to 0.75% failed with survival ranging from 1% to 13% (±0.41). Continued increases in CPA levels from 1.25 to 5% resulted in a significant increases in cell survival at each concentration evaluated ranging from 25% (±0.71) to 50% (±0.82) ($P < 0.001$). Me_2SO concentrations of 10 and 15% resulted in a decrease in cell survival in comparison with that of survival at 5% Me_2SO [41% (±1.02) and 36% (±0.75), respectively, ($P < 0.001$)]. As with the fibroblast system, a CPA dependent survival cap

Table 2
Time-course survival analysis of (A) human fibroblasts (NHDF) and (B) canine renal cells (MDCK) following cryopreservation in either media (FBM or DME) or HypoThermosol (HTS) supplemented with 5% Me_2SO

Time (h)	Control (37 °C)	FBM + 5% Me_2SO	HTS + 5% Me_2SO
(A) NHDF cells			
1	2550 (±48)	2330 (±43)	2510 (±47)
	(100% ± 1.8)	(91% ± 1.8)	(98% ± 1.8)
12	2370 (±56)	2030 (±85)	2350 (±55)
	(93% ± 2.4)	(79% ± 4.2)	(92% ± 2.3)
24	2400 (±61)	1250 (±78)	1910 (±61)
	(95% ± 2.5)	(49% ± 6.2)	(75% ± 3.2)
48	3120 (±65)	1400 (±59)	2340 (±65)
	(123% ± 2.1)	(55% ± 4.2)	(91% ± 2.7)
Time (h)	Control (37 °C)	DME + 5% Me_2SO	HTS + 5% Me_2SO
(B) MDCK cells			
1	5420 (±88)	5257 (±110)	5366 (±83)
	(100% ± 1.5)	(97% ± 2.1)	(99% ± 1.5)
12	5311 (±91)	3902 (±103)	5095 (±112)
	(98% ± 1.7)	(72% ± 2.7)	(94% ± 2.2)
24	5203 (±81)	1693 (±93)	4173 (±121)
	(96% ± 1.5)	(32% ± 5.5)	(77% ± 2.9)
48	7371 (±106)	2818 (±96)	5382 (±137)
	(136% ± 1.4)	(52% ± 3.4)	(99% ± 2.5)

Cell survival was assessed over a 48-h post-thaw interval by microscopy using a fluorescent membrane integrity dye (Calcien-AM). Reported cell number represents total cell number from $N = 6$ experimental repeats (±SD). Calculated percent survival (±SD) is based upon comparison with 1-h non-cryopreserved control samples.

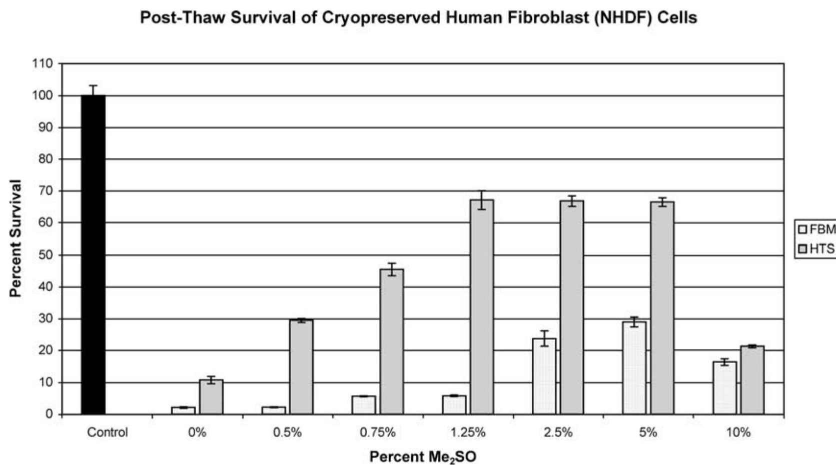

Fig. 1. Twenty-four hour post-thaw cell viability analysis of human fibroblast (NHDF) following cryopreservation. Cell survival percentage (±SEM) determination was based upon fluorescent metabolic activity analysis in comparison with non-cryopreserved controls (37 °C). Statistical significance (P-values) reported in text. $N \geqslant 32$ replicates for each experimental condition.

in the keratinocyte system was identified at media + 5% Me_2SO (50%±0.81).

