

## Me<sub>2</sub>SO- and serum-free cryopreservation of human umbilical cord mesenchymal stem cells using electroporation-assisted delivery of sugars

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### ABSTRACT

Cryopreservation is the universal technology used to enable long-term storage and continuous availability of cell stocks and tissues for regenerative medicine demands. The main components of standard freezing media are dimethyl sulfoxide (hereinafter Me<sub>2</sub>SO) and fetal bovine serum (FBS). However, for manufacturing of cells and tissue-engineered products in accordance with the principles of Good Manufacturing Practice (GMP), current considerations in regenerative medicine suggest development of Me<sub>2</sub>SO- and serum-free biopreservation strategies due to safety concerns over Me<sub>2</sub>SO-induced side effects and immunogenicity of animal serum.

In this work, the effect of electroporation-assisted pre-freeze delivery of sucrose, trehalose and raffinose into human umbilical cord mesenchymal stem cells (hUCMSCs) on their post-thaw survival was investigated. The optimal strength of electric field at 8 pulses with 100 μs duration and 1 Hz pulse repetition frequency was determined to be 1.5 kV/cm from permeabilization (propidium iodide uptake) vs. cell recovery data (resazurin reduction assay).

Using sugars as sole cryoprotectants with electroporation, concentration-dependent increase in cell survival was observed. Irrespective of sugar type, the highest cell survival (up to 80%) was achieved at 400 mM extracellular concentration and electroporation. Cell freezing without electroporation yielded significantly lower survival rates. In the optimal scenario, cells were able to attach 24 h after thawing demonstrating characteristic shape and sugar-loaded vacuoles. Application of 10% Me<sub>2</sub>SO/90% FBS as a positive control provided cell survival exceeding 90%. Next, high glass transition temperatures determined for optimal concentrations of sugars by differential scanning calorimetry (DSC) suggest the possibility to store samples at −80 °C. In summary, using electroporation to incorporate cryoprotective sugars into cells is an effective strategy towards Me<sub>2</sub>SO- and serum-free cryopreservation and may pave the way for further progress in establishing clinically safe biopreservation strategies for efficient long-term biobanking of cells.

### 1. Introduction

Stem-cell therapy is one of the most promising strategies in modern medicine for treatment of patients suffering from diverse disabilities or chronic diseases. Manufacturing of cell therapy products has to follow GMP standards to ensure safety and high reproducibility which is required by governmental institutions such as the Food and Drug

Administration in United States or the European Medicines Agency in Europe [4]. Cryopreservation of cells for the development of cellular products and cryopreservation of manufactured products are inevitable steps of cell therapy delivery chain. In clinical settings, repeated transplantations and flexible treatment scheduling are needed or unavoidable [31] and there are conflicting reports on the impact of cryopreservation procedures on cell functionality. Most clinical applications use frozen

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stocks of cells which requires detailed phenotypic characterization of fresh and cryopreserved cells [13]. In practice, the most frequently used freezing media in cell banking comprises the cryoprotective agent Me<sub>2</sub>SO and FBS. However, GMP-grade cryopreservation media must be animal-component free and provide high batch-to-batch consistency and quality of cell products stored under deep cryogenic temperatures. This is because of high immunogenicity, lot-to-lot variability, risk of contamination and bioethical issues associated with application of FBS. Therefore, there is considerable effort put into replacing FBS with different non-animal components. An often used example is human platelet lysate in both cell expansion [6] and cryopreservation media with [76,77] or without Me<sub>2</sub>SO [60].

Me<sub>2</sub>SO was first synthesized in 1866 by the Russian scientist Alexander Zaytsev and first used as a cryoprotective agent for cryopreservation of human and bovine red blood cells as well as bull spermatozoa by Lovelock and Bishop [42]. Until now, Me<sub>2</sub>SO was an indispensable component of most freezing solutions. However Me<sub>2</sub>SO may cause adverse effects after transplantation [69], alter cell pluripotency [36] and epigenetic profile [17,18,32]. Unfortunately, no consensus has been reached on which Me<sub>2</sub>SO concentration is considered safe for the infusion into patients and the issue of its depletion before infusion is still debated [49]. In recent years, the trend towards GMP-compliant cryopreservation led to development and commercialization of next generation cryopreservation media without Me<sub>2</sub>SO and FBS such as Bambanker™ DMSO Free, CryoSOfree™ or ibidi Freezing Medium DMSO-Free. However, lack of long-term studies, narrow spectrum of tested cell lines and potential risks associated with undisclosed components substituting Me<sub>2</sub>SO or FBS raise safety concerns. Thus, the search for new non-toxic cryopreservation formulations which would minimize or completely eliminate Me<sub>2</sub>SO and animal serum while maintaining high cryopreservation efficiency is urgently needed.

One strategy for mitigating Me<sub>2</sub>SO-related complications after transplantation is direct reduction of its concentration from routinely used 10%–5% or lower as it is suggested in some comparative studies [3, 47]. Alternatively, the replacement of Me<sub>2</sub>SO could be achieved by combining cryoprotective agents (CPAs) from different classes (penetrating, non-penetrating, sugars, alcohols) with various modes of action [5,40].

Some sugars, for example, trehalose and sucrose, and sugar alcohols such as mannitol and sorbitol, possess numerous protective benefits and are regarded as nature inspired, non-toxic CPAs. They accumulate in some extremophile species of fungi [58], plants [75], invertebrates [27], poikilothermic vertebrates such as frogs, reptiles and fishes [22] tolerating subzero temperatures. The exceptional protein stabilization by sugars is well known and for this reason various sugars have routinely been used as excipients in pharmaceutical (reviewed in Ref. [44]) and food [70] products. For medical purposes, sugars are being increasingly considered as compounds that can replace Me<sub>2</sub>SO in cryopreservation solutions or at least reduce its concentration. Examples include human umbilical cord blood stem cells [20,43], hematopoietic stem cells [59], mesenchymal stem cells [7], amniotic fluid-derived stem cells [65], human fetal liver hematopoietic stem/progenitor cells [53] and oocytes [79]. Another study has recently shown that a combination of sugars, sugar alcohols and small-molecule additives is superior to Me<sub>2</sub>SO in terms of post-thaw cell attachment, alignment of the actin cytoskeleton and epigenetic stability [56].

After overcoming the membrane permeability problem, sugars become much more attractive as CPAs. Various methods to introduce trehalose into cells for cryopreservation purposes with their advantages and drawbacks have been recently summarized [73]. Examples include endocytotic uptake [52] or freezing-induced uptake via membrane phase transitions [82], nanoparticle-mediated delivery [57], amphipathic polymer-mediated uptake [66] or using engineered forms of trehalose which are able to permeate into mammalian cells [1,9].

In addition to highlighted methods, electroporation deserves special attention as ‘the most mature in regard to industrial applications and clinical

translation’ [72]. Electroporation is a physical method for controlled introduction of membrane-impermeable molecules into cells through transient permeabilization of the plasma membrane by electric pulses [39]. Electroporation gained broad attention in transdermal drug delivery [16], electrochemotherapy [46], introduction of deoxyribonucleic acid (DNA) vaccines [62] and delivery of genes into cells [61].

