Cryobiology 72 (2016) 191–197

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Cryopreservation by slow cooling of rat neuronal cells

M. Celeste Robert [a, c, f], Leonardo Juan de Paz [a, b], Daniel A. Graf [b], Silvia Gazzin [c], Claudio Tiribelli [c, d], Hebe Bottai [e], Joaquín V. Rodriguez [a, f, *]

[a] Centro Binacional (Argentina-Italia) de Investigaciones en Criobiología Clínica y Aplicada (CAIC), UNR, Avda. Arijon 28 bis, Rosario, 2000, Argentina
[b] Servicio de Electrónica y Óptica, FCByF, UNR, Suipacha 531, 2000, Rosario, Argentina
[c] Fondazione Italiana Fegato (Italian Liver Foundation), AREA Science Park, BldgQ, SS14, Km 163.5, Basovizza, 34139, Trieste, Italy
[d] Liver Center, Department of Medical, Surgical, and Health Sciences, University of Trieste, Trieste, Italy
[e] Area Estadística y Procesamiento de Datos, FCByF, UNR, Suipacha 531, 2000, Rosario, Argentina
[f] Concejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

ARTICLE INFO

Article history:
Received 20 October 2015
Received in revised form
29 March 2016
Accepted 4 May 2016
Available online 7 May 2016

Keywords:
Neuronal cells
Slow cooling
Cryopreservation

ABSTRACT

Although primary neuronal cells are routinely used for neuroscience research, with potential clinical applications such as neuronal transplantation and tissue engineering, a gold standard protocol for preservation has not been yet developed. In the present work, a slow cooling methodology without ice seeding was studied and optimized for cryopreservation of rat cerebellar granular cells. Parameters such as cooling rate, plunge temperature and cryoprotective agent concentration were assessed using a custom built device based on Pye's freezer idea. Cryopreservation outcome was evaluated by post thawing cell viability/viable cell yield and in culture viability over a period of 14 days. The best outcome was achieved when 10% of Me_2SO as cryoprotective agent, a cooling rate of 3.1 ± 0.2 °C/min and a plunge temperature of −48.2 ± 1.5 °C were applied. The granular cells cryopreserved under these conditions exhibited a cell viability of 82.7 ± 2.7% and a viable cell yield of 28.6 ± 2.2%. Moreover, cell viability in culture remained above 50%, very similar to not cryopreserved cells (control). Our results also suggest that post-thaw viability (based on membrane integrity assays) not necessarily reflects the quality of the cryopreservation procedure and proper functionality tests must be carried out in order to optimize both post thaw viability/cell yield and in culture performance.

1. Introduction

Cryopreservation has been routinely used in prolonged storage of many mammalian cells/tissues such as blood, bone marrow cells, spermatozoa and embryos used both for research and therapeutic purposes. Rat neuronal cells are frequently used in neuroscience studies. In particular, cerebellar granule cells are a well characterized model for the study of mechanisms of survival, apoptosis, neurodegeneration and neuroprotection [2]. Successful cryopreservation of neuronal cells would reduce the waste of cells, and allow the pooling of cells from different donors. Cell storage is also of great value in scientific research; allowing archiving of material, repeated experiments from the same tissue source and facilitating research collaboration.

The first studies of nerve tissue cryopreservation were performed by Luyet and Gonzales in 1953 [8]. Since then, and particularly with the development of transplantation studies in the last 30 years, development and optimization of cryopreservation protocols have been a permanent necessity. Several studies have been performed to achieve cryopreservation of nervous tissuecells and neural stem cells [7,9], as summarized by Paynter [14]. However, cryopreservation has not been successful to be incorporated into routine clinical practice. This indicates that further optimization of cooling and thawing protocols is needed to improve the outcome of the cryopreservation procedure.

Slow cooling (or "freeze-thaw") techniques are currently used in a wide variety of cell types. Briefly, the procedure consists in loading the sample with a cryoprotective additive and decreasing the temperature at an optimal constant rate until a prefixed temperature is reached followed by a plunge in liquid nitrogen (LN_2) for

* Corresponding author.
E-mail addresses: celeste_robert@hotmail.com (M.C. Robert), ljuandep@fbioyf.unr.edu.ar (L. Juan de Paz), agraf@fbioyf.unr.edu.ar (D.A. Graf), silvia.gazzin@csf.units.it (S. Gazzin), ctliver@csf.units.it (C. Tiribelli), hbottai@fbioyf.unr.edu.ar (H. Bottai), jrodrig@fbioyf.unr.edu.ar (J.V. Rodriguez).

http://dx.doi.org/10.1016/j.cryobiol.2016.05.003
0011-2240/

preservation. Additionally, ice nucleation may be induced ("seeding") by different methods as summarized by Morris & Acton [13]. When ice seeding is performed, post-crystallization cooling rate is known to be critical to cell recovery after cryopreservation. The cooling rate determines the "fate of intracellular water during freezing" [10]directly affecting the size and number of intracellular ice crystals formed during the cooling of the sample to its storage temperature. This intracellular ice formation is directly associated with cellular damage observed during cryopreservation.

Our present work deals with the refinement of a slow cooling method to be applied in the cryopreservation of rat cerebellar granular cells. A rather simple apparatus [3] (based on Pye's idea [15]) is adopted as replacement for the expensive freezers usually used for freeze-thaw techniques. Spontaneous nucleation is used (i.e. no ice nucleation is induced) to simplify the procedure. With this approach, the sample is cooled at a constant rate until it reaches the spontaneous crystallization point. After crystallization, the sample starts cooling again following the temperature of the chamber until a pre-selected LN_2 plunge temperature is reached.

