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Trehalose to cryopreserve human pluripotent stem cells

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ABSTRACT

The successful exploitation of human pluripotent stem cells (hPSCs) for research, translational or commercial reasons requires the implementation of a simple and efficient cryopreservation method. Cryopreservation is usually performed with dimethylsulphoxide (DMSO), in addition to animal proteins. However, even at sub-toxic levels, DMSO diminishes the pluripotency capacity of hPSCs and affects their epigenetic system by acting on the three DNA methyltransferases (Dnmts) and histone modification enzymes. Our study aimed to test trehalose-based cryosolutions containing ethylene glycol (EG) or glycerol (GLY) on hESCs RC17, hiPSCs CTR2#6 and long-term neuroepithelial-like stem cells (lt-NES) AF22. Here, we demostrate the effectiveness of these cryosolutions in hPSCs by showing an acceptable rate of cell viability and high stability compared to standard 10% DMSO freezing medium (CS10). All cell lines retained their morphology, self renewal potential and pluripotency, and none of the cryosolutions affected their differentiation potential. Genotoxicity varied among different stem cells types, while trehalose-based cryopreservation did not sensibly alter the homeostasis of endoplasmic reticulum (ER). This study provides evidence that pluripotent and neural stem cells stored in trehalose alone or with other cryoprotectants (CPAs) maintain their functional properties, indicating their potential use in cell therapies if produced in good manufacturing practice (GMP) facility.

1. Introduction

Cell therapy products, including stem cells, have high clinical potentiality (Singh et al., 2015; Wang et al., 2015); indeed the global stem cells market size is expected to reach USD ($) 170,15 billion by 2020 (https://www.grandviewresearch.com/press-release/global-stem-cells-market). For their successful application, efforts have been made aiming to produce and bank large amounts of generated undifferentiated or partially differentiated cell lines, while maintaining consistent sample handling and quality (Li and Ma, 2012). However, despite advanced developments, many biobank stakeholders call for improving not only the standardization of derivation procedures but also the cryopreservation process, being the only available method for long-term preservation of the final cell product that maintains cell viability and biochemical functions (Chen, 2008; Li et al., 2009; Hunt, 2011; Liu and Chen, 2014). This remains a critical and significant drawback faced by cryobiologists before successful stem cell transplantation.

In order to avoid any damaging effect due to intracellular ice formation, several cryomedia that contain cryoprotectants (CPAs) are commercially available. The most popular cryoprotectant is dimethylsulphoxide (DMSO) in conjuction with fetal bovine serum or serum replacement which may induce zoonotic infection with unknown pathogens (Berz et al., 2007). DMSO was reported to be toxic to tissues and cells depending on exposure time, temperature and concentration (Rowley and Anderson, 1993; Katayama et al., 1997; Hunt et al., 2003). The degree of toxicity varies from cell type to cell type and adverse reactions have been reported in patients re-infused with thawed cells without DMSO removal (Katkov et al., 2011; Morris et al., 2014). Furthermore, DMSO is known to affect the epigenome of mouse embryoid body by modulating the transcription of three DNA methyltransferases (Dnmts) and by altering genome-wide methylation profile (Iwatani et al., 2006), determining an uncontrolled differentiation of murine stem cells (Adler et al., 2006).

Sugars have cryoprotective properties and are not toxic, hence trehalose has been investigated as an alternative CPA by virtue of its stability upon freezing (Buchanan et al., 2004). Trehalose is a disaccharide found at high concentration in a variety of organisms capable of surviving in complete dehydration, including bacteria, yeast tardigrades and nematodes (Crowe et al., 2001; Thompson, 2003). Mammals do not produce trehalose but indeed it is an effective cryoprotectant, decreasing the amount of cell injury by ice crystallization (Crowe et al.,

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1984; Crowe and Crowe, 2000). Its protective action is related both to osmotic effect and specific interactions with cell membrane phospholipids and labile proteins, preventing their damage and denaturation due to desiccation and oxidative stress (Benaroudj et al., 2001). Nevertheless, the precise mechanism by which trehalose provides such protection is yet to be elucidated.