In cryopreservation field electroporation was pioneered by the group of Katkov and Ostashko (former USSR). The authors demonstrated two important nuances that electroporation could be used both as a “surgical knife” (for loading of cells with non-penetrating CPAs, for example, sugars) and as a “stethoscope” (for diagnostics and prediction of the cell cryotolerance before freezing). The latter was shown in the cross-checking experiments with double treatments (freezing-thawing vs. electroporation). It was shown that spermatozoa resistant to freezing were also resistant to electroporation and *vice versa*. Moreover, Katkov and Ostashko found a good correlation between electromechanical stiffness of spermatozoan membranes and its cryoresistance. They explained this phenomenon by the difference in lipid content (cholesterol, some phospholipids and fatty acids) [34,35,37]. The results of early work on the application of electroporation in cryopreservation have recently been summarized and will be published elsewhere.

Thereafter, with implications for dry and cryo-preservation of mammalian cells, trehalose was successfully introduced into mouse myeloma Sp2 cell line by means of electroporation [68]. Later on, electroporation was used to load red blood cells with trehalose for freeze-drying purposes [84]. Electroporation was also used for delivery of β-galactosidase into smooth muscle cells as a model for introducing disaccharides into mammalian cells [10]. Nonetheless, the first actual data on high post-thaw survival of human stem cells after electroporation-induced loading of trehalose were published in 2017 where authors also tested cell differentiation potential [24].

Apart from trehalose, other sugars such as sucrose and raffinose possess excellent cryoprotective properties. Raffinose was loaded into mouse oocytes via microinjection and promoted high cryosurvival, fertilization, and development rates [25]. The incorporation of sucrose into mammalian cells using high intensity femtosecond laser pulses [38] and via endocytotic uptake [54] for biopreservation purposes has previously been demonstrated.

Thus, our primary goal was to evaluate the efficacy of electroporation for intracellular delivery of sucrose, trehalose and raffinose used as CPAs for Me<sub>2</sub>SO- and serum-free cryopreservation of human umbilical cord mesenchymal stem cells.

## 2. Materials and methods

### 2.1. Reagents

If not specifically mentioned, all chemicals were purchased from Sigma-Aldrich (Germany).

#### 2.1.1. Cell culture

HUCMSCs were derived from umbilical cord tissues of voluntary donors by explant culture after the approval of the Slovenian National Medical Ethics Committee (code 136/02/12) and donors’ signed informed consent. Cells were cultured in Phenol red-free DMEM/F-12 (Gibco, USA) supplemented with 10% (v/v) FBS (Gibco, USA), 2 mM L-glutamine, 100 U/ml penicillin/100 µg/ml streptomycin and 1 ng/ml of recombinant human FGF-basic (Peprotech, UK) in a humidified incubator at 37 °C and 5% CO<sub>2</sub>. After reaching about 70% confluency, cells were trypsinized using 0.05% trypsin-EDTA solution and further subcultured at the density 1 × 10<sup>4</sup> cells/cm<sup>2</sup>. For electroporation and cryopreservation experiments, cells of the 5-7th passages were used.

#### 2.1.2. Permeabilization and recovery assays

Low-conductive electroporation buffers were prepared from

10 mM  $K_2HPO_4$ , 10 mM  $KH_2PO_4$ , 1 mM magnesium chloride ( $MgCl_2$ ) including 250 mM of sucrose, trehalose or raffinose. To achieve a physiological pH of 7.4,  $K_2HPO_4$  and  $KH_2PO_4$  were mixed in the ratio of 40.5 to 9.5. A  $1 \times 10^6$  cells per 100  $\mu$ l cell suspension of hUCMSCs was prepared in the electroporation buffer which for permeabilization experiments was supplemented with 150  $\mu$ M propidium iodide (PI, Life Technologies, USA) before pulse application. To the control samples PI was added, but no pulses were delivered. Cells were transferred to disposable electroporation cuvettes (VWR International, Radnor, PA, USA) with a 2 mm gap size between the electrodes. By using the BTX™ Gemini  $\times 2$  Electroporation System (Harvard Apparatus, USA) 8 pulses (100  $\mu$ s duration and 1 Hz pulse repetition frequency) were generated at 0–2.5 kV/cm electric field. The electric field was estimated as the voltage applied divided by the distance between the electrodes. The pulse parameters were monitored using an AP015 current probe and an ADP305 high-voltage differential voltage probe connected to the oscilloscope (WaveSurfer 422, 200 MHz, all from LeCroy, USA). Following incubation for 2 min at room temperature, samples were washed by centrifugation for 1 min at 2000 g to remove extracellular PI. The cell pellet was resuspended in electroporation buffers containing respective sugars and transferred at the density of  $2.5 \times 10^5$  cells/well into a 96-well plate (TPP, Switzerland). Fluorescence intensity of PI-loaded and non-electroporated cells were read at 535/617 nm (Ex/Em) using an Infinite M200 plate reader (Tecan Austria GmbH). For cell recovery assay, electroporated and non-electroporated cells were seeded at a density of  $5 \times 10^3$  cells/well into 96-well culture plates and cultivated overnight. Cells were incubated with 44  $\mu$ M of resazurin dissolved in culture medium for 2 h and fluorescence was measured using the plate reader at 550/590 nm (Ex/Em). All experiments were performed at least in four independent repetitions.

### 2.1.3. Cryopreservation procedure

For cryopreservation of hUCMSCs, cells were electroporated as described in the section above but using 50–400 mM of sugars in electroporation buffers. After electroporation, cells in the concentration of  $0.5 \times 10^6$  cells/ml were then resuspended in the electroporation buffers containing the same concentrations of sugars and serving as freezing media. Samples with the volume 0.5 ml were placed in alcohol-free cell freezing container CoolCell (Biocision, USA) and stored overnight at  $-80^\circ\text{C}$ . Afterwards, cryotubes were transferred into liquid nitrogen containers and stored there for at least 24 h. Thawing of samples was performed in a water bath prewarmed to  $37^\circ\text{C}$  with gentle agitation. As a positive control, a standard cryopreservation medium composed of 10%  $Me_2SO$  (v/v) and 90% FBS (v/v) was used. Osmolality of freezing solutions was measured by freezing point depression with Osmomat 030 osmometer (Gonotec, Germany).

### 2.1.4. Assessment of post-thaw survival

Before and directly after electroporation and cryopreservation cell survival was assessed using trypan blue exclusion assay and flow cytometry. For the first assay, an aliquot of cell suspension was mixed with equal amount of 0.4% trypan blue stain (Invitrogen, USA) and incubated for 2 min at a room temperature. Cells were counted using hemocytometer and the percent of live cells was calculated as the number of live cells/total number of cells.

In the next step, after cryopreservation cell survival was analyzed using flow cytometry. Briefly, 100  $\mu$ l cell aliquot was resuspended in sample buffer supplemented with 100  $\mu$ g/ml PI. After incubation for 3 min at a room temperature samples were run on flow cytometer (Attune NxT; Life Technologies, USA) with laser excitation at 488 nm. Emission was collected with 574/526 nm band-pass filter. Gating was performed against PI-negative (untreated) cells and the cells frozen in high-conductivity electroporation buffer containing 150 mM NaCl where the majority of cells were PI-positive. The measurement was finished when 10,000 events were acquired. Obtained data was analyzed using the Attune NxT software, where cell survival was

assessed on fluorescence intensity histogram.