The optimal cooling rate and plunge temperature, along with the most favourable cryoprotective agent (CPA) concentration, were evaluated by post preservation cell viability and their capacity to remain in culture.

2. Material and methods

2.1. Animals

Sprague Dawley rats of both sexes were obtained from the animal facility of the Faculty of Medical Sciences of National University of Rosario (Rosario, Argentina). Animal care and procedures were conducted according to the guidelines approved by the Argentinean National Council for Scientific Research. The project was approved by the Local Bioethics Commission for the Management and Use of Laboratory Animals (Resolution Number: 573/2012), Faculty of Biochemical and Pharmaceutical Sciences of National University of Rosario. All efforts were made to minimize the number of animals and their suffering.

2.2. Cerebellar granular cells isolation

Animals of 8 days after birth (P8) were sacrificed by decapitation and their brains were rapidly excised and placed at room temperature (RT) in BME culture medium as described below. The cerebellum was immediately removed and cleaned of meninges and 4th ventricle choroid plexus as it was previously described [4].

Cerebellar Granular Cells (CGC) were obtained by dissecting the cerebella from six P8 Sprague Dawley rats as described previously by Mitchell [12], with minor modifications. Tissues were collected at RT in BME medium (Invitrogen, Life Technologies Corporation, Grand Island, NY) with 100 μg/mL Gentamycin (Sigma-Aldrich, St. Louis, MO, USA). After chopping, the tissue was transferred into 10 mL of Solution 1 (0.03% $MgSO_4$and 3 mg/mL BSA in Krebs buffer: 124 mM NaCl, 5.37 mMKCl, 1.01 mM $NaH_2PO_4 \cdot H_2O$, 2.7 μM Phenol Red, 25 mM Hepes, 14.5 mM D-glucose, pH 7.40) and centrifuged at 200 g for 1 min. The pellet was resuspended in 10 mL of Solution 2 (0.03% $MgSO_4$, 3 mg/mL BSA and 0.12 mg/mL trypsin in Krebs buffer) and incubated at 37 °C for 15 min with gentle agitation. Trypsin was inhibited by the addition of 10 mL of Solution 3 (0.03% $MgSO_4$, 3 mg/mL BSA, 0.16 mg/mL trypsin inhibitor, 0.014 mg/mL DNase I in Krebs buffer), and the suspension centrifuged at 200 g for 1 min. After decanting of the supernatant, 3 mL of Solution 4 (0.07% $MgSO_4$, 3 mg/mL BSA, 0.52 mg/mL trypsin inhibitor, 0.045 mg/mL DNase I in Krebs buffer) were added, and cells gently separated by pipetting through a sterile fire-polished glass Pasteur pipette. After 4% BSA gradient-separation (Gradient Solution: 4% BSA in 0.06% $MgSO_4$, 0.001% $CaCl_2$, 3 mg/mL BSA in Krebs buffer), 180 g for 5 min, the cell pellet was resuspended in 5 mL of Neuron Medium (BME, 10% heat-inactivated Foetal Bovine Serum (FBS), 2 mM L-glutamine, 25 mMKCl, 10 mMHepes, 44 mM $NaHCO_3$, 100 μg/mL Gentamycin, pH 7.40). Cells were counted using a Neubauer chamber, and cell viability was evaluated by 0.4% (w/v) trypan blue dye exclusion. The viability of the isolated cell suspension was always above of 94%.

Isolated cells were divided in two: controls (fresh isolated cells plated in culture) and cryopreserved cells (fresh isolated cells subjected to cryopreservation-rewarming procedure and then put into culture). Fig. 1 shows an outline of the experimental design.

2.3. Slow cooling and rewarming procedure

The fraction of isolated cells separated for cryopreservation was divided in three CryoTube Vials (NUNC™, Cat. No. 366656). Each tube containing a suspension of 20×10^6 viable cells in 1 mL of Neuron Medium with 10% Me_2SO (*Sintorgan®*, *Argentina*) was immersed in a 400 mL methanol bath at 10 °C during 10 min, to allow Me_2SO diffusion into the cells. A fourth tube containing the same cell suspension and a Type K thermocouple wire was also immersed in the bath and used as a sample temperature indicator. Sample and bath temperature were simultaneously recorded every 10 s using a TES-1384 electronic thermometer (*TES Electrical, Taiwan*) connected to a PC, and a graph temperature *vs*. time was generated.

After the incubation period, the temperature of the bath was decreased at a fixed cooling rate, using a liquid nitrogen reservoir as a "heat sink" in the configuration described by *Pye* [15] and modified by us [3]. Briefly, a cylindrical copper bar in contact with the methanol bath and the LN_2 was used as heat conductor. The heat transfer, and ultimately, the cooling rate of the bath, was fixed by selecting a proper copper bar diameter and taking special care on maintaining LN_2 in the Dewar Flask by constant refilling. Table 1 shows the cooling rates obtained using different diameters of the heat conductor. Sample cooling rate was calculated, using the generated temperature *vs.* time graph of the tube containing the type K thermocouple, by linear regression of the curve region previous to spontaneous crystallization. A typical temperature recording is showed in Fig. 2.