The results observed in both the fibroblast and keratinocyte cell lines led to the investigation of the cap concept with other cell lines. Accordingly, a human hepatocarsonma cell line (C3A) and human and canine renal cell lines (RPTEC and MDCK, respectively) were investigated. Preser-

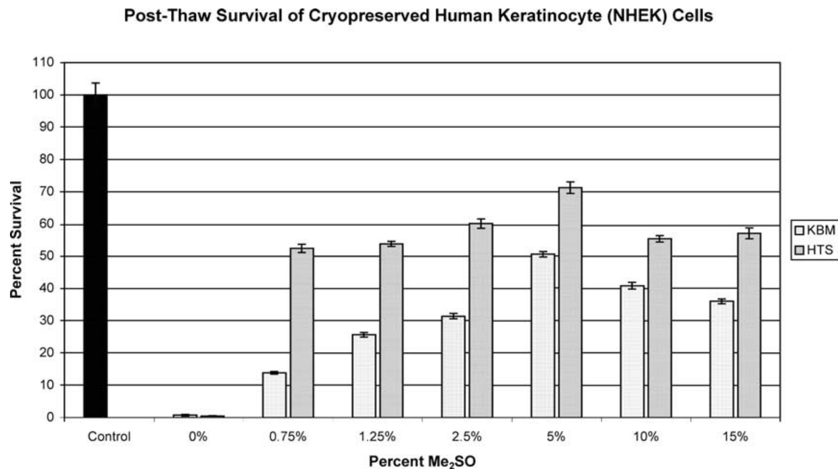

Fig. 2. Twenty-four hour post-thaw cell viability analysis of human keratinocyte (NHEK) cells following cryopreservation. Cell survival percentage (±SEM) determination was based upon fluorescent metabolic activity analysis in comparison with non-cryopreserved controls (37 °C). Statistical significance (*P*-values) reported in text. $N \geqslant 32$ replicates for each experimental condition.

vation of these three-cell types resulted in a similar survival profile to that of the NHDF and NHEK cells, but in all cases the maximal survival levels varied and the optimal Me_2SO concentrations shifted to 10%. Preservation of the hepatic cell line (C3A) resulted in a maximal survival of 56% (±3.14) following preservation in media with 10% Me_2SO (Fig. 3). This peak in survival was significantly higher than cell survival following preservation in either 5% or 15% Me_2SO [14% (±0.47) and 49% (±1.32), respectively, ($P < 0.002$)]. Evaluation of the survival of human renal cells (RPTEC) revealed peak survival of 34% (±0.80) following CP in media with 10% Me_2SO (Fig. 4). This peak represented a significant increase in cell survival over that of cells preserved in either media + 5% or 15% Me_2SO [20% (±0.83) and 26% (±0.79), respectively, ($P < 0.001$)]. CP cap elucidation in a canine renal cell line (MDCK) again revealed a post-thaw survival limit of 43% (±0.87)

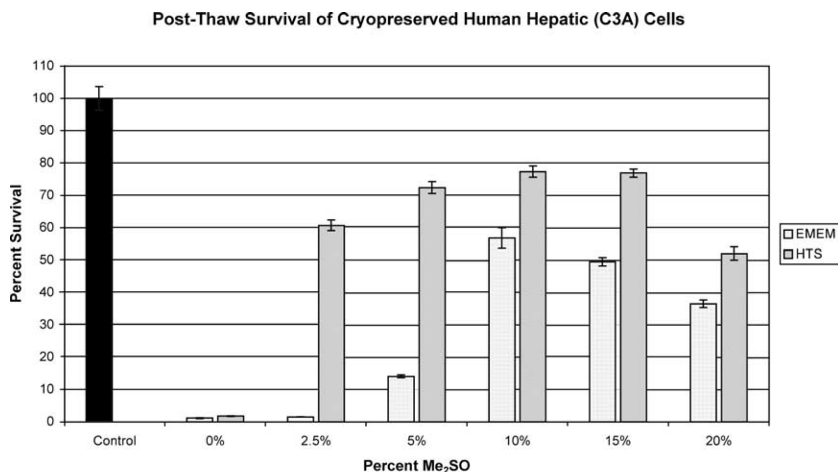

Fig. 3. Twenty-four hour post-thaw cell viability analysis of human hepatocarsonma (C3A) cells following cryopreservation. Cell survival percentage (±SEM) determination was based upon fluorescent metabolic activity analysis in comparison with non-cryopreserved controls (37 °C). Statistical significance (*P*-values) reported in text. $N \geqslant 32$ replicates for each experimental condition.