### 2.1.5. Lucifer Yellow uptake

To visualize sugar uptake and distribution within cell cytoplasm, Lucifer Yellow was used. Efficiency of electroporation was evaluated using Lucifer Yellow (LY) uptake. In this set of experiments, after trypsinization cells were allowed to settle to the bottom of 24-well culture plates for 15 min and electroporated using platinum-iridium wire electrodes with 0.8 mm diameter and 4 mm gap. Pulses of 1.5 kV/cm voltage-to-distance ratio were applied to hUCMSCs suspension in buffers containing 400 mM of respective sugars (prepared as indicated in 'Permeabilization and recovery assays') and 0.5 mg/ml of Lucifer Yellow CH dilithium salt (molecular weight of 521.6 g/mol) using the  $\beta$ tech electroporator (Electro cell B10, Betatech, France). Electroporated samples were allowed for resealing for 10 min at room temperature followed by washing with PBS by centrifugation. Negative control samples were treated in the same manner but without electroporation. The LY dye was excited with 425 nm and emission detected from the band pass filter at 605 nm (D605/55 m, Chroma, Rockingham, USA). Bright field and fluorescent images were randomly acquired from at least 10 fields of view in the middle between the electrodes using inverted microscope AxioVert 200 (Zeiss, Germany) equipped with VisiCam 1280 camera (Visitron, Germany) and the MetaMorph 7.1.1 PC software (Molecular Devices, USA). The total number of cells and the number of LY-positive cells were manually counted on each image using cell counter plugin in ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA). The efficiency of electroporation was calculated as the percentage of the LY-positive cells from total number of cells on one image.

### 2.1.6. Differential scanning calorimetry

In order to determine glass transition and melting temperatures, samples were analyzed using DSC with a Netzsch DSC 204F1 Phoenix instrument (Netzsch-Geraetebau GmbH, Selb, Germany). Freezing solutions (approximately 10 mg) were added into a 25- $\mu$ l aluminum pans and hermetically sealed whereas an empty pan was used as a reference sample. For DSC measurements, the cooling/heating protocol was the same as that used in the cryomicroscopy studies with the exception of an isothermal annealing step. Thermal events were determined from the obtained DSC thermograms using Netzsch software.

### 2.1.7. Cryomicroscopy

To evaluate ice crystallization and recrystallization behavior of sugar-containing electroporation buffers cryomicroscopy was performed. Briefly, a cell-free sample aliquot of 2  $\mu$ l was pipetted on a quartz crucible (Resultec Analytic Equipment, Germany) and covered with a 12-mm cover glass (A. Hartenstein, Germany) to inhibit evaporation inside a FDSC196 freeze-drying cryostage (Linkam, UK) which was mounted on an AxioVert M1m microscope (Carl Zeiss, Germany). Samples were incubated at  $20^\circ\text{C}$  for 2 min, cooled with a ramp of 20 K/min down to  $-180^\circ\text{C}$  and after holding at  $-180^\circ\text{C}$  for 10 min heated with a ramp of 10 K/min to annealing temperature  $-3^\circ\text{C}$ . After isothermal annealing for 30 min the samples were melted with a ramp of 10 K/min back to  $20^\circ\text{C}$ . Images were acquired with a frequency of 6 frames/s using Linkam Software (Linksys32, Linkam, UK).

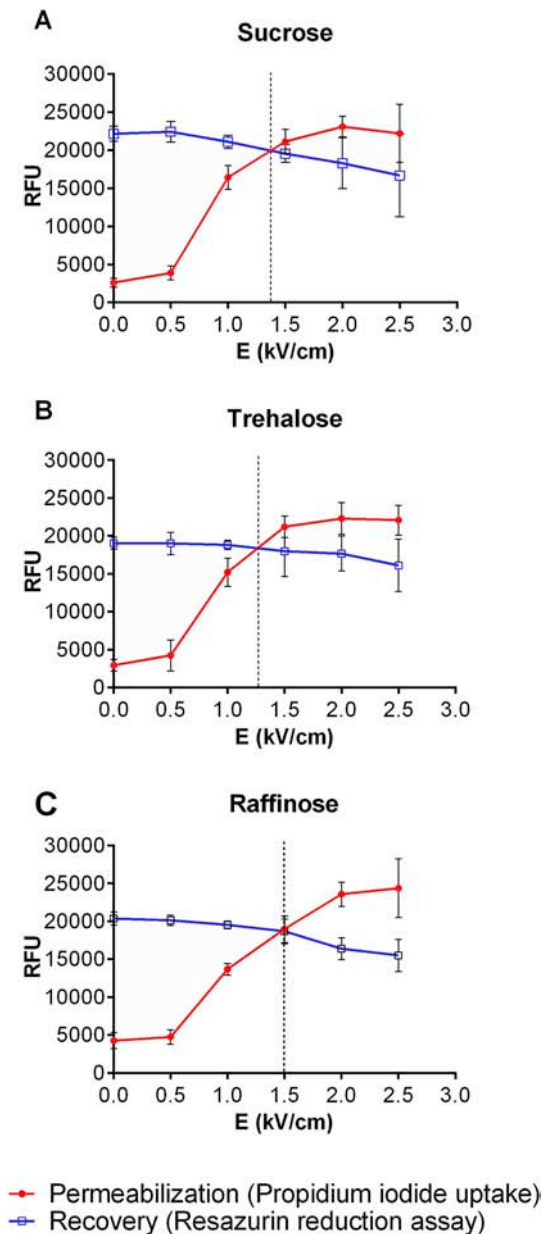
### 2.1.8. Statistical analysis

To test the obtained data for normality, a Shapiro–Wilk test was performed. Normally distributed data were reported as mean  $\pm$  standard deviation (SD) and analyzed using parametric one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. For representation of quantitative data and their statistical analysis Graph Pad Prism 8.0 (GraphPad Software, USA) was used. Cut-off  $p$ -values less than 0.05 were considered as statistically significant.

### 3. Results

#### 3.1. Evaluation of cell permeabilization and recovery

In the first set of experiments, pulses with different strength of electrical field were tested to provide effective permeabilization of hUCMSCs while not compromising their recovery. To minimize the effect of osmotic stress, all sugars were used in 250 mM concentration. Cell recovery was evaluated by resazurin reduction assay 24 h after electroporation. Varying the electric field in the range of 0–2.5 kV/cm, respective permeabilization vs. recovery curves were generated. With the increasing pulse amplitude, higher PI fluorescence was detected. The uptake of PI increased with increasing electric field up to 2 kV/cm (Fig. 1



**Fig. 1.** Cell recovery and PI uptake during electroporation. The fluorescence intensity of PI and resazurin after electroporation of hUCMSCs using (A) 250 mM of sucrose, (B) trehalose and (C) raffinose (8 pulses of 100  $\mu$ s duration and 1 Hz pulse repetition frequency) are presented. Uptake of PI increased, and cell recovery decreased with increased pulse amplitudes. Mean values with standard deviation of at least four independent experiments are shown. RFU stands for relative fluorescence unit.

