



Effects of freezing profile parameters on the survival of cryopreserved rat embryonic neural cells

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ABSTRACT

The ability to successfully cryopreserve neural cells would represent an important advance with benefits to neural tissue engineering, neural transplantation, and neuroscience research. We have examined key factors responsible for damage to rat embryonic neural cells during cryopreservation using a two-step temperature profile, with an emphasis on the effects of cooling rate and plunge temperature. Our results indicate that the initial addition of 8% dimethyl sulfoxide (DMSO) and seeding of extracellular ice do not significantly decrease viable cell yield. However, subsequent freezing resulted in significant cell losses for all profile parameter combinations examined. A maximum post-thaw survival of 56% (compared to unfrozen controls) was observed after cooling at 2 °C/min to –80 °C followed by direct immersion in liquid nitrogen. Single-step removal of DMSO after thawing was associated with an additional 40–70% loss of viable cells, and the number of viable cells was further reduced by approximately 70% after 2 days of cell culture (resulting in a net viable cell yield of $9.6 \pm 0.4\%$). Nonetheless, the cryopreserved neurons that did survive displayed a normal morphology, including formation of neurites. Trends in neuronal viability conformed with predictions of existing theoretical models of cell freezing, with reduced survival for rapid cooling rates or high plunge temperatures (attributable to intracellular ice formation), and decreasing viability with increasing profile duration (consistent with the known effects of cell dehydration at suboptimal cooling rates). These observations suggest that neural cells are good candidates for further refinement of freezing profile design using a physics-based approach to parameter optimization.

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1. Introduction

Effective cryopreservation strategies for neural cells would be of benefit in various clinical and research applications. For example, transplantation of neural cells has shown promise for treatment of traumatic brain injury (Soares et al., 1995; Muir et al., 1999; Shear et al., 2004), Parkinson's disease (Lindvall et al., 1990; Ben-Hur et al., 2004; Inden et al., 2005), and other neurodegenerative diseases (Bond et al., 1989; Dinsmore, 1998; Watts and Dunnett, 2000; Lindvall and Björklund, 2004; Totoiu et al., 2004; Ben-Hur et al., 2005; Oliveira and Hodges, 2005; Wernig et al., 2008; Walker et al., 2009). However, these strategies often require pooling of tissue from multiple donors to generate a sufficient quantity of cells

for effective treatment (Brundin et al., 2000). The ability to cryopreserve brain tissue would facilitate pooling of tissue from distinct donors, and would also allow time for testing to ensure safety and function of the cells prior to transplantation. Successful cryopreservation would also be useful for neural tissue engineering, because it would allow long-term storage for purposes of inventory control, quality control and product distribution (Karlsson and Toner, 1996). Moreover, cryopreservation of primary neural cells would greatly facilitate the logistics of basic neuroscience research, by allowing researchers to perform extended experiments using the same tissue source, and by reducing the required frequency of cell isolation (Otto et al., 2003).

Rat embryonic brain tissue is commonly used for neuroscience research. Because of the short shelf-life of isolated brain tissue, cells are typically used in experiments immediately after isolation, or may be refrigerated for a short time before use. The ability to cryopreserve neural cells in liquid nitrogen (LN₂) would yield significant practical benefits to neuroscience research, including more efficient use of isolated brain tissue (thereby decreasing the number of animals used in experiments), as well as advantages associated with making available cells from a single isolation for use over time in long-term experiments (thus mitigating the

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confounding effects of harvest-to-harvest variability). In addition to its value in neuroscience research, rat embryonic brain tissue is a convenient model system for analyzing the effect of cryopreservation on the function and viability of neural cells, with implications for clinical applications of neural transplantation. Various researchers have investigated cryopreservation of rat embryonic brain tissue, with viable cell recovery ranging between 8% and 62% of unfrozen controls, depending on the method used to assess viability and the gestational age and anatomical region of the donor tissue (Jensen et al., 1984; Collier et al., 1988, 1993; Mattson and Kater, 1988; Sauer et al., 1992; Swett et al., 1994; Sautter et al., 1996, 2000; Negishi et al., 2002; for a review, see also Paynter, 2008). Although these previous studies suggest that cryopreservation of rat embryonic brain cells may be feasible, improvements in viable cell recovery are needed before the full potential of cryogenic banking can be realized. Embryonic cortical neurons, the focus of our present study, appear to be particularly sensitive to the cryopreservation process, with a post-thaw viability of only 8.2% reported previously (Negishi et al., 2002).

The development of cryopreservation procedures is a challenging undertaking, which involves selection of the optimal medium composition, cooling and warming procedures, as well as cryoprotectant additive (CPA) loading and dilution methods. The problem is further complicated by the fact that the processing steps are interdependent, and thus cannot be optimized independently. For example, the optimal cooling rate is known to depend on the CPA concentration (Mazur, 1970), whereas the optimal warming rate depends on the cooling procedure (Karlsson, 2001). As a result, rigorous empirical optimization requires a prohibitively large number of experiments (Karlsson and Toner, 2000). However, previous studies have demonstrated that biophysical models can be used to simulate the outcome of cryopreservation procedures, dramatically decreasing the effort required to identify the optimal method (Karlsson et al., 1996, 2009). Nonetheless, because some cell types are known to exhibit idiosyncratic behavior that is not accounted for in existing mathematical models, it is prudent to first confirm that the basic responses of neural cells to cryopreservation are consistent with the key predictions of physics-based models, before attempting to adapt computer-aided optimization techniques to neural cryopreservation procedures. In particular, if the dependence of neural cell survival on freezing profile parameters such as cooling rate and plunge temperature (i.e., the temperature from which samples are immersed into LN₂) does not match the qualitative trends expected from biophysical models of cell freezing (Mazur, 1984; Karlsson et al., 1996), then any invalid modeling assumptions must be identified and corrected before a theoretical approach to profile design can be used with neurons.