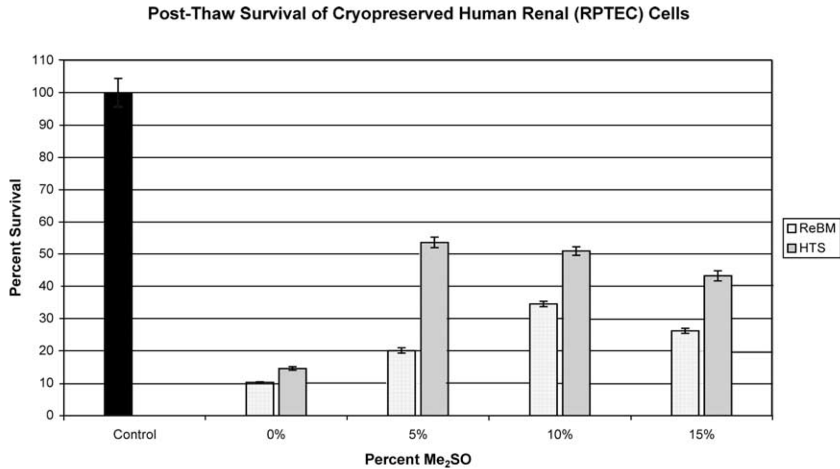

Fig. 4. Twenty-four hour post-thaw cell viability analysis of human renal (RPTEC) cells following cryopreservation. Cell survival percentage (±SEM) determination was based upon fluorescent metabolic activity analysis in comparison with non-cryopreserved controls (37 °C). Statistical significance (P-values) reported in text. $N \geqslant 32$ replicates for each experimental condition.

following preservation in media + 10% Me_2SO (Fig. 5), flanked by significantly lower survival of cells preserved in media + 5% Me_2SO [29% $(\pm 0.36), P < 0.001$] and a slight decrease in survival at 15% and 20% Me_2SO [42% (±1.04), $P \geqslant 0.06$].

The observed survival cap in each of the cell lines described warranted investigation into the possible effect of residual Me_2SO affecting cell post-thaw survival particularly following CP protocols utilizing elevated CPA levels (10–20%). Accordingly, cryopreserved samples were diluted 1:12 and 1:24 in culture media following thawing and cell survival was evaluated. Evaluation of the 1:12 and 1:24 dilutions in the NHDF, C3A, and MDCK cell systems revealed no significant difference in cell survival 24 h post-thaw at all Me_2SO concentrations evaluated (Table 3). In addition, the effect of elevated Me_2SO concentrations (5–20%) on cell viability due to the single-