When the predefined final cooling temperature (plunge temperature, -24.0 ± 0.5 °C or -48.2 ± 1.5 °C) was reached, CryoTube vials were removed from the bath, immediately immersed in LN_2, and finally stored for preservation. The cooling rate of this step was estimated around 221 ± 19 °C/min ($n = 3$). The sample containing the thermocouple wire was discarded in cases that were not used to measure the rewarming rate.

At the cooling rate of 0.40 °C/min, the methanol bath temperature was only able to reach a minimum temperature of ~ −25 °C. This limitation is related to the heat exchange between the cooling chamber and the lab environment. When temperature reaches ~ −25 °C the heat flow between the LN_2 and the methanol bath equals the heat flow between the methanol bath and the environment. This led to a thermal equilibrium slowing down the cooling rate to virtually 0 °C/min.

After the cryopreservation time (from 5 days up to 2 years of LN_2 storage) cryovials were rewarmed in a 37 °C bath. The three Cryovial tubes of each experiment were pooled and the cryopreserved cell suspension was diluted 1:5 with fresh Neuron Medium (for diluting the Me_2SO) and centrifuged 70 g for 4 min. After removing the supernatant, the cell pellet was resuspended in 2 mL of fresh Neuron Medium. Cryopreserved cells were counted, and the viability evaluated by trypan blue dye exclusion, as

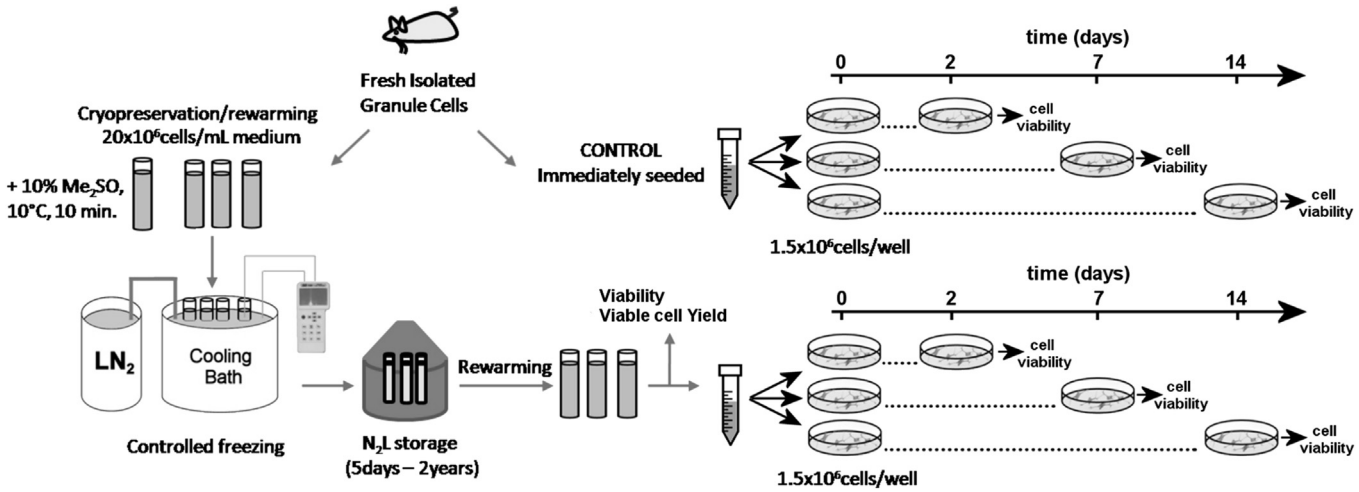

Fig. 1. Outline of the experimental design. *div*: days *in vitro*.

Table 1
Mean cooling rate estimated for the different copper bars (*S.E.M.: standard error of mean; n: number of replicates*) used as heat transfer units. 3/8 + 5/8 and 5/8 + 5/8 represent different bars used simultaneously.

Copper bar diameter (inches)	Sample cooling rate (°C/min)	S.E.M. (°C/min)	n
1/4	0.40	0.01	5
3/8	1.20	0.05	11
5/8	3.10	0.15	13
3/8 + 5/8	4.30	0.24	7
5/8 + 5/8	7.00	0.78	6

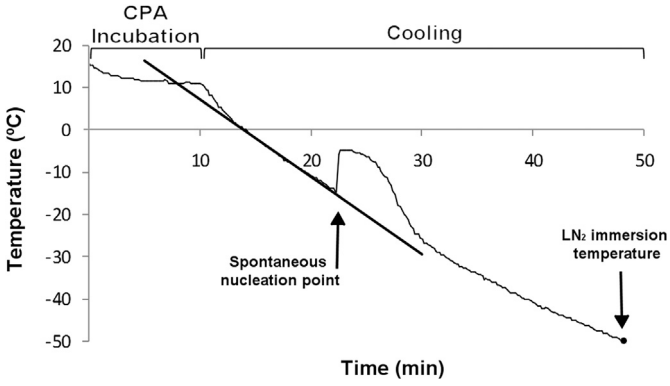

Fig. 2. Temperature profile for a given sample treated as described in text. For the first 10 min the sample is incubated at 10 °C for CPA diffusion. Then, methanol bath and the samples are cooled until LN_2 immersion temperature is reached. Thick line represents estimated cooling rate.

described below. Finally the cryopreserved cells were cultured and in culture studies were performed.