Few previous investigations have examined the factors that cause damage to rat embryonic neural cells during the cryopreservation process. Only the effects of cooling rate (Das et al., 1983), CPA concentration (Das et al., 1983; Kawamoto and Barrett, 1986; Fang and Zhang, 1992; Negishi et al., 2002), and thawing procedure (Das et al., 1983; Fang and Zhang, 1992) have been reported. However, none of these previous studies induced extracellular ice formation under controlled and reproducible conditions, which makes it difficult to interpret the published results in the context of theoretical models of the freezing process. Moreover, we are not aware of any prior systematic investigation of the effect of the LN₂ plunge temperature on neural cell freezing.

In the present study, we examined the effect of various factors of a common cryopreservation technique on the viability of rat embryonic brain cells frozen using DMSO as the CPA. In particular, we have focused our investigation on the effects of cooling rate and plunge temperature in a two-step freezing profile. Significantly, we have seeded the extracellular ice under consistent conditions at the start of each profile, in order to ensure reproducible results

that comply with the assumptions of existing theoretical models of the cellular response to freezing (Mazur, 1984; Toner et al., 1990; Karlsson et al., 1993). We have also examined the effects of the CPA addition and removal processes, whereas these factors have not been adequately investigated in previous work on neural cell cryopreservation. Our experiments resulted in improved post-thaw survival of cortical neurons compared with previous studies, and our findings lay the groundwork for further optimization of neural cryopreservation processes using physics-based models.

2. Materials and methods

2.1. Isolation of neural tissue

Cerebral cortices were harvested from embryonic day 17 rat fetuses in accordance with the Georgia Institute of Technology Institutional Animal Care and Use Committee regulations. Timed-pregnant Sasco Sprague-Dawley rats (Charles River, Wilmington, MA) that had been anesthetized with halothane (Halocarbon, River Edge, NJ) were euthanized by decapitation, and the uterus was immediately removed by Caesarian section and placed in ice cold Hanks Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA). Rat fetuses were removed from the amniotic sac and sacrificed. The brains were subsequently excised, and the cerebral cortices were isolated. Cortical tissue was either dissociated immediately for cryopreservation experiments, or stored overnight at 4 °C in L15 medium (Invitrogen) supplemented with 2% (v/v) B-27 (Invitrogen), before dissociation the following day. To prepare the tissue for dissociation, cortices were incubated in a pre-warmed solution of 0.25% (w/v) trypsin and 1 mM EDTA solution (Invitrogen) for 10 min at 37 °C. After rinsing with HBSS, 0.15 mg/ml deoxyribonuclease I (Sigma, St. Louis, MO) in HBSS was added, and the tissue was dissociated by trituration with a flame narrowed Pasteur pipet. The resulting cell suspension was centrifuged at 200 × g for 3 min, and resuspended in either Neurobasal medium (Invitrogen), L15 medium or B-27 supplemented L15 medium at a density of 5–10 × 10⁶ viable cells/ml, as determined by trypan blue exclusion. The samples were then stored on ice for 2–8 h until the start of cryopreservation experiments. For each of four separate isolations, an aliquot of the cell suspension was set aside as an unfrozen control. These unfrozen control aliquots were stored on ice and assayed for viability by trypan blue exclusion directly before initiation of the cryopreservation protocol.

2.2. Cryopreservation of neural cells

Cell suspensions prepared as described above were diluted 1:4 with 10% (v/v) DMSO (EM Science, Gibbstown, NJ) in L15 medium, to achieve a final concentration of 8% (v/v) DMSO. Aliquots of 0.5–1 ml of this cell suspension were transferred into 1.5-ml cryovials (Corning, Corning, NY) and kept at 4 °C for 30 min to allow for DMSO equilibration. A subset of samples was then plunged directly into LN₂. The remaining cryovials were cooled to –5 °C, at which temperature extracellular ice was seeded using chilled forceps. After ice seeding, samples were equilibrated at –5 °C for 10–15 min. Samples were then either thawed for viability assessment, plunged directly into LN₂, or cooled in a controlled rate freezer (CryoMed IVF Freezer, Thermo Electron) at 0.1, 0.5, 1, 2, 5, or 10 °C/min to a temperature of –40 °C, –80 °C or –120 °C prior to plunging into LN₂. After storage in LN₂ for 2–13 days, samples were thawed in a 37 °C water bath for 10–15 min, and the concentration of viable cells was determined by trypan blue exclusion. In addition, for a subset of the samples frozen at 2 °C/min, further viability studies were undertaken. After thawing, these cells were centrifuged at 200 × g for 3 min, the supernatant was aspirated to remove DMSO, and the

cell pellet was resuspended in 440 μ l neuronal medium; neuronal medium consists of 2% (v/v) B-27 and 500 μ M L-glutamine (Invitrogen) in Neurobasal medium. A 50 μ l aliquot of this suspension was assayed by trypan blue exclusion to determine the cell viability after CPA removal. The remaining suspension was then used for cell culture experiments, as described below.