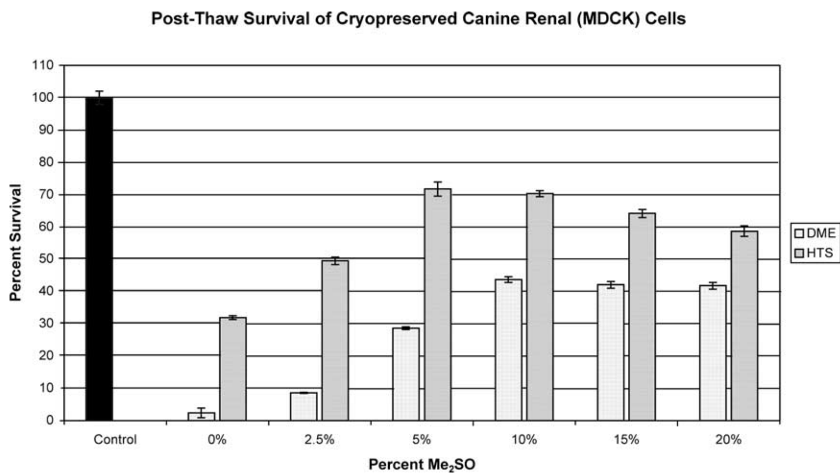

Fig. 5. Twenty-four hour post-thaw cell viability analysis of canine renal (MDCK) cells following cryopreservation. Cell survival percentage (±SEM) determination was based upon fluorescent metabolic activity analysis in comparison with non-cryopreserved controls (37 °C). Statistical significance (P-values) reported in text. $N \geqslant 32$ replicates for each experimental condition.

Table 3
Twenty-four post-thaw evaluation of (A) human fibroblasts (NHDF), (B) human liver cells (C3A) and (C) canine renal cells (MDCK) survival comparing post-thaw dilution ratios of 1:12 and 1:24 following cryopreservation in either media (FBM, EMEM, or DME) or HypoThermosol (HTS) supplemented with Me_2SO

Cell type	Me_2SO (%)	Dilution			
		Media		HTS	
		1:12	1:24	1:12	1:24
NHDF					
	2.5	23.7% (±2.4)	25.1% (±2.9)	66.8% (±1.6)	65.9% (±2.1)
	5	28.9% (±1.57)	31.2% (±1.9)	67.5% (±1.6)	68.4% (±2.0)
	10	16.4% (±1.06)	18.2 (±1.3)	21.1% (±0.45)	23.4% (±0.9)
C3A					
	2.5	1.5% (±0.04)	1.8% (±0.09)	60.6% (±1.6)	61.3% (±1.2)
	5	14.1% (±0.47)	15.3% (±0.93)	72.3% (±1.8)	72.9% (±1.4)
	10	57.6% (±3.1)	58.6% (±2.5)	77.3% (±1.7)	76.1% (±1.4)
	15	49.5% (±1.3)	51.2% (±1.4)	76.8% (±1.2)	78.1% (±1.5)
	20	36.5% (±1.1)	39.3% (±1.2)	52% (±2.0)	54.9% (±1.8)
MDCK					
	2.5	8.6% (±0.12)	9.9% (±0.4)	49.5% (±1.11)	48.9% (1.9)
	5	28.6% (±0.3)	30.2% (±0.6)	71.7% (±2.2)	73.2% (±1.9)
	10	43.7% (±0.87)	43.4% (±1.1)	70.3% (±0.92)	71.6% (±1.4)
	15	42.1% (±1.0)	43.2% (±1.4)	64.1% (±1.2)	66.3% (±1.6)
	20	41.7% (±1.04)	40.9% (±1.6)	58.7% (±1.6)	61.1% (±2.0)

Percent survival was determined in comparison with non-cryopreserved controls. $N = 32$ experimental repeats (±SEM) for each condition.

step addition and dilution protocol was evaluated in CPA-exposed non-frozen controls in comparison with non-frozen media controls (data not shown). This investigation revealed no significant decrease in cell viability in comparison with non-frozen controls in any of the systems, at any of the Me_2SO concentrations evaluated ($P \geqslant 0.075$ for each sample in comparison with non-frozen media controls).

Cryopreservation cap elevation

Recent reports on the benefits of improved cell survival following CP through the incorporation of HTS as the CP medium [1–3] along with the identification of the CP cap led us to hypothize that the incorporation of HTS as the CP medium might yield an improvement in survival. To test this hypothesis, all cell systems were cryopreserved in the same manner as described in the media-based studies with the substitution of HTS as the carrier medium. Fibroblasts cryopreserved in Me_2SO-free HTS failed to adequately survive the preservation process yielding only 10% (±1.17) survival (Fig. 1). Addition of 0.5% Me_2SO to the HTS-base solution resulted in a significant increase in cell survival over that of HTS alone [29% (±0.63) vs 10% (±1.17), $P < 0.001$]. Continued increases in Me_2SO concentrations up to 5% resulted in a significant improvement in survival over that of HTS + 0.5% Me_2SO to 66% (±2.95)($P < 0.001$), followed by a substantial drop in cell survival to 21% (±0.45) following preservation in HTS + 10% Me_2SO($P < 0.001$). Preservation of the NHDF cells in Me_2SO concentrations ranging from 1.25 to 5% resulted in a plateau in cell survival around 66% ($P \leqslant 0.85$).