2.4. Cryoprotective agent concentration

Evaluation of the best combination of cooling rate and plunge temperature was performed using 10% of Me_2SO as CPA. Then, we further investigate the effect of a higher (14%) and a lower (7%) CPA concentrations on post rewarming viability, viable cell yield and *in vitro* cell viability.

2.5. Cell culture

Control and Cryopreserved cells were seeded in 5 μg/mL poly-L-lysine (molecular weight 30,000–70,000) pre-coated 40 mm culture plate at the density of 1.5 × 10^6 cells/well in Neuron Medium, and incubated at 37 °C, in a 5% CO_2atmosphere and90% humidity. After 24 h the medium of control cells was renewed with the addition of D-glucose (5.6 mM) and cytosine-β-D-arabino-furanoside (Ara-C, 10 μM), to prevent the growth of non-neuronal cells. The medium of cryopreserved cells was renewed with the only addition of D-glucose (5.6 mM) at 24 h; Ara-C (10 μM) was added 48 h after putting them in culture. From that moment on, the medium was changed twice a week. The cell culture was maintained for as long as 14 days.

2.6. Cell viability

The effectiveness of the cryopreservation and rewarming process was assessed measuring the post-rewarming viability and the capacity for survival in culture for "*x days*". The cultures were monitored daily, always by the same operator, by direct observation with an inverted microscope. Cultures were discontinued when the cells that remained attached to the culture plate were less than 20% of the original seeded cells, or when cells were not attached to the culture plate 24 h after seeding. The cell viability (viable cells/total cells) of control and cryopreserved cells (before and immediately after cryopreservation-rewarming); and of control and cryopreserved cell cultures at 2, 7 and 14 days were evaluated by trypan blue dye exclusion test. Briefly, 10 μl of control and cryopreserved cell suspensions were diluted 1:50 and 1:10 in 0.4% (w/v) trypan blue solution, respectively, living and dead (blue) cells were counted. In the case of cell cultures, 800 μl of 0.4% (w/v) trypan blue solution was added to the plate and incubated for 5 min, after that the culture was washed with phosphate buffered saline. Finally, living and dead (blue) cells were counted in ten different fields per well on an inverted microscope [1].

2.7. Viable cell yield

The viable cell yield was also calculated at the end of the cryopreservation– rewarming process, defined as the number of viable cells post-cryopreservation-rewarming, normalized to the number of viable cells before the cryopreservation. The number of viable cells was also determined using the trypan blue exclusion test, as described above. The viable cell yield reflects the outcome of the cryopreservation procedure, as it represents the fraction of cells that survive the cryopreservation process; taking into account the cells that were lost throughout the procedure [6].

2.8. Statistical analysis

Independent experiments were repeated at least three times in duplicate each. Significant differences between groups were determined by one or two-way ANOVA (as appropriate), with a Tukey Multiple Comparison post-test. When evaluating the survival at different percentage of Me_2SO a Kruskal-Wallis test was applied, due to failure to comply the assumptions. Differences were considered statistically significant at a p value lower than 0.05. All results were expressed as mean ± S.E.M.

3. Results

We initially studied the effect of the cooling rate and the plunge temperature on the granular cell post-rewarming viability using a CPA concentration of 10%. The cell viability was not significantly affected by the cooling rate at the two plunge temperatures applied. However, at the lower plunge temperature (−48.2 ± 1.5 °C) we observed a significant improvement of cell viability, respect to the plunge temperature of −24.0 ± 0.5 °C ($p < 0.05$) at the tested cooling rates, excepted at 7.00 °C/min (Fig. 3).

When we evaluated the outcome of the cryopreservation procedure by means of the viable cell yield, we observed that the viable cell yield at a plunge temperature of −24.0 ± 0.5 °C was less than 4% for all the cooling rates tested, except for the cooling rate of 7.0 °C/min which reached 12.2± 1.8%. At a plunge temperature of −48.2 ± 1.5 °C, the viable cell yield was significantly increased at a cooling rate of 3.1 °C/min respect to the viable cell yield obtained at 1.2 °C/min. Nevertheless, at a cooling rate of 1.2 °C/min, 3.10 °C/min and 4.4 °C/min the yield was significantly improved (14.3± 3.4%; 28.6± 2.2%; 17.0± 3.7%,$p < 0.05$) when the plunge temperature was −48.2 ± 1.5 °C (Fig. 4), compared with a plunge temperature of 24.0 ± 0.5 °C.

The primary culture of control and cryopreserved granular cells was monitored daily to check its capacity for survival in culture by "x days". When the plunge temperature was −24.0 ± 0.5 °C, the cryopreserved granular cells were able to develop in culture only if the cooling rate was 3.1 °C/min or higher, but these cultures did not survive more than 7 days on average. When a lower plunge temperature was applied (−48.2 ± 1.5 °C) the cryopreserved granular cells were able to grow in culture, surviving more than 8 days on average, arriving to 14 days in some cases (Fig. 5). The survival was also significantly improved compared to that obtained at the higher plunge temperature (−24.0 ± 0.5 °C, $p < 0.05$) regardless of the cooling rate used.