2.3. Cell culture

Neurons were cultured at 37 °C under a humidified 5% CO₂ atmosphere. Cells were plated in tissue culture 48-well plates which had been pretreated with 0.25 ml/well of 0.5 mg/ml poly-L-lysine overnight at 37 °C, and subsequently with 0.25 ml/well of 0.6 mg/ml Matrigel (Becton Dickinson Biosciences; Bedford, MA) in Neurobasal medium for 2–4 h at 37 °C. Immediately prior to plating, excess Matrigel solution was removed, leaving a thin film of matrix to promote neuronal attachment. Freshly dissociated cells (unfrozen controls) were diluted in neuronal media to a concentration of 8×10^5 viable cells/mL, and 250- μ l aliquots of the resulting suspension were seeded into 3 replicate wells (seeding density $\sim 2 \times 10^5$ cells/cm²). Frozen-thawed samples were diluted 2:3 in neuronal media, and 250- μ l aliquots of the resulting suspension were seeded; 3 replicates were seeded for each freezing protocol. At 48 h following plating, the viability and density of cultured cells were assessed as described below.

2.4. Viability assessment

Cell suspensions were assayed for viability using the trypan blue exclusion technique, as follows. Neural cell suspensions were combined with 0.4% (w/v) trypan blue solution (Sigma) in a ratio ranging from 1:1 to 2:1, and unstained cells were counted manually using a hemocytometer. The viability and density of cultured cells were determined using a fluorescence-based live-dead assay (calcein-AM and ethidium homodimer-1, Molecular Probes, Eugene, OR), as follows. Cultures in 48-well plates were incubated in Dulbecco's phosphate-buffered saline (DPBS, Invitrogen) containing 4 μ M ethidium homodimer-1 and 2 μ M calcein-AM for 30 min at 37 °C, and then rinsed with DPBS before imaging. The imaging system consisted of a microscope with epifluorescence illumination (Eclipse TE300, Nikon, Melville, NY), a digital camera (DKC-ST5/DMC, Sony, Tokyo, Japan), and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). For each well, fluorescence images of 1–3 randomly selected fields were acquired, and the numbers of live (green) and dead (red) cells counted.

2.5. Data analysis

Two different quantitative measures of viability were used to analyze the data: viable cell yield and percent viability. The viable cell yield was defined as the density of viable cells in the frozen-thawed samples, normalized to the density of viable cells in the unfrozen control for the corresponding isolation, after correcting for any dilution and/or concentration of samples during experimental processing. Viable cell density was determined using either the trypan blue exclusion assay or live-dead staining, as described above. Percent viability was calculated from live-dead assays by dividing the number of viable (green fluorescent) cells counted in a given sample by the total number of cells observed in that sample. The viable cell yield is superior to the percent viability as a metric for evaluating the outcome of cryopreservation procedures, because the former represents the fraction of cells that ultimately survive the cryopreservation process. The percent viability, on the other hand, only reflects the fraction of viable cells among the subpopulation that was recovered, and does not account for the cells that are lost during the cryopreservation process. Nonetheless, we

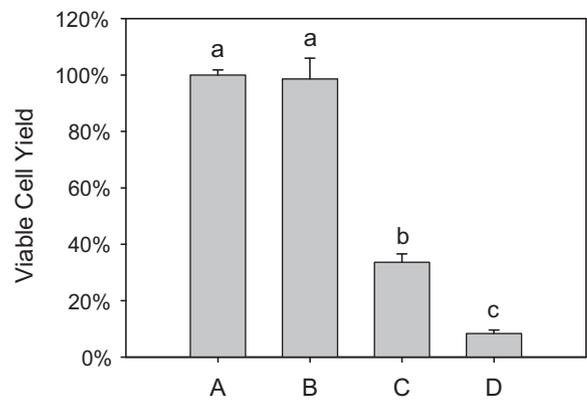


Fig. 1. Post-thaw yield of viable cells at various points of the cryopreservation process. (A) Unfrozen control cells. (B) Cells thawed after DMSO loading and seeding of ice. (C) Cells thawed after DMSO loading, seeding of ice, cooling at 1 °C/min to -80 °C, followed by plunging into LN₂. (D) Cells thawed after DMSO loading followed directly by plunging into LN₂. Significant differences are denoted by distinct labels (a, b, c). Sample size: $n = 2$.

have calculated both the percent viability and the viable cell yield, because the percent viability is commonly used to evaluate cryopreservation procedures in the published literature. All data are reported as the average and standard error of the mean. Experiments were analyzed by one-way or two-way ANOVA, and Fisher's least significant difference test was used for pairwise comparisons. Effects were considered statistically significant at a level $p = 0.05$.

3. Results

Initially, we investigated the response of neural cells to the individual steps of a conventional cryopreservation procedure (controlled cooling at 1 °C/min in the presence of 8% DMSO). As shown in Fig. 1, the processes of CPA addition and extracellular ice seeding did not cause a significant reduction in the viable cell yield. However, subsequent cooling at 1 °C/min, followed by a LN₂ plunge from -80 °C, reduced the viable cell yield by $66 \pm 3\%$. As a negative control, samples were plunged into LN₂ directly after DMSO addition; the resulting viable cell yield was only $8 \pm 1\%$, illustrating the importance of ice seeding and controlled cooling for minimization of damage during cryopreservation of neural cells.

To determine the effects of cooling rate and plunge temperature on post-thaw survival, we conducted a factorial design experiment exploring cooling rates in the range 0.1–10 °C/min and plunge temperatures ranging from -40 °C to -120 °C. The resulting viable cell yields are shown in Fig. 2. Analysis by two-way ANOVA indicated that the main effects of cooling rate and plunge temperature, as well as the interaction between these parameters, were all statistically significant.