The observed increase in CP efficacy through the incorporation of HTS as the carrier medium prompted investigation into whether the 24-h nadir in NHDF cell survival remained following CP in HTS + 5% Me_2SO. Post-thaw time course analysis revealed that the 24-h interval remained the survival nadir despite the incorporation of HTS as the carrier medium (Table 2).

The elevation in survival following the incorporation of HTS in the fibroblast cell system was also observed in the NHEK cell system (Fig. 2). As with the NHDF cell line, HTS-base (Me_2SO-free) failed to yield successful preservation of NHEK cells [1% (±0.067)]. Addition of 0.75%

Me_2SO to HTS resulted in a significant increase in cell survival over that of HTS-base [52% (±1.29) vs 1% (±0.067), $P < 0.001$]. This improvement in cell survival continued with increasing Me_2SO concentrations to 5% [71% (±1.77), $P < 0.001$]. Further elevation in Me_2SO concentrations to 10 and 15% yielded significant decreases in cell survival [56% (±1.02) and 57% (±1.71), respectively, $P < 0.001$]. This peak in survival established a CP survival cap at 5% Me_2SO for the NHEK system when HTS was used as the CPA diluent.

As with the NHDF and NHEK cell lines, utilization of HTS as the carrier medium resulted in a significant increase in cell survival following CP of RPTEC, MDCK, and C3A cell systems. C3A cells preserved in HTS-base (Me_2SO-free) again failed to adequately survive the preservation process (2%, ±0.10). The addition of 2.5% Me_2SO resulted in a significant increase in cell survival over that of HTS-base [60% (±1.59) vs 2% (±0.10), respectively, ($P < 0.001$)] (Fig. 3). Increases in the Me_2SO concentration to the 10–15% range yielded continued enhancement in overall cell survival to 76% (±1.72) ($P < 0.001$). The addition of 20% Me_2SO, however, resulted in a substantial decline in post-thaw cell survival [52% (±2.02) ($P < 0.001$)]. In the RPTEC cell system, incorporation of HTS improved post-thaw survival (Fig. 4). Me_2SO-free HTS yielded a low RPTEC post-thaw survival rate of 14% (±0.53). Addition of 5 and 10% Me_2SO elevated cell survival to 52% (±1.61) ($P < 0.001$). As with the other systems examined, continued elevation of CPA concentration to 15% yielded a substantial decline in RPTEC cell survival to 43% (±1.53) ($P < 0.001$). Examination of MDCK cell survival in HTS-base carrier media revealed a significant increase following Me_2SO addition (all concentrations) (Fig. 5). Preservation of MDCK cells in HTS-base yielded a post-thaw survival rate of 32% (±0.57). Addition of Me_2SO to HTS resulted in improved survival up to the 5–10% concentration range yielding peak survival of 71% (±2.21) ($P < 0.001$). Further addition of Me_2SO to 15 and 20% resulted in a decrease in post-thaw survival [64% (±1.23) and 59% (±1.66), respectively, $P < 0.01$]. As with the NHDF cells, post-thaw time course assessment of cell survival over the first 48-h interval revealed the 24-h interval as the nadir in MDCK cell survival (Table 2).

With the observed elevation in survival following preservation in HTS-based CP solutions and the potential influence of residual Me_2SO in the recovery media on cell survival, dilution investigations were undertaken on HTS + Me_2SO samples (Table 3). As with the media with Me_2SO dilution experiments, no significant difference in cell survival was observed in the NHDF, C3A, and MDCK cell systems. In addition, there was no observed decline in cell viability in CPA-exposed non-frozen controls at any of the Me_2SO concentrations in the systems evaluated in comparison with non-frozen media controls ($P \geqslant 0.075$) (data not shown).