In vitro cell viability of control and cryopreserved cells (plunge temperature of −48.2 ± 1.5 °C) was evaluated at 2, 7 and 14 days on cultures. The mean cell viability of control cultures, was significantly higher ($p < 0.05$) at 2, 7 and 14 days compared to the cells cryopreserved at a cooling rate of 1.2, 4.4 and 7.0 °C/min. No difference on cells viability at 2, 7 and 14 days was observed in respect to controls when the cooling rate applied was of 3.1 °C/min. (Fig. 6). For every tested condition the *in vitro* cell viability significantly decreases by increasing the time of culture ($p < 0.05$).

Cryopreservation at a cooling rate of 3.1 °C/min, and plunge temperature of −48.2 ± 1.5 °C were performed at two different CPA concentrations. No significant difference was observed at a lower (7%) and higher (14%) concentration of CPA on cell post-rewarming

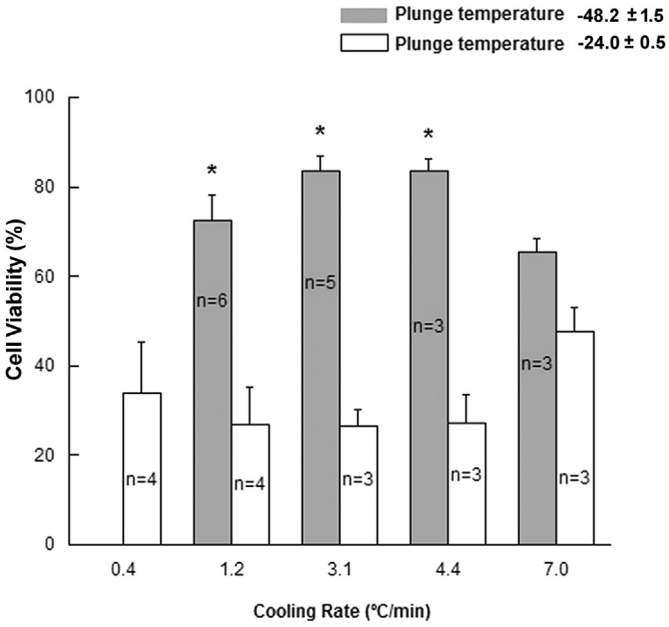

Fig. 3. Effect of plunge temperature and cooling rate on post-rewarming viability. Data are expressed as mean ± SEM. Statistical significance: *$p < 0.05$ when comparing cell viability at the two plunge temperatures. The absence of data at a plunge temperature of −48.2 °C and a cooling rate of 0.4 °C/min is explained in the text. (*n: number of replicates).*

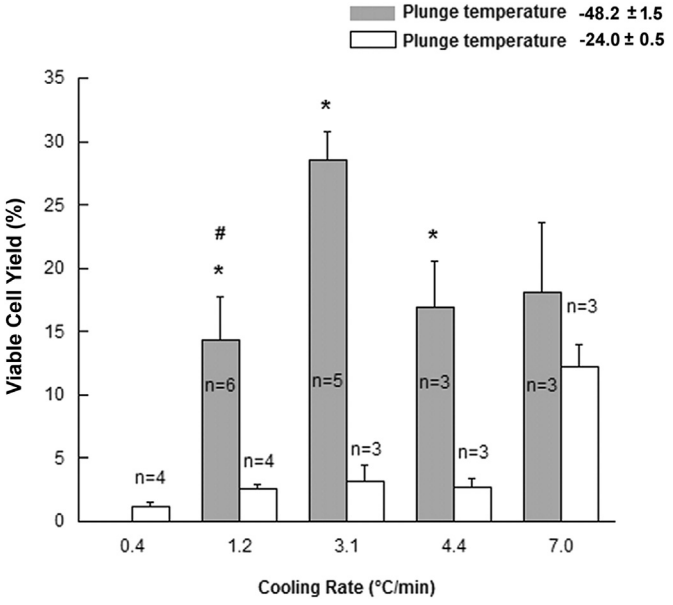

Fig. 4. Effect of plunge temperature and cooling rate on viable cell yield after cryopreservation in LN_2 and rewarming at 37 °C. Data are expressed as mean ± SEM. Statistical significance: *$p < 0.05$ when comparing viable cell yield at the two plunge temperatures, and #$p < 0.05$ when comparing the viable cell yield (plunge temperature of −48.2 °C) at the cooling rate of 3.1 °C/min respect to the cooling rate of 1.2 °C/min. The absence of data at a plunge temperature of −48.2 °C and a cooling rate of 0.4 °C/min is explained in the text. (*n: number of replicates).*