A-C, red lines). At the same time, cell recovery declined starting from 1.5 kV/cm as shown by the blue lines in Fig. 1 A-C. The uptake and recovery trends were similar for all three sugars in the range of pulse parameters used.

The two parameters (permeabilization vs. recovery) for all sugars had their interception very close to 1.5 kV/cm. Since at about 2.0 kV/cm no further increase in PI intensity was observed and from 1.5 kV/cm cell recovery started to decrease, the 1.5 kV/cm voltage-to-distance ratio was considered as being optimal.

Cells showed similar shape and attachment when an electric field of 1.5 kV/cm (Fig. 2 D-F) was used for permeabilization in comparison to control cells, i.e. 0 kV/cm (Fig. 2 A-C). At 2.5 kV/cm far less cells were able to attach and their shape was altered (see Fig. 2 G-I).

#### 3.2. Evaluation of electroporation-assisted cryopreservation on post-thaw cell survival

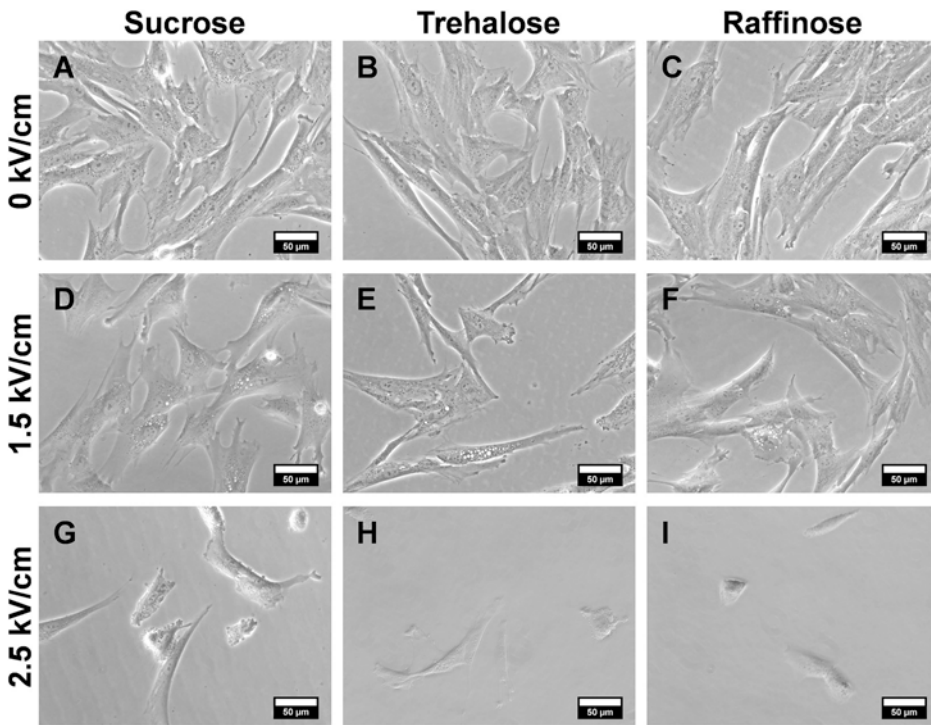
Since the optimal field strength was determined, the next step was to investigate the effect of different sugar concentrations loaded into hUCMSCs on their protective effect during freezing. For this purpose, low-conductivity electroporation buffers containing 0–400 mM of corresponding sugars were used as the sole cryoprotectants. As a positive control, the standard freezing solution containing 10% Me<sub>2</sub>SO and 90% FBS was used. Cell survival before and after cryopreservation was analyzed by trypan blue exclusion assay and flow cytometry using PI.

The effect of different sugar concentrations on post-thaw survival of hUCMSCs based on membrane integrity is shown in Fig. 3. Using the trypan blue exclusion assay (Fig. 3 A), a sugar concentration dependent increase in survival is shown. The highest percentage of viable cells was found to be for sucrose 76  $\pm$  8%, trehalose 77  $\pm$  8% and raffinose 82  $\pm$  9%, respectively, using the highest tested concentration of 400 mM. Similar results were obtained using flow cytometry (Fig. 3 B). Again, with increasing sugar concentrations, post-thaw survival increased and suggests a correlation between the survival of hUCMSCs and concentration of sugars used for the electroporation. In addition, flow cytometry data corroborated that all 3 sugars are equally effective in preserving hUCMSCs without animal serum and Me<sub>2</sub>SO.

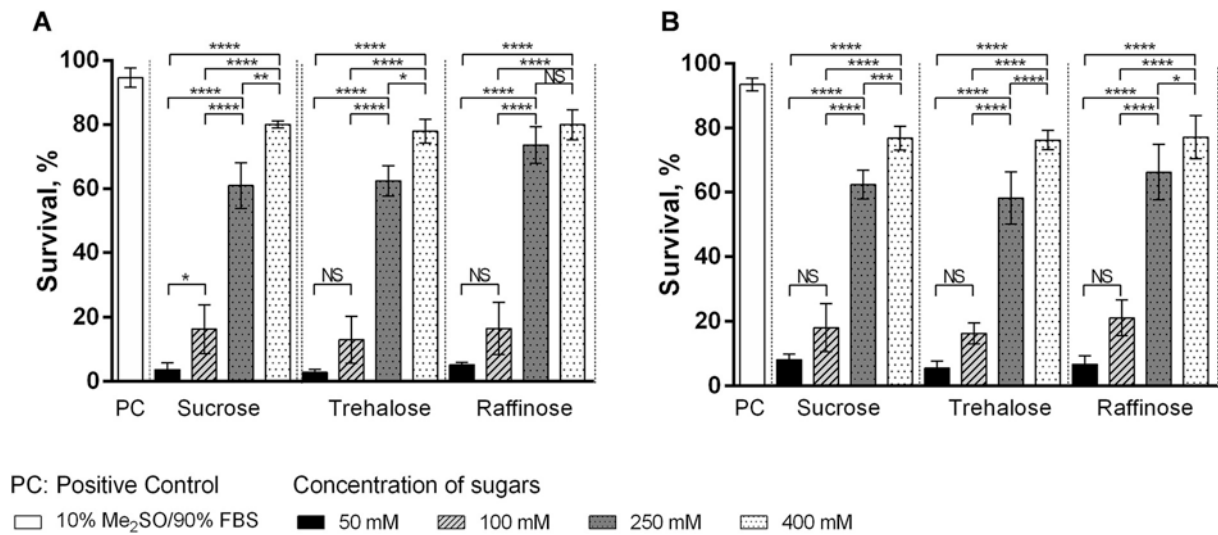
The cryopreservation efficiency using 400 mM of sugars with electroporation was reproducibly statistically higher than in other groups tested, thus this concentration was then used in further experiments. In general, cryopreservation of hUCMSCs using 10% Me<sub>2</sub>SO lead to significantly higher survival (95  $\pm$  4%) compared to the other groups independent of the analysis method.

Since osmolality plays an important role in resealing after electroporation and in cryopreservation, the osmolality of the electroporation/freezing solutions was determined. A 400 mM sucrose freezing solution exhibited an osmolality 497 mOsm/kg, trehalose 503 mOsm/kg and raffinose 592 mOsm/kg compared to 10% Me<sub>2</sub>SO/90% FBS which had an osmolality of 2098  $\pm$  mOsm/kg.