As shown in Fig. 2(a), post-thaw survival was low for slow cooling rates, reached a maximum for intermediate cooling rates, and decreased for fast cooling rates. A maximum viable cell yield of $56 \pm 4\%$ was observed for a cooling rate of 2 °C/min and a plunge temperature of -80 °C. For cooling rates of 2 °C/min or slower, the survival after plunging into LN₂ at -40 °C was significantly lower than the survival for plunge temperatures of -80 °C and -120 °C.

The effect of cooling at supraoptimal rates (>2 °C/min) is illustrated in Fig. 2(b). For plunge temperatures of -80 °C and -120 °C, post-thaw survival was significantly reduced for cooling rates higher than 2 °C/min. In particular, for a plunge temperature of -120 °C, there was a clear trend toward lower viability as the cooling rate increased, with a significantly lower viable cell yield for cooling at 5 °C/min compared to 2 °C/min, and a significantly lower viable cell yield for cooling at 10 °C/min compared to 5 °C/min.

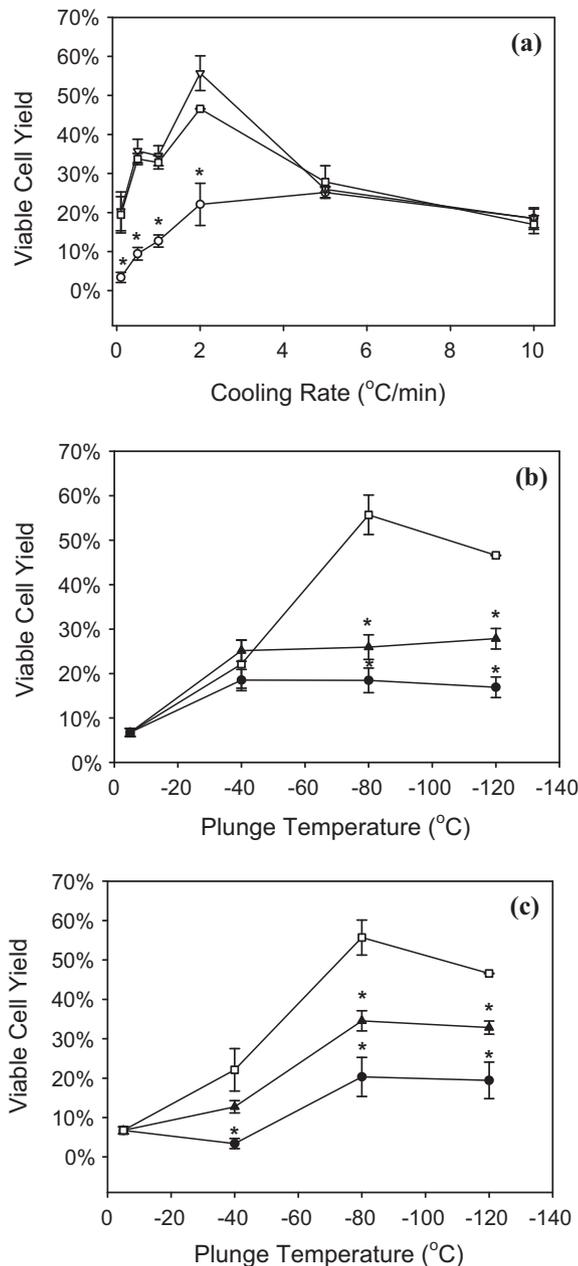


Fig. 2. Effect of cooling rate and plunge temperature on post-thaw yield of viable cells. (a) Effect of cooling rate at plunge temperatures of -40°C (circles), -80°C (triangles) and -120°C (squares); asterisks indicate a statistically significant difference compared to the other plunge temperatures. (b) Effect of plunge temperature at rapid cooling rates, including $2^{\circ}\text{C}/\text{min}$ (squares), $5^{\circ}\text{C}/\text{min}$ (triangles) and $10^{\circ}\text{C}/\text{min}$ (circles); asterisks indicate a statistically significant difference compared to samples cooled at $2^{\circ}\text{C}/\text{min}$. (c) Effect of plunge temperature at slow cooling rates, including $2^{\circ}\text{C}/\text{min}$ (squares), $1^{\circ}\text{C}/\text{min}$ (triangles) and $0.1^{\circ}\text{C}/\text{min}$ (circles); asterisks indicate a significant difference compared to samples cooled at $2^{\circ}\text{C}/\text{min}$. Sample size: $n=3$, except for samples cooled at $2^{\circ}\text{C}/\text{min}$ and plunged at -120°C ($n=2$), and for samples cooled at $10^{\circ}\text{C}/\text{min}$ ($n=9$).

Fig. 2(c) illustrates the effect of cooling at suboptimal rates ($<2^{\circ}\text{C}/\text{min}$). In general, the viable cell yield decreased with decreasing cooling rate in this regime. The survival of cells frozen at $1^{\circ}\text{C}/\text{min}$ was significantly lower than the survival of cells frozen at $2^{\circ}\text{C}/\text{min}$ for plunge temperatures less than -40°C . There was also a significant decrease in viable cell yield for cells cooled at $0.1^{\circ}\text{C}/\text{min}$ compared to $1^{\circ}\text{C}/\text{min}$ for plunge temperatures lower than -5°C .