Discussion

Reports on CP efficacy assessed within hours post-thaw have yielded highly variable results. For example, reports on MDCK cell survival assessed 1–6 h following thawing vary from 30–95% [1,13]. The high variability in reported survival has been recently linked with the phenomena of *CIDOCD*, cell death continuing over a period of many hours (up to 24 h) following CP (3,4,12). These reports demonstrated that *CIDOCD* contributes to high variability and survival overestimates with early post-thaw assessment. Assessment of survival for both MDCK and NHDF cells over the initial 48-h post-thaw interval verified reports on the contribution of *CIDOCD* in the MDCK system as well as the nadir of survival at 24-h post-thaw for the fibroblast system. Accordingly, we elected to evaluate CP protocol efficacy 24 h post-thaw to account for *CIDOCD*. Additionally, comparison of cell survival calculations between those obtained from calcein-AM vs alamarBlue revealed that the assays corresponded well to one another with the alamarBlue assay yielding a more stringent assessment of cell survival. Based upon our observations as well as previous reports [1,3,27,39], the alamarBlue assay was selected as the primary assessment assay for cell survival.

Utilization of traditional CP methodologies (serum-free culture medium with 0–20% Me_2SO) resulted in a CPA dependent survival limit (cap) in the cell systems tested. Evaluation of survival allowed for the determination of an "optimal" CPA concentration, yielding maximal cell survival. For the cell systems tested, peak survival was obtained from media with 5% Me_2SO (fibroblast and keratinocyte cells) and media with 10% Me_2SO (renal and hepatic cells). Preservation of the cell systems evaluated under sub- or supra-optimal Me_2SO concentrations in the culture medium-based solutions resulted in decreased

survival. We attribute the low cell survival at sub-optimal Me_2SO concentrations to a reduction in the protective capacity of the CP medium due to the inability of the CPA to effectively alleviate cellular damage due to solution effects and prevent intracellular ice formation, ultimately leading to cell death [9,20,22,23]. At CPA concentrations above optimal, we attribute the reduction in cell survival to CPA toxicity [6,9,23]. Concern that elevated Me_2SO concentrations may have resulted in cellular toxicity during the preservation interval as well as following dilution, due to the single-step addition and dilution procedure utilized, arose warranting further investigation. The effect of residual Me_2SO concentrations during the initial 24-h post-thaw interval revealed the residual Me_2SO had no significant effect on cell survival. In addition, there were no observed decreases in cell viability following the single-step addition and dilution of the Me_2SO in non-frozen CPA exposed controls in any of the systems or solutions utilized. The single-step addition and dilution protocol was selected in order to develop and evaluate simplified CP methodologies to facilitate practical integration into research and clinical areas such as cell and tissue engineering and regenerative medicine.

Optimal protocol determination in these studies corresponded with independent reports on CP protocol development for each respective cell system examined [10,16,20,26]. Additionally, the protocols correspond with CP protocols utilized in association with commercial and clinical CP applications [7,14,16,41]. Despite protocol agreement, evaluation of the various cell systems under their optimal CP regime resulted in "sub-optimal" cell survival in comparison with referenced reports. We attribute this difference in reported cell survival to our 24-h post-thaw evaluation in contrast with reports assessing survival 1–6 h post-thaw. As stated previously, the discrepancy between survival reports can be accounted for by *CIDOCD*. We believe that the survival estimates obtained 24-h post-thaw allowed for the systems under evaluation to reach a "survival equilibrium" following the peak of delayed-onset cell death [3] and prior to significant culture repopulation [1,3]. We have found that 24-h post-thaw estimates provide a more reliable assessment of cell survival.

Recent reports on the improvement in cell survival following the substitution of HTS for standard culture media as the CP carrier medium in a canine renal model [1,3] prompted investigation into the effects of HTS on the CP cap. Accordingly, we hypothesized that the utilization of HTS would elevate survival above that of the media-based solutions. Preservation of fibroblast and keratinocyte cells in HTS with 5% Me_2SO resulted in increases in cell survival over that of media-based counterparts (130% and 40%), respectively, ($P < 0.001$). These observations represent a substantial elevation in survival at the determined "optimal" CPA concentrations over media-based systems. As with the fibroblasts and keratinocytes, incorporation of HTS for the preservation of the renal and hepatic cell models resulted in significant increases in cell survival at optimal CPA concentrations over their media-based counterparts. Preservation of the RPTEC cells in HTS + 10% Me_2SO improved survival 30% over that of media + 10% Me_2SO ($P < 0.001$). Utilization of HTS + 10% Me_2SO in the preservation of both the C3A and MDCK cell systems yielded a greater than 50% increase in cell survival over media-based counterparts ($P < 0.001$).