Since it was found that hyperosmolar pulse media (400 mM) preserved significantly higher number of hUCMSCs in the covered concentration range, the next question was whether it also provides high electroporation efficiency. In these experiments Lucifer Yellow uptake was used. As opposed to PI (nucleic acid binding fluorophore), LY accumulates in cell cytoplasm and present an effective tool for visualization of intracellular delivery of molecules. It is a small, polar and non-permeant fluorescent tracer with a molecular mass of 522 g/mol and a net charge of  $-2$ . In the context of this study, LY is widely used in both electroporation and cryopreservation as a tool for detection of otherwise membrane-impermeable molecules after exposure to electric field [50], endocytosis [52] or freezing-induced osmotic forces [82]. Fig. 4 shows, that the vast majority of electroporated hUCMSCs exhibit green fluorescence due to LY in hyperosmolar medium containing sucrose (A), trehalose (B) and raffinose (C). On the contrary, no or negligible number of cells were fluorescent in all negative control samples (not exposed to electric pulses; D, E, F), respectively.



**Fig. 2.** Influence of electric field and sugar type on cell morphology. Panel of light microscopy images of hUCMSCs before and 24 h after electroporation with 250 mM sucrose, trehalose and raffinose, respectively. Control (A, B, C) and electroporated at 1.5 kV/cm (D, E, F) cells exhibit typical fibroblast-like morphology, attachment and spreading characteristics. However, cells electroporated at 2.5 kV/cm (G, H and I) demonstrate visibly altered cellular morphology and decreased cell numbers in all three groups of sugars. Scale bars represent 50  $\mu$ m.



**Fig. 3.** Post-thaw survival of hUCMSCs electroporated with sugars compared to positive control. Column charts showing the results on determination of viable cell counts using trypan blue (A) and flow cytometry (B) after electroporation and cryopreservation using different concentrations of sugars. 10% Me<sub>2</sub>SO/90% FBS served as a positive control (PC). Data are reported as mean  $\pm$  standard deviations. Here NS - not significant; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001 followed by one-way ANOVA.

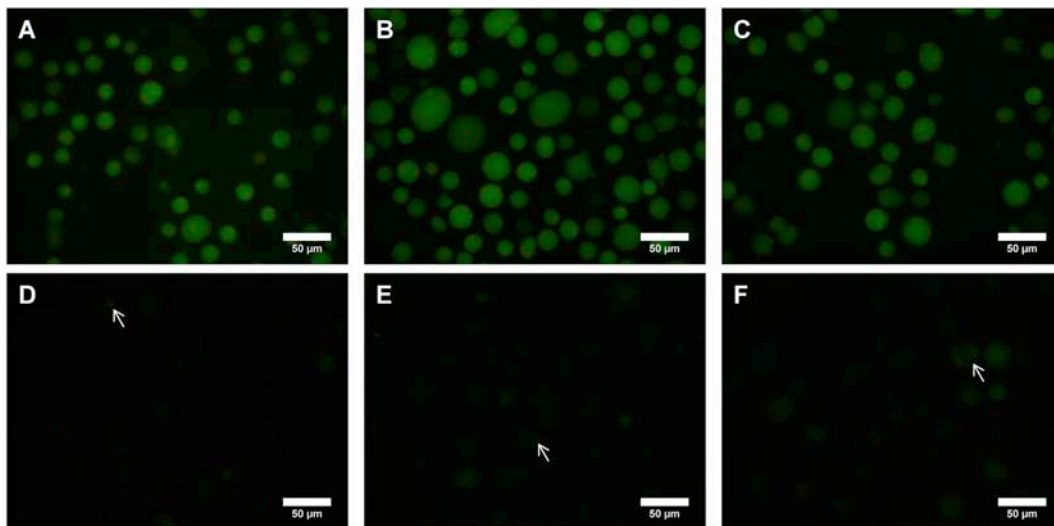
Lucifer Yellow was homogeneously distributed throughout the cell cytoplasm, but in non-electroporated cells LY started to accumulate in endocytotic vesicles.

The number of LY-positive cells was calculated from fluorescent images and compared with the total number of cells determined from respective phase contrast images. High electroporation efficiency (91  $\pm$  6, 89  $\pm$  5, 90  $\pm$  5% of LY-positive cells for sucrose, trehalose and raffinose, respectively) was accompanied by high post-electroporation survival of hUCMSCs evaluated by trypan blue exclusion (93  $\pm$  5, 92  $\pm$  5, 94  $\pm$  5% of membrane-intact cells for sucrose, trehalose and raffinose, respectively).

To compare the efficiency of cryopreservation with and without

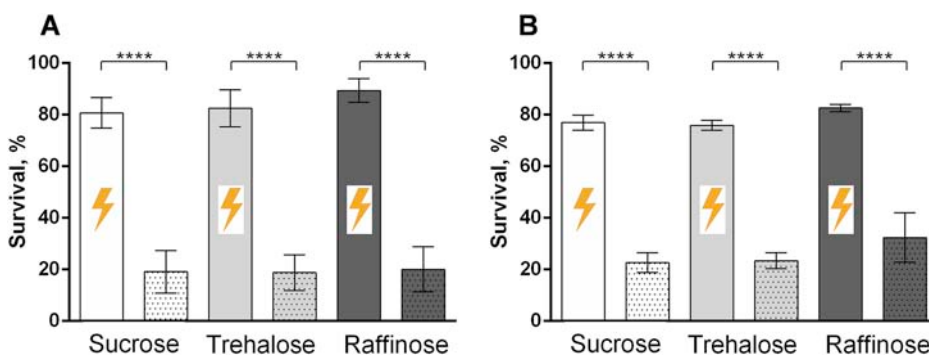
electroporation, electroporated and non-electroporated hUCMSCs were frozen in buffers containing 400 mM of each sugar as an extracellular cryoprotectant. Indeed, electroporation of cells with sugars significantly increased the cell survival after thawing as analyzed by trypan blue exclusion assay and flow cytometry (see Fig. 5 A, B). The first analysis revealed that hUCMSCs frozen with 400 mM extracellular sucrose have a post-thaw survival of 81  $\pm$  6% viable cells compared to non-electroporated cells (19  $\pm$  8%). Similar results were found for trehalose (83  $\pm$  7% vs. 19  $\pm$  7%) and raffinose (89  $\pm$  5% vs. 20  $\pm$  9%) (Fig. 5 A). The same trend was found also using flow cytometry with PI (Fig. 5 B).

Not only the post-thaw survival is an important parameter but also



**Fig. 4.** Detection of electroporation of hUCMSCs by Lucifer Yellow uptake.

All fluorescent images were acquired either 10 min after incubation in LY or after applying a train of eight 100  $\mu$ s rectangular pulses delivered with the repetition frequency 1 Hz at 1.5 kV/cm voltage-to-distance ratio. Cells electroporated with LY in sucrose- (A), trehalose- (B) and raffinose-based pulse media (C) exhibit bright green fluorescence, respectively. In some negative control cells (D, E, F), i.e. not exposed to electric pulses, accumulation of LY in endocytic vesicles was observed (white arrows). Scale bar represents 50  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 5.** Post-thaw survival of sugar-loaded with electroporation or non-loaded hUCMSCs after cryopreservation.