It is necessary to remove cryoprotectant chemicals from the cell suspension after freezing and thawing, because long-term expo-

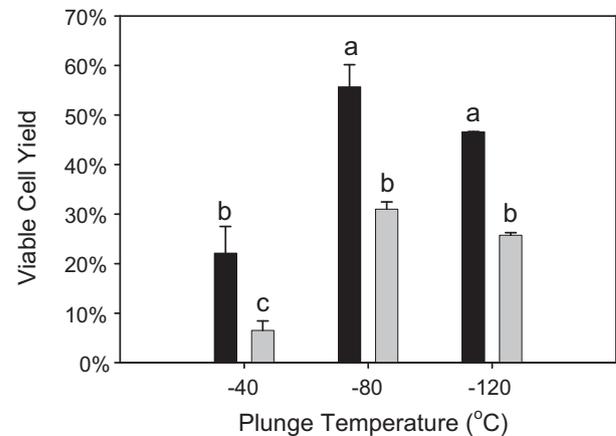


Fig. 3. Effect of DMSO removal on viable cell yield for various plunge temperatures. Viable cell yield was determined for frozen-thawed samples before (black bars) and after (grey bars) removal of DMSO by a single-step centrifugation procedure. All samples were cooled at $2^{\circ}\text{C}/\text{min}$ before plunging into LN_2 at the indicated temperature. Significant differences are denoted by distinct labels (a, b, c). Sample size: $n=2-3$.

sure to CPAs such as DMSO can be deleterious. Thus, for a subset of the cryopreserved samples, we removed the 8% DMSO freezing solution using a single-step dilution process. As shown in Fig. 3, the CPA removal process was associated with a statistically significant loss of viable cells in the frozen-thawed sample. For samples that had been cryopreserved using the most favorable freezing conditions (cooled at $2^{\circ}\text{C}/\text{min}$ and plunged at -80°C), the viable cell yield was reduced from $56 \pm 4\%$ immediately after thawing to $31 \pm 2\%$ after CPA removal. The plunge temperature had a statistically significant effect on the percentage of viable cells lost during the CPA removal procedure ($p < 0.05$), which suggests that the freeze-thaw process affects susceptibility to damage during CPA removal. Viable cell loss during CPA removal was lowest ($44 \pm 3\%$) for samples plunged at -80°C , whereas the largest reduction in viable cell yield ($71 \pm 9\%$) was seen during removal of DMSO from samples that had been plunged at -40°C .

Finally, we re-examined the viable cell yield and the percent viability of cryopreserved neurons after culture for 48 h. After 48 h, the percent viability of neurons cultured from cell suspensions that had been frozen at $2^{\circ}\text{C}/\text{min}$ ranged from 15% to 45%, depending on plunge temperature. In contrast, in unfrozen control cultures, 90% of neurons were viable, indicating that standard harvest and culture conditions were favorable. Fig. 4 shows the viable cell yield after culture of cells that had been frozen at $2^{\circ}\text{C}/\text{min}$ and plunged at -40°C , -80°C or -120°C . The highest yield of viable cells ($9.6 \pm 0.4\%$) resulted from culture of cells that had been cryopreserved using a plunge temperature of -80°C . The reduction of viable cell yield from the end of the CPA removal procedure (Fig. 3) to the end of the 48-h culture period (Fig. 4) was not significantly affected by plunge temperature ($p = 0.72$); the average reduction of viable cell yield in culture was approximately 70%. However, despite the drastic reduction in cell survival after 48 h of culture, the viable neurons from cryopreserved samples had morphology similar to that of unfrozen controls, including extension of neurites to begin formation of an interconnected network, as shown in Fig. 5.

4. Discussion

Although several previous studies have investigated the cryopreservation of neural cells, in many cases it is difficult to make direct comparisons to the present study due to differences in the species, age, and anatomical region of the donor tissue. For example, the viability of rat embryonic neural tissue after cryopreservation has

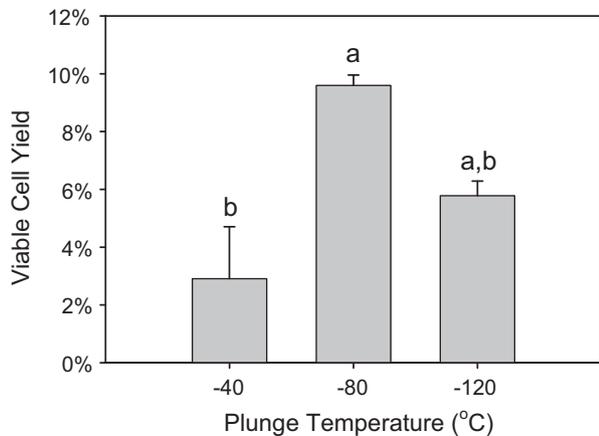


Fig. 4. Viable cell yield of frozen-thawed neurons after 48 h of culture. All samples were cooled at 2 °C/min and plunged into LN₂ from the indicated temperature, before thawing and DMSO removal. Significant differences are denoted by distinct labels (a, b). Sample size: $n = 2-3$.

been shown to vary widely depending on the gestational age of the donor (Sørensen et al., 1986). Comparison between studies is also confounded by differences in the methods used to measure the success of the cryopreservation procedure. Membrane integrity assays such as trypan blue exclusion are commonly used, and the results

are generally reported as a percent viability only (i.e., the percentage of recovered cells that are viable) (Collier, 1987; Mattson and Rychlik, 1990; Sauer et al., 1992; Archer et al., 1994; Sautter et al., 1996, 2000; Cameron et al., 1997; Decherchi et al., 1997). However, the total number of cells recovered after cryopreservation can be significantly lower than the starting cell count (Collier et al., 1988, 1993; Swett et al., 1994; Negishi et al., 2002). For example, Negishi et al. (2002) reported a post-thaw cell yield of only 26% compared to the total number of cells in the unfrozen control, whereas the percent viability was 89% within the cell population that was recovered after thawing. In the present study, we have evaluated outcomes using the viable cell yield, defined as the number of viable cells in the cryopreserved sample, normalized to the number of viable cells in the unfrozen control sample (with appropriate corrections for sample dilutions). The viable cell yield reflects the proportion of the initial cell population that ultimately survives the cryopreservation process, and is therefore a more suitable metric than percent viability for evaluating cryopreservation protocols.