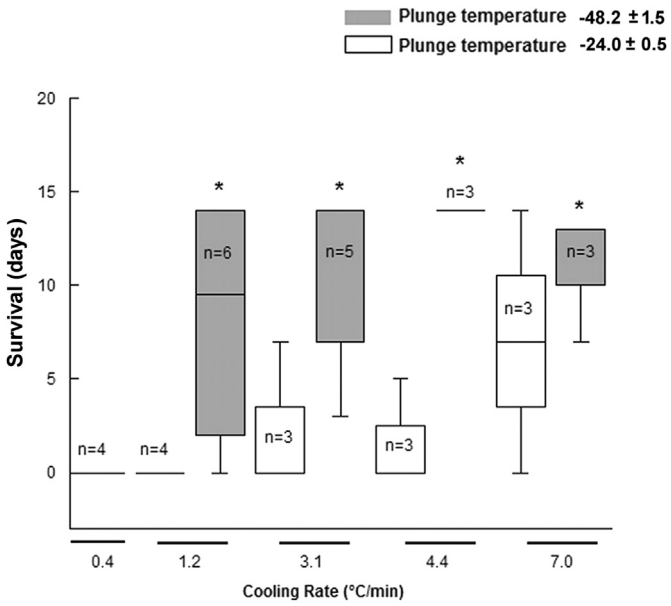

Fig. 5. Box Plot comparison of the effect of plunge temperature and cooling rate on the number of days that cells were able to survive in culture after cryopreservation in LN_2 and rewarming at 37 °C. Boxes indicate the upper and lower quartiles of the data of at least 3 independent experiments and bars indicate the median values. The whiskers indicate the minimum and maximum. Statistical significance: *p < 0.05 when comparing the number of days that cells were able to survive in culture at the two plunge temperatures. Absence of data at a plunge temperature of −48.2 °C and a cooling rate of 0.4 °C/min is explained in the text.

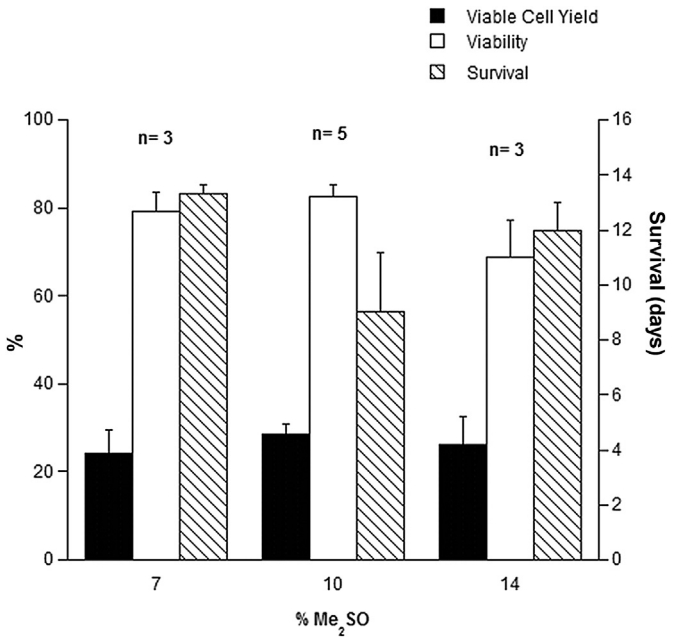

Fig. 7. Effect of CPA concentration on cell viability and viable cell yield after the cryopreservation/re-warming process at a cooling rate of 3.1 °C/min and a final plunge temperature of −48.2 ± 1.5 °C, and on the number of days that cells were able to survive in culture. The data from Figs. 3, 4 and 5 for samples cryopreserved in 10% Me_2SO at a cooling rate of 3.1 °C/min are replotted here to facilitate comparison with samples cryopreserved in 7% and 14% Me_2SO. Data are expressed as mean ± SEM. No statistical differences were found. (*n: number of replicates).*

viability, percentage of viable cell yield, and on survival (days that cells were able to be maintained in culture, Fig. 7). Nevertheless, the mean *in vitro* cell viability of control cultures at 7 and 14 days, was significantly higher ($p < 0.05$) compared to the cells that were cryopreserved with 7 and 14% of Me_2SO. Moreover, in vitro cell viability of samples cryopreserved with 10% of Me_2SO was significantly higher (#$p < 0.05$) when comparing with cells treated with 7% of Me_2SO. No statistical differences on culture cell viability at 2, 7 and 14 days were observed respect to controls when the CPA (Me_2SO) concentration was 10% (Fig. 8).

4. Discussion

In line with previous reports [11], the present results clearly indicate that the plunge temperature plays a significant role in the outcome of the cryopreservation of cerebellar granular cells. Post-rewarming viability and viable cell yield assays exhibit a better performance when cells were cooled at a controlled rate to a lower temperature (−48.2 ± 1.5 °C) and then immersed in LN_2.

Data obtained from cell culture showed a higher viability for lower plunge temperatures while cells cooled at controlled rates until −24.0 ± 0.5 °C and then rapidly cooled to −196 °C did not survive when plated. These observations seem to be related to a reduction in the cell water content when controlled cooling is maintained until lower temperatures, reducing the amount of intracellular ice formation and the associated damage.

The influence of the cooling rate was evaluated within the range of speeds achievable with the proposed freezer device (~0.4 °C/min to ~7.0 °C/min). At these cooling rates, no influence was observed in post-rewarming viability or viable cell yield assays, with the exception of the viable cell yield at a cooling rate of 1.2 °C/min compared to that observed at a cooling rate of 4.4 °C/min (Figs. 3 and 4). On the contrary, cell viability in culture showed a significant decrease at all the cooling rates tested, with the exception of 3.1 °C/min. Using a cooling rate of 3.1 °C/min, the average viability of the cultures was above 50% for as long as 14 days.