Trehalose does not present cytotoxicity and has been efficiently used for cryopreservation of murine spermatological (Lee et al., 2013), adult hematopoietic (Rowley and Anderson, 1993; Buchanan et al., 2004; Berz et al., 2007), mesenchymal (Liu et al., 2011; Kusuma et al., 2016), adipose-derived (Rao et al., 2015; Yong et al., 2015; Lopez et al., 2016), human embryonic (hESCs) (Imaizumi et al., 2014) and human induced pluripotent stem cells (hiPSCs) (Nishigaki et al., 2011); in addition it was used on cord blood, bone marrow, human transplantable hepatocytes (Katenz et al., 2007) and human pancreatic islets (Beattie et al., 1997). Moreover, it is used by food and cosmetic industries (Ohtake and Wang, 2011; Feofilova et al., 2014), in pharmaceutical applications, in ophthalmology (Luyckx and Baudouin, 2011; Aragona et al., 2014) and for the treatment of Huntington's chorea and Alzheimer's disease (Tanaka et al., 2004; Liu et al., 2005; Crowe, 2008; Du et al., 2013; Fernandez-Estevez et al., 2014; Tien et al., 2016). Even if trehalose is considered a nontoxic CPA, the major barrier that prevents its wide-spread application in biopreservation is the difficulty associated with its intracellular delivery (Ha et al., 2005). Several methods have been previously applied such as osmotic shock (Satpathy et al., 2004), liposomal delivery (Holovati et al., 2009), thermal poration (Beattie et al., 1997), electroporation (Shirakashi et al., 2002), microinjection (Eroglu et al., 2002; Eroglu et al., 2003), engineered pores (Lynch et al., 2011)and genetic engineering (Kikawada et al., 2007). However, the abovementioned approaches require laborious, time consuming and very often cumbersome manipulation that can lead to significant cell damage.

Here we show the successful cryopreservation of human pluripotent cell lines using trehalose alone or in combination with ethylene glycol (EG) or glycerol (GLY) as extracellular CPAs, in the absence of animal serum or albumin to reduce the likelihood of zoonotic infections. In detail, we examined four different trehalose-based cryopreservation cocktails on hESCs, hiPSCs and long-term neuroepithelial-like stem (lt-NES) cells, providing a more comprehensive study of several parameters to determine eventual effects on important biochemical pathways. Outcome measures included cell morphology, post-thawing viability, pluripotency marker evaluation, genomic stability, endoplasmic reticulum (ER) homeostasis (Koenig and Ploegh, 2014) as well as DNA damage response by H2A histone family, member X (γH2AX) phosphorylation, which represents an early event in the cellular response against DNA double strand breaks (DSBs) (Sanchez-Flores et al., 2015). In the conditions here standardized, all different stem cell types displayed good viability, genomic stability, pluripotency and differentiation capacity after thawing.

2. Materials and methods

2.1. Cell culture

All cell lines obtained from ISENET biobank, Milan, Italy (www.isenet.it). hESCs RC17 were maintained in StemPro Medium on vitronectin (VTN-N)-coated cell culture plates and passaged routinely with ethylenediaminetetraacetic acid (EDTA) solution (Canham et al., 2015; De Sousa et al., 2016). Human lt-NES AF22 cells were cultured as described previously (Falk et al., 2012). Briefly, cells were maintained in 0,01% Poly-L-Ornithine (Sigma-Aldrich) and 10 μg/mL laminin (Sigma-Aldrich) coated flask, using DMEM-F/12 medium (Euroclone) supplemented with 1% N2 and 0,1% B27 (Life Technologies), 10 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast growth factor (FGF2) (Peprotech EC). hiPSCs CTR2#6 derived from healthy control (HC) subjects using the generation of reprogramming viral particles from Sendai virus encoding the pluripotent transcription factors OCT4, KLF4, SOX2 and cMYC (CytoTune iPSC Reprogramming Kit, Life Technologies). Cells maintained on matrigel-coated dishes (BD) for expansion in serum free medium mTeSR1 (Stemcell) in the presence of Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor Y-27632 (Millipore), passaging them with enzymatic approach in accutase solution (Innovative Cell Technologies) at ratio 1:6. Medium was replaced daily.