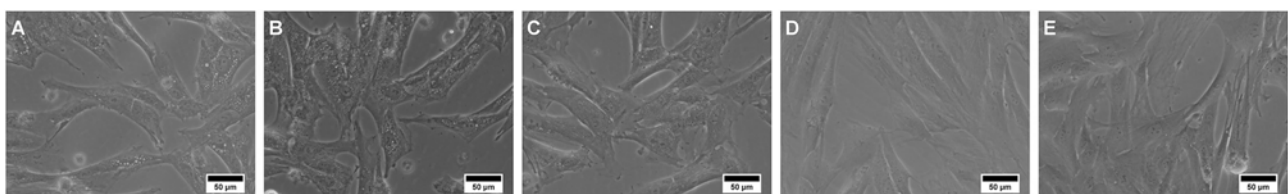
Column charts showing the results on determination of viable cell counts using trypan blue (A) and flow cytometry (B) in electroporated (symbol of high voltage) and non-electroporated samples cryopreserved using 400 mM sugar concentration. Data are reported as mean  $\pm$  standard deviations and asterisks represent significant differences between groups at  $p < 0.0001$  followed by one-way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

attachment and morphology of the cells. To analyze the cell shape, attachment and spreading after cryopreservation, hUCMSCs were thawed, plated and cultured for 24 h before light microscopy images were acquired. No morphological changes were found regardless of CPA after attachment. Fig. 6 A-E presents morphological features of cells cryopreserved using sucrose (A), trehalose (B), raffinose (C) and Me<sub>2</sub>SO (D) in comparison to fresh cells (E). All cryoprotectants employed preserved well the cell ability to adhere and spread as well as their typical fibroblast-like morphology. Electroporated cells frozen under the protection of sugars accumulated characteristic bright vacuoles absent in fresh and positive control cells.

Representative light microscopy images of *in vitro* cultures of cryopreserved vs. fresh hUCMSCs. Cells electroporated and cryopreserved using sucrose (A), trehalose (B) and raffinose (C) display comparable morphology to cells cryopreserved in Me<sub>2</sub>SO (D) and fresh cells (E). No negative impact of electroporation-assisted cryopreservation on cell attachment 24 h post-thaw was observed. Scale bar represents 50  $\mu$ m.

### 3.3. Determination of glass transition temperatures and ice recrystallization behavior of sugar-containing electroporation buffers

In order to understand the effect of different sugars on the



**Fig. 6.** Morphology of cells 24 h after cryopreservation using either sugars or Me<sub>2</sub>SO compared to non-cryopreserved cells.

Representative light microscopy images of *in vitro* cultures of cryopreserved vs. fresh hUCMSCs. Cells electroporated and cryopreserved using sucrose (A), trehalose (B) and raffinose (C) display comparable morphology to cells cryopreserved in Me<sub>2</sub>SO (D) and fresh cells (E). No negative impact of electroporation-assisted cryopreservation on cell attachment 24 h post-thaw was qualitatively observed. Scale bar represents 50  $\mu$ m.

cryopreservation process, DSC and cryomicroscopy investigations were necessary.

Determination of glass transition and melting temperatures in electroporation buffers used for cryopreservation was performed. Fig. 7 demonstrates representative DSC scans for all three sugar-containing electroporation buffers whereas in Table 1 quantitative data are reported.

### 3.4. Ice crystallization and recrystallization behavior

Ice crystallization and recrystallization are typical processes occurring during freezing/thawing. Crystallization temperatures determined using cryomicroscope for 400 mM sucrose, trehalose and raffinose were  $-19.67 \pm 2.90$  °C,  $-18.57 \pm 1.70$  °C and  $-17.20 \pm 0.40$  °C, respectively. Much lower values were obtained for 10% Me<sub>2</sub>SO/90% FBS group:  $24.83 \pm 0.60$  °C.

The next logical step was to assess the ice recrystallization behavior of sugar-containing electroporation buffers. All known types of recrystallization processes were evident in sugar solutions. Fig. 8 shows crystals formed in freezing solutions containing 400 mM sucrose (A, D), trehalose (B, E) and raffinose (C, F) at 5 and 30 min during isothermal annealing when crystals were fairly separated from each other. Isomass recrystallization which refers to changes in ice crystal shape in a way that a crystal with irregular-shape tends to become smoother due to minimization of energy is exemplified by sucrose (A vs. D). Migratory recrystallization or so-called Ostwald ripening is based on the growth of larger crystals at the expense of smaller ones. Larger ice crystals have higher specific surface energy in virtue of what water molecules tend to diffuse from the surface of smaller ice crystal to that of larger ones (C vs. F, example of raffinose). However, in our studies the most prominent was accretive recrystallization which occurs when two adjacent ice crystals fuse together to form a single larger crystal (B vs. E, example of trehalose).

## 4. Discussion

Adverse reactions induced by Me<sub>2</sub>SO present a significant problem in stem cell transplantation practice [23,71]. Elimination of Me<sub>2</sub>SO-related side effects might be achieved through its depletion before transplantation [for review see [30]. In turn, conventional methods of removing Me<sub>2</sub>SO from cell suspensions based on centrifugation are associated with considerable cell loss which is disadvantageous given the high cell doses needed for transplantation. On the other hand, use of animal serum in freezing media is connected with the immunogenicity,

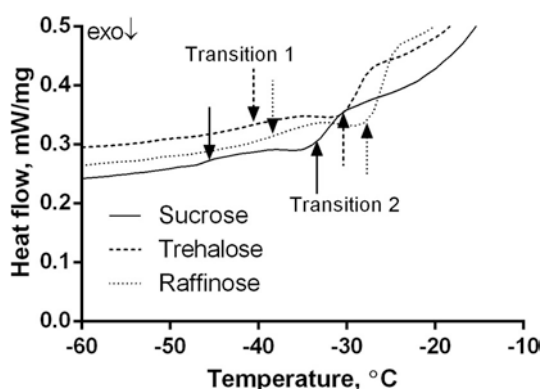


Fig. 7. Thermogram of different sugars analyzed with DSC.

A typical DSC thermogram of sugar-containing electroporation buffers obtained at heating rate of 10 °C/min. Two endothermic events representing glass transition points (Transition 1) and onset of melting (Transition 2) for sucrose (solid line and arrows), trehalose (dashed line and arrow) and raffinose (dotted line and arrow) are shown on the DSC curves.