To the authors' knowledge, there are only three previous investigations of cryopreservation of neural cells from rat embryonic cortices at gestational ages similar to the donor age used in our present study (Das et al., 1983; Fang and Zhang, 1992; Negishi et al., 2002). In two of the published studies, only qualitative outcome measures were used — i.e., survival in culture (Fang and Zhang, 1992) and survival after transplantation (Das et al., 1983) — so it is impossible to make quantitative comparisons to the present work. However, Negishi et al. (2002) measured cell membrane integrity after cryopreservation and reported their results in terms of viable cell yield (as well as percent viability), allowing for comparison with the present work. They performed experiments with intact cortical tissue as well as with neural cell suspensions, and in the former case, the frozen-thawed tissue was enzymatically disaggregated before performing viability assays. When freezing intact cortical tissue, Negishi and co-workers achieved a viable cell yield of 28% (corresponding to a percent viability of 89% among the cells recovered after thawing), following cryopreservation using 10% DMSO, a cooling rate of ~1 °C/min, and a plunge temperature of -80 °C (Negishi et al., 2002). However, when they used this same procedure to cryopreserve neural cell suspensions, they were only able to achieve a percent viability of 8.2% (viable cell yield was not reported for this case; however, 8.2% represents an upper bound for the viable cell yield) (Negishi et al., 2002). In contrast, we have cryopreserved suspensions of isolated neural cells, achieving a viable cell yield of 31% when using a procedure consisting of equilibration with 8% DMSO, cooling at 2 °C/min after extracellular ice seeding at -5 °C, and plunging at a temperature of -80 °C. The superior results obtained in our present study may have been due to the documented benefits of ice seeding (Diller, 1975), or to the reduced time of exposure to inhospitable conditions afforded by the faster rate of cooling (Mazur et al., 1972). For example, in our experiments, the post-thaw viable cell yield after freezing at 2 °C/min was over 70% higher than the viable cell yield for freezing at 1 °C/min. Thus, simply optimizing the rate of cooling resulted in significant improvement of cell survival.

Although our cryopreservation technique represents an improvement compared to previous methods, the ultimate yield of viable neurons after cryopreservation remains unsatisfactory, indicating that further optimization of the cryopreservation process is needed. Whereas physics-based models of the cryopreservation process have been shown to facilitate optimization of procedures for other cell types (Karlsson et al., 1996, 2009), it is unknown whether these models would also be effective for rat embryonic neural cells. To evaluate the feasibility of adapting existing physics-based models for use with rat embryonic neural cells, we have compared our results with theoretical expectations. Our models are based on Mazur's two-factor hypothesis of cryoinjury,

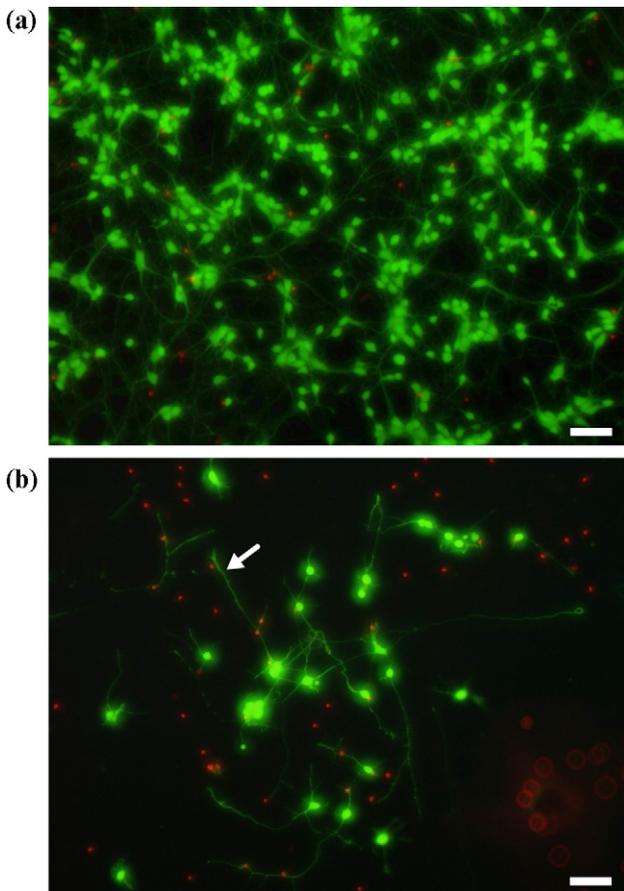


Fig. 5. Fluorescence micrographs of (a) control and (b) cryopreserved neural cells, stained with calcein-AM (green) and ethidium homodimer-1 (red) after 48 h in culture. Cryopreserved cells were cooled at a rate 2 °C/min to a plunge temperature of -80 °C, then immersed into LN₂. Neurons that survived cryopreservation exhibited normal neuronal morphology, including formation of neurites (arrow). Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