Post crystallization cooling rate has been conventionally

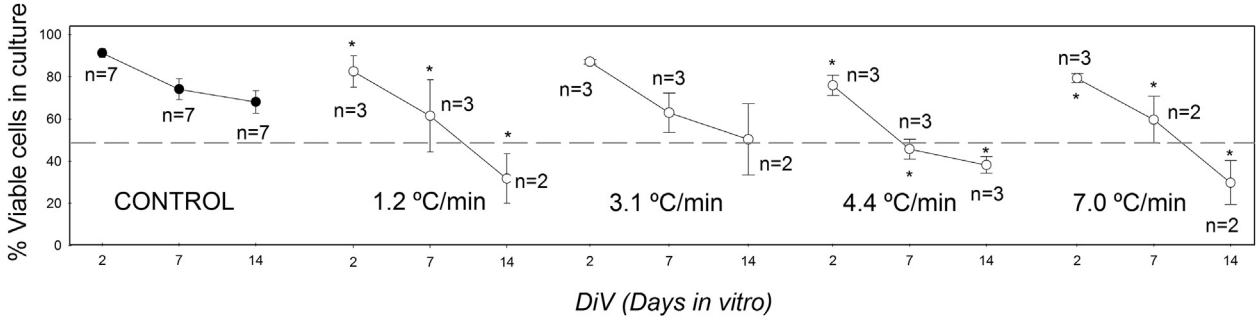

Fig. 6. Effect of cooling rate on *in vitro* cell viability of cryopreserved granular cells when the plunge temperature was −48.2 ± 1.5 °C. Data are expressed as mean ± SEM. Statistical significance: *p < 0.05 when comparing the cell viability at 2, 7 and 14 days in culture at each freezing rate used *vs.* cell viability on control cultures. (*n: number of replicates).*

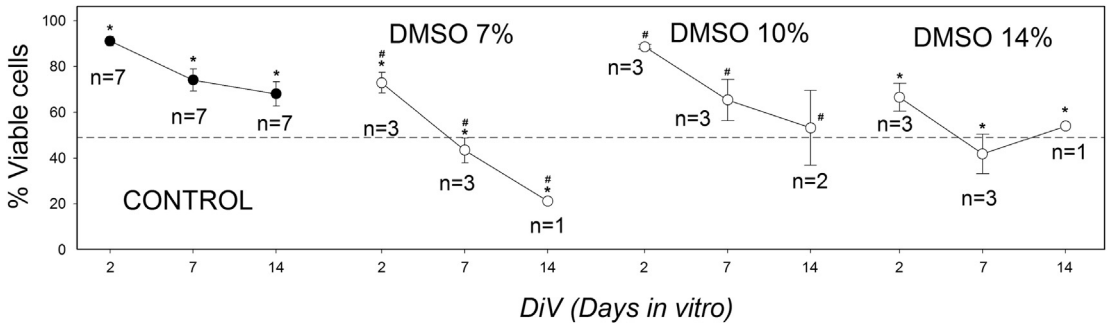

Fig. 8. Effect of CPA concentration on *in vitro* cell viability of cryopreserved granular cells after the cryopreservation/rewarming procedure at a cooling rate of 3.1 °C/min and a final plunge temperature of −48.2 ± 1.5 °C/min. The viability from Fig. 6 for samples cryopreserved in 10% Me_2SO at a cooling rate of 3.1 °C/min is replotted here to facilitate comparison with samples cryopreserved in 7% and 14% Me_2SO. Data are expressed as mean ± SEM. Statistical significance: *p < 0.05 when comparing with control culture at the same day and #p < 0.05 when comparing with cells cryopreserved using 10% of Me_2SO. (*n: number of replicates).*

associated to cryopreservation damage. In the present work we decided to measure cooling rate before ice nucleation due to the methodological difficulties of its measurement after spontaneous nucleation. Under such stochastic circumstances, measurement after ice nucleation would require to record all samples temperature and to perform the regression analysis individually, increasing experimental complexity. Pre-crystallization cooling rate serve in this case as a more representative measurement of the thermal history of all samples.

Nevertheless, the proposed equipment may also be used to perform controlled nucleation studies with proper modifications of the protocol. In such scenario, all samples are cooled under the same thermal history and post-nucleation cooling rate may be considered the same for samples treated simultaneously.

The concentration of the CPA (Me_2SO) did not significantly influence the post-rewarming viability, viable cell yield or survival in culture (Fig. 7). However, the use of a lower (7%) or a higher (14%) CPA concentration resulted in a decrease on the *in vitro* cell viability (Fig. 8). These results also suggest that low concentrations may be insufficient to achieve appropriate intracellular Me_2SO levels during the incubation time, while higher concentrations may be toxic.

In summary, the best outcome in the cryopreservation of rat cerebellar granular cells was achieved by using 10% of Me_2SO as CPA, a cooling rate of 3.1 ± 0.2 °C/min and a plunge temperature of −48.2 ± 1.5 °C. These conditions yielded an 82.7 ± 2.7% cell viability and28.6± 2.2% viable cell yield. In culture, cell viability remained above 50% for as long as 14 days exhibiting the most "control-like" behaviour.

In culture assays proved to be particularly useful to evaluate the quality of the recovered cells. Examining these results together with the membrane integrity tests suggests that, in the case of granular cells, a high post re-warming cell viability does not necessarily reflect the ability of the cells to develop a healthy and differentiated culture with proper neurite outgrowth. This is in agreement with previous observations about the impact on neurite grow of cooling conditions [5]. A number of cell functions which are not exclusively related to membrane integrity may be affected during the pre-conditioning/cooling/rewarming procedure influencing the ultimate functionality of recovered cells. More refined methods, such as immunocytochemical imaging, morphological and electrophysiological studies may be used in order to evaluate effects of cryopreservation over the functional quality of the culture [16].