For the differentiation of AFF2 cells, $3{,}5 \times 10^4$ cells/cm^2 plated directly after splitting into Poly-L-Ornithine and laminin coated wells in N2B27 medium, composed by Neurobasal (GIBCO) and D-MEM/F12 with GlutaMAX (Invitrogen) 1:1, supplemented with 1% B27 (GIBCO) and 0,5% N2 (GIBCO). Immunostaining for the differentiation markers βIII-tubulin and O4 was carried out after 2 weeks of culture.

2.2. Preparation of cryoprotectant solutions

For the preparation of the freezing cocktails we used the following CPAs: Trehalose (Sigma-Aldrich), EG (Sigma-Aldrich), GLY (Sigma-Aldrich), DMSO (Sigma-Aldrich) and CS10 (Cryostor™ 10, BioLife Solution, USA) or (Knockout Serum Replacement) KSR (GIBCO). Final concentrations of cocktail constituents were as follow: Freezing Medium A: 0,5 M Trehalose, Freezing Medium B: 0,5 M Trehalose +2,5% EG, Freezing Medium C: 0,5 M Trehalose +10% EG and Freezing Medium D: 0,5 M Trehalose +10% GLY. All freezing media were prepared just before use and diluted in phosphate-buffered saline (PBS). As a reference condition in all experiments we used cells cryopreserved in CS10. CS10 is a serum, animal component-free, defined cryopreservation medium containing 10% DMSO and it is recommended for cryopreservation of human pluripotent stem cells (hPSCs).

2.3. Cell freezing and thawing

For cryopreservation, cells were cultured under specific conditions, detached with 0,5 mM EDTA for hESCs RC17 and accutase for hiPSCs CTR2#6 and AF22 and processed in cold fresh-prepared freezing cocktails (A,B,C and D). $2{,}0 \times 10^6$ cells were resuspended in 1,5 mL of each freezing medium and then transferred to cryogenic vials (Nunc). Cryogenic vials cooled using the Nalgene “Mr Frosty” isopropanol freezing container that offers a simple and ideal cooling rate close to minus 1 °C/min required for effective cryopreservation of cells and transferred overnight to −80 °C. After 24 h the cryovials transferred to liquid nitrogen (LN_2) and stored for at least one week before analysis.

For thawing, cryovials were warmed in a water bath at 37 °C until the icy masses disappeared and cell suspensions diluted with warm culture medium. Cells were collected by centrifugation at 200*g* for 3 min and seeded onto appropriate for each cell type-coated culture vessels at the indicated seeding densities. RC17 and CTR2#6 cells recovered after thawing in presence of ROCK inhibitor. Media was changed every day.

2.4. Cell viability assay

Cells were thawed and seeded into 24-well plates (5×10^4 cells per well) under appropriate culture conditions for each cell line and incubated at 37 °C. Cell proliferation was quantitatively measured using the Alamar blue assay for cell numbers (Invitrogen). This assay utilizes a water-soluble fluorometric viabilty indicator based on the detection of metabolic activity, specifically, an oxidation- reduction (redox) indicator that both fluoresces and changes colour, from dark blue to red, in response to chemical reduction of the growth medium caused by cell metabolism. The culture medium was removed and fresh medium containing 5% Alamar blue was added before culturing overnight at 37 °C. The day after, the relative fluorescence units of Alamar blue-containing medium were measured at 590 nm using the Synergy 2