**Table 1**  
Characteristics of hyperosmolar electroporation buffers.

Parameters studied	Sugar type		
	Sucrose	Trehalose	Raffinose
Glass transition temperature (T <sub>g</sub> ' midpoint), °C	$-45.23 \pm 0.21$	$-39.73 \pm 0.83$	$-37.97 \pm 0.40$
Tonset of melting, °C	$-32.50 \pm 0.71$	$-29.53 \pm 0.60$	$-26.67 \pm 0.50$
Melting peak (T <sub>m</sub> ), °C	$5.78 \pm 0.57$	$6.23 \pm 0.35$	$5.47 \pm 0.87$

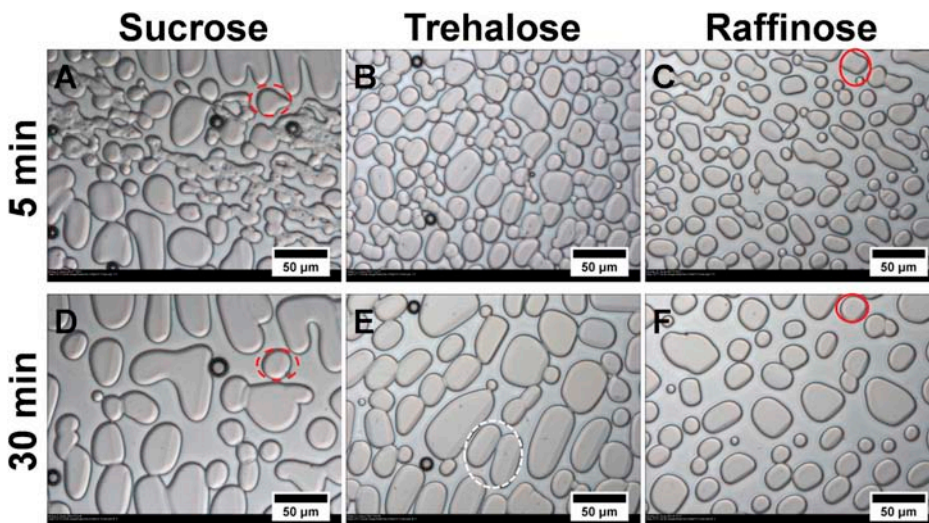
contamination risks as well as process and product irreproducibility. Therefore, to conform to GMP requirements, alternatives to Me<sub>2</sub>SO and serum in cryomedia as well as development of technical approaches enabling Me<sub>2</sub>SO- and serum-free cryopreservation are of high value [41, 67].

Owing to multiple cryoprotective properties, selected disaccharides, such as sucrose and trehalose, are widely used in cryopreservation practice as additives to various freezing [2,51] and vitrification solutions [15,33,83]. Conceptually, combined introduction of sugars into cryopreservation media and their pre-freeze loading into cells serves as an alternative to conventional cryopreservation workflow. In cryopreservation, among diverse techniques for sugar loading electroporation offers high performance, reproducibility, safety and accuracy [24]. Thus, the main idea of this work was to validate an electroporation-based approach for incorporation of sugars into cells for serum- and Me<sub>2</sub>SO-free cryopreservation.

Electroporation is a well-established and long used method to introduce membrane-impermeable molecules into cells and has thus found its application also in cryopreservation. In the electroporation field, PI uptake is used to determine the efficiency of permeabilization. PI is a non-permeant and non-permeable fluorescent molecule widely used for electroporation detection. The molecular mass of PI corresponds to 668 g/mol, however, it is believed that only propidium ions with the molecular mass 542 g/mol are detected [50]. Sucrose, trehalose dihydrate and raffinose pentahydrate used in these studies have a molecular weight of 342, 378 and 594 g/mol, respectively. Although indicated sugars and PI are of similar size, they vary significantly in charge and structure. Therefore, their interactions when passing through the permeabilized membrane are expected to be different. For instance, propidium ion has two positive charges and is hydrophilic dye whereas the tested sugars are nonionic amphiphilic molecules. Despite these differences, Mohr et al. shows in the context of our article that PI and trehalose could be effectively loaded into human embryonic stem cells at the same pulse parameters [48]. However, as recent review by Kotnik et al. highlights, in fact, only few molecular dynamic simulations were performed to model the transport of both large and small molecules across permeabilized cell membranes and little is known about the exact molecular mechanisms behind and timescale involved in these processes [39].

First, we determined the optimal strength of electric field as a compromise between high PI uptake and cell recovery. All sugars were delivered to the cells using eight 100 μs pulses of 0–2.5 kV/cm voltage-to-distance ratio. Under conditions tested here, 1.5 kV/cm was chosen as optimal voltage-to-distance ratio based on PI uptake and cell recovery data (Fig. 1). These observations were in agreement with data on cell attachment and morphology in all three sugar groups. Cell morphology was not altered when using 1.5 kV/cm (Fig. 2 D-F) compared to control cells (Fig. 2 A-C). However, using an electric field of 2.5 kV/cm caused visible alterations in cell shape and spreading characteristics probably due to affected cytoskeleton [14,39]. The pulse parameters (pulse duration, number and pulse repetition frequency) were used based on the previous work [24] and only pulse amplitude was optimized.

For cryopreservation purposes, cells were electroporated with sucrose, trehalose and raffinose in 0–400 mM extracellular concentration range and frozen in the same sugar concentrations to mitigate osmotic stress. Non-electroporated cells cryopreserved with 10% Me<sub>2</sub>SO/90%



**Fig. 8.** Cryomicroscopic analysis of ice crystal growth during isothermal annealing. Cryomicroscopy images showing dynamics of ice crystal growth in the presence of electroporation buffers containing 400 mM sucrose (A, D), trehalose (B, E), raffinose (C, F), Isomass (red dashed circles), accretive (white dashed circle) and migratory (red solid circles) types of recrystallization are shown in sucrose, trehalose and raffinose, respectively. Scale bar represents 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

FBS were used as a positive control. Among all sugar concentrations that were tested, the highest percentage of viable cells around 80% was obtained at a sugar concentration of 400 mM. Still the survival in positive control group was around 15% higher as determined by trypan blue exclusion test and flow cytometry (see Fig. 3). This was despite similar pre-freeze survival of electroporated (in case of pre-optimized strength of electric field) and non-electroporated cells. It has to be noted that *a priori* volumetric response of sugar-loaded cells and cells equilibrated with Me<sub>2</sub>SO to constant slow cooling will be different. Cells equilibrated with Me<sub>2</sub>SO having much higher osmolality would apparently dehydrate faster. It might be assumed that initial volume of sugar-loaded cells would be bigger than that of unloaded. Therefore, direct comparison of such cryopreservation group is associated with certain limitations.

Although trehalose is the most widely used and investigated sugar with respect to its intracellular delivery for cryopreservation purposes, this study clearly demonstrates that sucrose and raffinose are as effective as trehalose. Our study is consistent with previous work by Petrenko et al. [54] in which no difference between sucrose, trehalose and raffinose was found in Me<sub>2</sub>SO- and xeno-free cryopreservation of hMSCs.

To visualize electroporation process and its impact on cell survival in the presence of hyperosmolar sugar-containing pulse media, LY uptake and trypan blue studies were followed. Fluorescence imaging revealed higher than 90% efficiency of LY incorporation and cell survival after electroporation in all groups tested (see Fig. 4). This suggests that pulse media based on 400 mM sugars did not affect cell permeabilization and viability. In this study LY was not used as a predictive measure of intracellular sugar content depending on extracellular one. However, it is known from previous work and literature that small molecules tend to equilibrate across the permeabilized membrane, i.e. intracellular concentration becomes equal to extracellular for ions, for larger molecules intracellular concentration becomes progressively lower with respect to extracellular concentration [39].