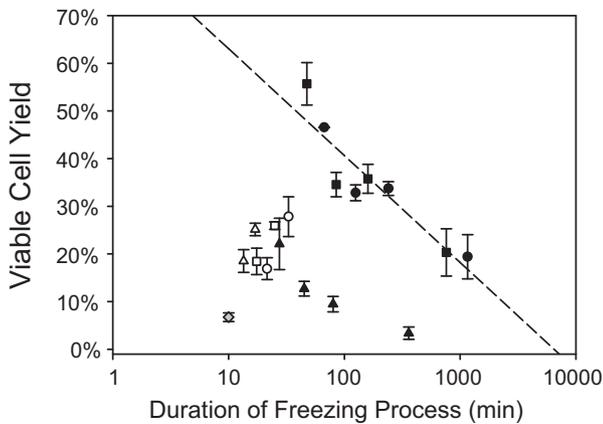


Fig. 6. Effect of freezing process duration on post-thaw viable cell yield. Samples were cooled slowly ($\leq 2^\circ\text{C}/\text{min}$, closed symbols) or rapidly ($> 2^\circ\text{C}/\text{min}$, open symbols) to temperatures of -40°C (triangles), -80°C (squares), or -120°C (circles) before plunging into LN_2 . Also shown are data for samples plunged into LN_2 directly after ice seeding at -5°C (grey diamond). The dashed line is a logarithmic fit to the data from samples cooled slowly to plunge temperatures lower than -40°C (i.e., closed circles and closed squares).

which posits that cell damage during freezing results from two distinct mechanisms (Mazur et al., 1972): (1) intracellular ice formation, and (2) so-called solution effects (a class of damage mechanisms that includes cytotoxicity of the freeze-concentrated aqueous solution). Our results match the trends predicted by the two-factor hypothesis, with low post-thaw survival observed for slow cooling rates (attributed to solution effects), a decrease in survival at fast cooling rates (attributed to intracellular ice formation), and a survival maximum at an intermediate cooling rate (here, $2^\circ\text{C}/\text{min}$). We also observed a drop-off in cell survival with increasing plunge temperatures; this type of trend is consistent with injury predicted to occur due to formation of intracellular ice during the LN_2 plunge, caused by insufficient cell dehydration during cooling to the plunge temperature (Karlsson et al., 1996). Overall, our findings suggest that ice formation in neural cells can be inhibited if cells are cooled at a rate of $2^\circ\text{C}/\text{min}$ or lower to a plunge temperature of -80°C or lower. However, even in samples that were cooled slowly to avoid deleterious intracellular crystallization, we observed loss of over 40% of viable cells after thawing, representing cryoinjury attributable to solution effects.

To analyze the contribution of solution effects to injury of cryopreserved neural cells, we have examined the dependence of post-thaw survival on the duration of the freezing process, because this exposure time is known to correlate with the extent of damage due to solution effects (Mazur et al., 1972; Karlsson et al., 1996). Thus, in Fig. 6, the viable cell yield data from Fig. 2 have been re-plotted as a function of freezing process duration (which was defined as the time elapsed between ice seeding and plunging into LN_2). As explained above, for cells cooled slowly ($\leq 2^\circ\text{C}/\text{min}$) to low plunge temperatures ($\leq -80^\circ\text{C}$), damage due to solution effects is expected to be the dominant mechanism of cell injury. The survival data for the corresponding experimental groups (closed circles and closed squares in Fig. 6) follow a logarithmic trend ($R^2 = 0.86$). The logarithmic fit to these data predicts a reduction in viable cell yield by 22% for each ten-fold increase in freezing process duration. Likewise, the best-fit logarithmic trend predicts that solution effects will be negligible for a process duration of 13.5 s. Samples cooled rapidly ($> 2^\circ\text{C}/\text{min}$) and those plunged from high temperatures ($> -80^\circ\text{C}$) exhibited post-thaw viable cell yields falling below the solution effects trend-line in Fig. 6, suggesting additional injury due to formation of intracellular ice crystals (Mazur et al., 1972; Karlsson et al., 1996). Together, our results demonstrate that the response of the rat embryonic neural cells to freezing is consistent

with the predictions of existing biophysical models, suggesting that model-based optimization represents a viable strategy for developing effective cryopreservation procedures for these cells.

In the meantime, other approaches to improving the survival of cryopreserved rat embryonic neural cells can be identified based on our finding that solution effects cryoinjury appears to be a limiting factor in the survival of neural cells frozen using a two-step profile. For example, because cell injury due to solution effects is dependent on the composition of the freezing medium, it may be possible to mitigate this damage by using DMSO at a different concentration, or by using a different CPA altogether. Previous studies of neural cell cryopreservation have reported reductions in viability when DMSO concentrations were increased beyond 15% (Kawamoto and Barrett, 1986; Fang and Zhang, 1992; Negishi et al., 2002). However, those studies used single-step CPA addition and removal protocols, which are known to result in deleterious cell volume excursions at high CPA concentrations (Karlsson et al., 2009). Therefore, we cannot rule out the possibility that higher DMSO concentrations might be beneficial during the freeze-thaw process if osmotic injury were prevented by use of optimized multi-step CPA addition and removal procedures (Karlsson et al., 2009). In addition to testing DMSO as a cryoprotectant for rat neural cells, Fang and Zhang (1992) investigated the use of glycerol and polyvinylpyrrolidone; these alternative CPAs were found to be less effective than DMSO. However, to our knowledge, cryopreservation of rat neural cells using other known cryoprotective substances, such as ethylene glycol or propylene glycol, has not yet been attempted. Further investigation and optimization of cryopreservation media for neural cells will be needed to improve viable cell yield.