A refined protocol should optimize the recovery of the highest amount of cells to reduce the number of animals, and preserve the ability of such cells to remain healthy in culture for their use in final applications. These requirements are essential when dealing with primary cerebellar granular cell cultures, considering that upon plating, the majority of these cells become postmitotic, initiate a differentiation program (axon extension and dendrite formation), and ultimately die. In contrast to what happen with immortal cell lines, the cell number in primary cerebellar granular cells cultures is, at best, virtually constant during the entire culture period, with a very low division rate. Therefore, when dealing with cryopreservation of such cells, special care must be taken to pursue the recovery of maximum number of living cells with maximum functionality and quality.

5. Statement of funding

MCR was supported by a PhD fellowship from the Italian Ministry of Foreign Affairs, Rome, Italy and by a Postdoctoral fellowship from CONICET, Argentina. JdPL was supported by a fellowship from National University of Rosario- UNR, Argentina. JVR is member of CONICET, Argentina. The work was supported by an in house grant and a grant from Relazioni Internazionali Regione Friuli-Venezia Giulia to the "Fondazione Italiana Fegato" (Italian Liver Foundation), Italy [Prot 19096/PT] and from Secretaria de Estado de Ciencia, Tecnología e Innovación-SECTEI- [N°-1010-008-11], Santa Fe, Argentina. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We wish to thank Dr. Edgardo E. Guibert for the critical revision of the study and manuscript, and Mr. Juan M. Gargallo (Gases Rosario, Santa Fe, Argentina) for the valuable support to this research. The project was supported by an in house grant and a grant from Relazioni Internazionali Regione FVG to the "Fondazione Italiana Fegato" (Italian Liver Foundation) and from Secretaria de Estado de Ciencia, Tecnología e Innovación-SECTEI- [N°-1010-008-11], Santa Fe, Argentina. MCR and JVR are members of CONICET, Argentina.

References

[1] M. Aras, K. Hartnett, E. Aizenman, Assessment of cell viability in primary neuronal cultures, Curr. Protoc. Neurosci. (2008) (Chapter 7), Unit 7.18.
[2] A. Contestabile, Cerebellar granule cells as a model to study mechanisms of neuronal apoptosis or survival in vivo and *in vitro*, Cerebellum 1 (2002) 41–55.
[3] L.J. de Paz, M.C. Robert, D.A. Graf, E.E. Guibert, J.V. Rodriguez, Design of a slow cooling device for cryopreservation of small biological samples, CryoLetters 36 (2015) 363–371.
[4] S. Gazzin, N. Strazielle, C. Schmitt, M. Fevre-Montange, J.D. Ostrow, C. Tiribelli,

et al., Differential expression of the multidrug resistance-related proteins ABCb1 and ABCc1 between blood-brain interfaces, J. Comp. Neurol. 510 (2008) 497–507.
[5] M. Götte, G. Hofmann, A.I. Michou-Gallani, J.F. Glickman, W. Wishart, D. Gabriel, An imaging assay to analyze primary neurons for cellular neurotoxicity, J. Neurosci. Methods 192 (2010) 7–16.
[6] A.Z. Higgins, D.K. Cullen, M.C. LaPlaca, J.O.M. Karlsson, Effects of freezing profile parameters on the survival of cryopreserved rat embryonic neural cells, J. Neurosci. Methods 201 (2011) 9–16.
[7] J. Ichikawa, R. Yamada, R. Muramatsu, Y. Ikegaya, M. Noorio, R. Koyama, Cryopreservation of granule cells from the postnatal rat hippocampus, J. Pharmacol. Sci. 104 (2007) 387–391.
[8] B. Luyet, F. Gonzales, Growth of nerve tissue after freezing in liquid nitrogen, Biodynamica 7 (1953) 171–174.
[9] X.-H. Ma, Y. Shi, Y. Hou, Y. Liu, L. Zhang, W.-X. Fan, et al., Slow-freezing cryopreservation of neural stem cell spheres with different diameters, Cryobiology 60 (2010) 184–191.
[10] P. Mazur, Freezing of living cells: mechanisms and implications, Am. J. Physiol. 247 (1984) C125–C142.
[11] L.E. McGann, Optimal temperature ranges for control of cooling rate, Cryobiology 16 (1979) 211–216.
[12] P.J. Mitchell, J.C. Hanson, A.T. Quets-Nguyen, M. Bergeron, R.C. Smith, A quantitative method for analysis of in vitro neurite outgrowth, J. Neurosci. Methods 164 (2007) 350–362.
[13] G.J. Morris, E. Acton, Controlled ice nucleation in cryopreservation–a review, Cryobiology 66 (2013) 85–92.
[14] S.J. Paynter, Principles and practical issues for cryopreservation of nerve cells, Brain Res. Bull. 75 (2008) 1–14.
[15] J. Pye, M.C. Holmes, A. Crawford, Simple piece of equipment for controlled cooling and freezing of mouse embryos, in: G.H. Zeilmaker (Ed.), Frozen Storage Lab. Anim. Proc. A Work. Harwell, U.K., May 6th–9th 1980, Gustav Fischer, 1981, pp. 61–66.
[16] K. Quasthoff, S. Ferrea, W. Fleischer, S. Theiss, A. Schnitzler, M. Dihné, J. Walter, Freshly frozen E18 rat cortical cells can generate functional neural networks after standard cryopreservation and thawing procedures, Cytotechnology 67 (2015) 419–426.