The next question was what contribution has electroporation of cells with sugars to their cryoprotection. Cryopreservation studies involved two comparison groups for respective sugars: cells frozen solely in extracellular sugar solutions and cells frozen employing extracellular sugars and electroporation. After cryopreservation using extracellular sugars without electroporation the survival levels were around 60% lower than that of in the group with electroporation (Fig. 5).

Being used as sole extracellular CPAs, sugars produce multiple cryoprotective actions. According to the most accepted 'water replacement hypothesis' formulated by Carpenter and Crowe [12], sugars stabilize lipid membranes through direct interaction with polar groups of membrane lipids and proteins by hydrogen bonding. In addition, we believe that directly after electroporation sugars may be homogeneously

distributed within the cell cytoplasm and organelles as demonstrated by LY delivery (Fig. 4) and inhibit intracellular ice formation and propagation throughout a cell. Additional experiments are needed to verify this assumption and to determine the intracellular content of sugars providing the highest cryopreservation outcome. All cryoprotectants that were employed here preserved cell morphology and the cell's ability to adhere and spread which on its own is a good indicator of post-thaw cell recovery (Fig. 6). However, the determination of actual number of attached cells would give more evidence on the efficacy of such a cryopreservation approach. Electroporated cells that were protected by sugars accumulated characteristic bright vacuoles after recultivation that are often observed in the case of endocytosis-mediated introduction of sucrose [8] or trehalose [11]. Such inclusions were absent in fresh and positive control cells. In this context, some studies suggest that sucrose, trehalose and raffinose when internalized promote autophagy in mammalian cells [21,29,45,64]. Other studies showed that non-reducing disaccharides could block autophagy [78]. Investigations on the role of internalized sugars on post-thaw cell fate would provide further insights into the safety of electroporation-assisted cryopreservation of stem cells at the molecular level.

The issue of post-thaw sugar elimination from cells is scarcely addressed in the literature and was beyond the scope of these investigations. Eroglu et al. revealed rapid elimination of microinjected trehalose from developing mouse embryos presumably occurred by exocytosis [26]. Zhang et al. showed that intracellular trehalose washes progressively out of cells during cell culturing at a somewhat slower rate compared to endocytic uptake [81]. In case of electroporated cells, further studies would be of great value to determine the kinetics of sugar efflux from cells.

It is generally admitted that for stable long-term storage frozen samples must be stored well below the glass transition point (T<sub>g</sub>) of CPA when no or limited molecular mobility takes place. Sugars are known to possess excellent glass forming properties which is a major advantage in terms of storage stability. In view of an emerging interest in the use of -80 °C freezers for storage of cells and tissues in biobanks or, importantly, safe specimens shipment on dry ice, some recent studies address the feasibility of cell storage at -80 °C by elevating the T<sub>g</sub> of cryoprotective medium using for instance sucrose [74]. Yuan et al. showed that cryoprotective medium comprised of Ficoll 70 and Me<sub>2</sub>SO provided reliable cryopreservation of various types of human and porcine pluripotent stem cells at -80 °C via increased devitrification temperature of the CPA (-67 °C) and increased system thermal stability [80]. In our DSC studies, we observed two transitions differently interpreted in the literature. Schawe reports that the transition 1 at lower temperature (at around -44 °C) is T<sub>g</sub>' (glass transition of freeze-concentrated



solution when glass transforms to liquid) and the transition 2 (at around  $-34^{\circ}\text{C}$ ) represents the onset of ice melting for sucrose-water mixtures [63]. Hauptmann et al. determined  $T_g$  in sucrose-containing samples at  $-35^{\circ}\text{C}$  and revealed no second glass transition in DSC scans near  $-42^{\circ}\text{C}$  [28]. In the physical aging studies of the frozen  $\text{Me}_2\text{SO}$ -sucrose-water using DSC it was observed that the enthalpy relaxation peak, which is an attribute of the glass transition, was observed only in the case of Transition 1 and not 2 confirming that the second transition is not associated with the glass transition [74].

Since the measured  $T_g$ 's of all the sugar solutions used for electroporation and introduction into freezing solutions are much lower  $-80^{\circ}\text{C}$  (see Fig. 7 and Table 1), we hypothesize that samples frozen with sugars might potentially be long term stored at this temperature in ordinary laboratory freezers. This would reduce high maintenance costs associated with liquid nitrogen handling and risk of contamination through liquid nitrogen. Long-term studies would also be necessary to provide a direct comparison between sugars in terms of storage stability.

Ice recrystallization is one of the key mechanisms of cryodamage during transient warming and thawing and in particular at multiple freeze-thaw cycles and is manifested by enlargement of ice crystals with time. The results on ice recrystallization behavior are presented on Fig. 8. Of note, smaller ice crystals were revealed in the raffinose group as compared to sucrose and trehalose. The study by Chaytor et al. on ice recrystallization inhibition activity of diverse sugars showed that disaccharides inhibit ice recrystallization better than monosaccharides and that 200 mM galactose provided the best cell viability after cryopreservation at the level comparable to 5%  $\text{Me}_2\text{SO}$  [19]. The authors also demonstrated that the cryoprotective benefits of galactose were a result of its internalization and ability to mitigate osmotic stress, prevent IIF and/or inhibit ice recrystallization. Moreover, introduction of ice recrystallization inhibitors into cells is a novel approach to modulate intracellular ice growth as has recently been shown by Poisson et al. [55]. We believe that upon delivery into cells by means of electroporation sugars may prevent IIF and intracellular ice recrystallization which requires further experimental verification.

Thus, in this study relatively high post-thaw cell survival was obtained by introducing of different sugars into human stem cells using one method – electroporation. This was achieved so far by varying only one pulse parameter – strength of electric field while keeping other parameters constant. Further optimization of pulse parameters such as duration and repetition frequency, number of pulses as well as resealing time are needed to improve cryopreservation outcome. It is reasonable to coordinate such a multiparameter study with parallel determination of intracellular sugar content to find out optimal interplay between pulse parameters, cell survival after electroporation, intracellular sugar content and post-thaw cell survival. Other considerations for optimizing cryopreservation protocol include investigation of controlled-rate freezing and induced nucleation. Apart from conventional  $\text{Me}_2\text{SO}$ -containing media, comparing of optimized electroporation approach with commercial available  $\text{Me}_2\text{SO}$ - and serum-free media would provide additional insights into potential of electroporation-assisted cryoprotection of stem cells.

## 5. Concluding remarks

Elimination of xenogeneic serum from freezing solutions and potential decrease of concentration or complete elimination of cytotoxic  $\text{Me}_2\text{SO}$  while retaining high cell viability and functionality after thawing will definitely be advantageous for GMP-certified cryopreservation of clinically relevant cell types. In this work, we show that electroporation even with only roughly optimized pulse parameters seems to be a cell-friendly method for intracellular sugar delivery and enables serum- and  $\text{Me}_2\text{SO}$ -free cryopreservation. Although not statistically significant, electroporation with raffinose showed a trend towards higher cryopreservation efficiency as compared to sucrose and trehalose. The results of our study will hopefully stimulate new scientific ideas to cover wider

range of applications in the field of cryobiology. Further studies are needed to shed light on the intracellular sugar content providing improved cryopreservation outcome and overall long-term stability and safety of electroporation-assisted cryopreservation.

## Declaration of competing interest

The authors declare that they have no conflict of interests.

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