We observed significant cell losses in frozen-thawed samples after removal of CPA using a single-step dilution protocol. One possible explanation for the loss of viable cells is mechanical damage due to excessive cell swelling upon replacement of the 8% DMSO solution by isotonic medium (Karlsson et al., 2009). Dong et al. (1993) reported that single-step removal of 7% DMSO from human neural cells resulted in 30–40% lower viability than multi-step DMSO removal; this result is consistent with the 40–70% loss of viable cells observed in the present study after single-step DMSO removal. It is also possible that the cells in our study were damaged by forces resulting from centrifugation. Kawamoto and Barrett (1986) reported that cryopreserved rat neural cells are especially sensitive to centrifugation injury, whereas minimal damage occurred when frozen-thawed cells were initially incubated in a 5% DMSO solution, which was subsequently replaced with isotonic cell culture media after 30 min in culture. Thus, it may be possible to reduce cell loss during CPA removal by considering protocols that reduce cell swelling (e.g., multistep protocols) and by eliminating other potential sources of mechanical damage, such as centrifugation.

Although the current study demonstrated that some of the cryopreserved neural cells were able to survive in tissue culture, the number of viable cells decreased by approximately 70% during the first 48 h of culture. This loss of viable cells may be due to delayed manifestation of injuries that were triggered during the cryopreservation process (e.g., apoptosis); it has been suggested that such latent damage may be mitigated by blocking apoptotic enzymes, e.g., using caspase inhibitors (Baust et al., 2000). On the other hand, it is also possible that the observed reduction in viability can be attributed to an inadequate seeding density. In the present study, the unfrozen control cells were seeded at a density of 2×10^5 cells per well. However, when culturing cryopreserved cells, the volume of the cell suspension aliquot used for seeding was selected such that the seeding density would match that of the control cultures if no viable cells had been lost during cryopreservation (or post-thaw processing). As a consequence of this seeding protocol, the effective seeding density of the frozen-thawed neurons was

always lower than the seeding density of the control cultures, due to cell losses associated with cryopreservation. Because the viable cell yield after thawing and CPA removal was at most $\sim 30\%$ for cryopreserved cell suspensions, the effective seeding density of frozen-thawed neurons was less than or equal to 6×10^4 viable cells per well, which may be insufficient for effective tissue culture. Thus, it may be possible to improve survival of cryopreserved cells in culture by adjusting the concentration of the thawed cell suspensions to account for cell loss during cryopreservation. This strategy for culturing cryopreserved neurons has been successful in previous studies (Kawamoto and Barrett, 1986; Seggio et al., 2008).

In summary, we have demonstrated improved recovery of rat embryonic brain cells after cryopreservation, and we showed that cryopreserved neurons survived in culture and had a morphology that was comparable to that of unfrozen controls. However, we recognize limitations of our work that must be addressed before cryopreserved brain cells can be used routinely for applications in neural transplantation, neural tissue engineering or neuroscience research. For example, in this study, the probability of cell damage was assessed by membrane integrity assays (trypan blue exclusion and live-dead fluorescence staining) and morphological appearance after 48-h culture, whereas future studies are required to evaluate the effect of cryopreservation on neural cell function during long-term culture. Possible approaches to such functional assessment following freezing and thawing include evaluation of maturation using cytoskeletal markers (Steinschneider et al., 1996), or electrophysiological assays for synaptic integration and formation of functional neuronal networks (Steinschneider et al., 1996; Cullen et al., 2010) as well as the detection of inhibitory GABA-induced currents (Cancedda et al., 2007; Deng et al., 2007). Furthermore, in the experiments reported here, we have not distinguished between neuronal subtypes when assessing viability. If some subtypes are more vulnerable to cryopreservation damage than are others, then the distribution of neuronal phenotypes in frozen-thawed cell suspensions would differ from the distribution in freshly isolated neuronal preparations. Thus, future studies will determine whether specific neuronal phenotypes preferentially survive the cryopreservation process. Nonetheless, the present results demonstrate that the response of neural cells to cryopreservation can be understood based on current knowledge of the biophysical mechanisms of cryoinjury. Therefore, we consider it likely that further improvements in cell yield and function will result from adaptation of physics-based computer models that can predict optimal cryopreservation process parameters (Karlsson et al., 1993, 1996, 2009; Karlsson, 2010). The next steps required to implement this strategy will be to measure the cell-specific biophysical properties of rat embryonic neural cells, including key parameters such as osmotically active and inactive volumes, the membrane permeability, as well as the catalytic activity of intracellular ice nucleators (Karlsson et al., 1993; Karlsson and Toner, 1996, 2000). Some of these biophysical parameters have recently been measured in murine primary neural brain cells (Paynter et al., 2009).

5. Conclusions

Of the cryopreservation procedures investigated in this study, the most favorable consisted of equilibration in 8% DMSO, followed by cooling at $2^\circ\text{C}/\text{min}$ to a LN_2 plunge temperature of -80°C . Although this protocol improves significantly upon the performance of previously published methods, the ultimate yield of viable cells in cultures of cryopreserved neurons was only $\sim 10\%$ of the theoretical maximum yield. Nonetheless, those cells that did survive cryopreservation displayed typical neuronal morphology after 48 h of culture. Our findings show that the response of neural cells

to cryopreservation is consistent with expectations based on current understanding and models of cell biophysics, suggesting that computer-aided optimization strategies may be an effective tool for further refinement of neuronal freezing profiles. Moreover, our analysis suggests that additional opportunities for improving the survival of rat neural cells include reformulation of cryopreservation media, and more careful attention to post-thaw processing methods. By enhancing cryopreservation techniques for embryonic neural cells isolated from the rat cortex, our work has the potential to benefit pre-clinical neuroscience research. More importantly, our study also provides a starting point for the future development and optimization of effective cryopreservation procedures for human neural cells, which promise to greatly facilitate therapeutic interventions that require neural transplantation.

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