The improvements in cell survival following the incorporation of HTS as the carrier medium at optimal Me_2SO concentrations led us to investigate whether a shift in the CP cap was plausible. Accordingly, fibroblasts and keratinocytes were cryopreserved in HTS with Me_2SO concentrations ranging from 0 to 15%. The utilization of HTS in conjunction with low Me_2SO concentrations yielded significant improvements in cell survival over that of media-based counterparts. Specifically in the fibroblast system, preservation in HTS + 1.25% Me_2SO yielded a 130% increase in survival at a quarter the CPA concentration necessary for peak NHDF preservation in media-based solutions (5% Me_2SO) ($P < 0.001$). As with the fibroblast system, keratinocyte preservation in HTS was accomplished with a 7-fold reduction in Me_2SO concentrations to 0.75% yielding survival equivalent to 5% Me_2SO in media-based solutions ($P = 0.36$). The utilization of HTS as the carrier medium in the renal and hepatic models also supported a reduction in the CPA concentration levels (25–50%) while maintaining survival at or above those of peak media-based survival [C3A: HTS + 2.5% ($P = 0.88$), RPTEC: HTS + 5% ($P < 0.001$), and MDCK: HTS + 2.5% ($P < 0.001$) in comparison to media + 10% Me_2SO].

Utilization of HTS as the carrier medium resulted in a substantial elevation in cell survival over the media-based CP survival cap. We have previously demonstrated that the utilization of HTS as the CP carrier medium significantly re-

duces the level of *CIDOCD* through the modulation of apoptotic and necrotic cell death [4]. Additionally, recent independent reports have implicated the induction of apoptosis and necrosis by preservation induced oxidative stress in a hypothermic model (i.e., sub-lethal stress) [5]. The formulation of HTS is designed to alleviate sublethal stresses experienced by biologists during hypothermic preservation [29,31,32] and has been subsequently shown to modulate the levels of apoptosis and necrosis [3,4]. This, coupled with the identification of *CIDOCD*, leads us to hypothesize that the benefit in preservation (elevation and shift in the survival cap) afforded by the incorporation of HTS was due to a reduction in the level of sub-lethal stress experienced by a cell during the CP process, thereby reducing the level of *CIDOCD*.

The data presented in this study verifies the existence of a CPA dependent limit in cell survival following the utilization of standard CP methodologies. This study further illustrates that the utilization of HTS (an intracellular-like preservation medium) during CP yields a significant improvement in cell survival. Three essential findings supporting this concept are (1) CP efficacy can be improved through the use of HTS with Me_2SO, (2) with an intracellular-like carrier solution, equivalent CP survival can be achieved with significantly reduced Me_2SO levels, and (3) appropriately formulated preservation solutions offer potential protective benefit during CP without Me_2SO utilization.

The observations on the benefits of HTS in CP may prove critical to the advancement of the emerging fields of tissue engineering, gene therapy, and regenerative medicine. For example, the development of engineered liver assist devices often rely on the use of hepatocellular systems (i.e., porcine hepatocytes, C3A cells, etc.). Current preservation strategies pose a problem in both efficacy and Me_2SO-related toxicity issues for the preservation of these devices [15,16,18,41]. Similarly, the emergence of regenerative medicine, specifically cell and gene therapy, requires CP methodologies that are compatible with direct injection of the transformed biologic in comparatively large volumes of CP solution [14,40]. Today, this volume dependence necessitates both timely and complex washing procedures to reduce and/or eliminate Me_2SO levels. These example applications would clearly benefit from a CP regime based upon trace/or low levels of Me_2SO, or preferably a Me_2SO-free preservation strategies, rather than on the molar concentrations currently utilized. The HTS model detailed in this study provides a basic strategy whereby effective, essentially Me_2SO-free CP may be achieved.

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