J Membrane Biol (2017) 250:1–9
DOI 10.1007/s00232-016-9916-z

Cryopreservation of Human Adipose-Derived Stem Cells in Combination with Trehalose and Reversible Electroporation

Barbara Dovgan[1] · Ariana Barlič[1] · Miomir Knežević[1] · Damijan Miklavčič[2]

Received: 20 October 2015 / Accepted: 25 June 2016 / Published online: 6 July 2016

Abstract New cryopreservation approaches for medically applicable cells are of great importance in clinical medicine. Current protocols employ the use of dimethyl sulfoxide (DMSO), which is toxic to cells and causes undesirable side effects in patients, such as cardiac arrhythmias, neurological events, and others. Trehalose, a nontoxic disaccharide, has been already studied as a cryoprotectant. However, an efficient approach for loading this impermeable sugar into mammalian cells is missing. In our study, we assessed the efficiency of combining reversible electroporation and trehalose for cryopreservation of human adipose-derived stem cells. First, we determined reversible electroporation threshold by loading of propidium iodide into cells. The highest permeabilization while maintaining high cell viability was reached at 1.5 kV/cm, at 8 pulses, 100 μs, and 1 Hz. Second, cells were incubated in 250 or 400 mM trehalose and electroporated before cryopreservation. After thawing, 83.8 ± 1.8 % (mean ± SE) cell recovery was obtained at 250 mM trehalose. By using a standard freezing protocol (10 % DMSO in 90 % fetal bovine serum), cell survival after thawing was about 91.5 ± 1.6 %. We also evaluated possible effects of electroporation on cells' functionality before and after thawing. Successful cell growth and efficient adipogenic and osteogenic differentiation were achieved. In conclusion, electroporation seems to be an efficient method for loading nonpermeable trehalose into human adipose-derived stem cells, allowing long-term cryopreservation in DMSO-free and xeno-free conditions.

Keywords Electroporation · Trehalose · Cryopreservation · Stem cell therapy · Adipose-derived stem cells

Introduction

Human mesenchymal stem/progenitor cells (MSCs) from bone marrow, adipose tissue, placenta, umbilical cord, and other tissues are currently being administered to large number of patients in different clinical trials (Syed and Evans 2013; Fang et al. 2007; Lendeckel et al. 2004; Puissant et al. 2005; Rada et al. 2009). For stem cell-based therapies to be used routinely in a clinical setting, these cells must be stored. The most widely used method for long-term storage of cells and tissues at subzero temperatures, while preserving their vital function after thawing, is cryopreservation. Since 1949, when Polge and coworkers published an article describing a method for successful freezing and thawing of mammalian spermatozoa (Polge et al. 1949), cryopreservation methods began to develop extensively (Mazur 1984; Crowe and Crowe 2000; Eroglu et al. 2000). Cryopreservation seeks to reach low temperatures while protecting cellular structures from damage caused by formation of ice crystals during freezing and thawing. The most traditional approach relies on protocols that include addition of cell membrane penetrating cryoprotectants such as dimethyl sulfoxide (DMSO) (Mazur 1984). DMSO is a small amphiphilic molecule with a hydrophilic sulfoxide group and two hydrophobic methyl groups. Due to its properties, DMSO can readily penetrate cell membranes. It acts as a solvent, which loosens cell

✉ Damijan Miklavčič
damijan.miklavcic@fe.uni-lj.si

[1] Educell Ltd, Prevale 9, 1236 Trzin, Slovenia

[2] Faculty of Electrical Engineering, University of Ljubljana, Tržaška 25, 1000 Ljubljana, Slovenia

membrane and generates "pores" through which it enters into the cell (Gurtovenko and Anwar 2007) and at the same time increases the intracellular concentration of solutes (Karlsson and Toner 1996). However, despite its efficiency in cryopreservation, DMSO is toxic to cells at room temperature (Katkov et al. 2006; Wang et al. 2011). The toxicity of DMSO is even more damaging in clinical settings when stem cells are thawed and infused directly into patients. In these cases, DMSO can cause serious side effects or can be even fatal (Zenhausern et al. 2000; Grigg et al. 2000; Higman et al. 2000; Syme et al. 2004; Sharp et al. 2013; Cox et al. 2012).

In nature, a wide variety of organisms and animals are able to tolerate low temperatures or dehydration stress by accumulation of large amounts of disaccharides, like trehalose and sucrose. These include plant seeds, bacteria, insects, yeast, crustaceans, and also vertebrates (Crowe and Crowe 2000; Brockbank et al. 2010; Larson et al. 2014; Tsujimoto et al. 2016). These sugars are believed to protect cells in two ways. The first proposed mechanism is "the water replacement hypothesis" or stabilization of biological membranes and proteins by direct interaction of sugars with polar residues through hydrogen bonding. Addition of disaccharides, especially trehalose, depresses phase transition temperature (T_m) so that the membrane remains in the liquid crystalline state even when dried. Upon rehydration, no phase transition takes place and no leaking of cytoplasmic contents occurs (Crowe et al. 1988, 1989; Konov et al. 2015). The second proposed mechanism includes the ability of sugars to form a stable glass (vitrification) upon loss of water. Loss of water within cells results in high molecular packing and slowing of molecular mobility, providing protection of cells until water is available (Kanias and Acker 2006).

To be effective as a cryoprotectant, trehalose has to be present on both sides of the membrane (Eroglu et al. 2000). Therefore, its use in mammalian cells has been limited because these cells do not metabolize trehalose, and their cell membranes are almost impermeable to disaccharides or larger sugars (Campbell and Brockbank 2012; Brockbank et al. 2010). To circumvent this obstacle, various experimental approaches have been tested to introduce trehalose into mammalian cells. Some of these techniques make use of the leakiness of cell membranes during phase transition (Beattie et al. 1997), the fluid-phase endocytosis (Wolkers et al. 2001), or with a genetically engineered mutant of pore forming toxin (Eroglu et al. 2000). In a different approach, mammalian cells were transfected with genes for trehalose synthesis using viral vectors (Guo et al. 2000). Another possibility to increase intracellular trehalose concentration seems to be culturing cells in vitro in the presence of trehalose (Campbell and Brockbank 2012; Petrenko et al. 2014). Despite a variety of described approaches, these methods exhibit low efficiency and poor control of trehalose loading into cells.

An alternative approach for introducing trehalose into cells is electroporation (Shayanfan et al. 2013; Phoon et al. 2008; Zhou et al. 2010). The principles of electroporation are known since more than 40 years. The method is based on temporary increase in the electrical conductance and permeability of biological membranes for various molecules upon application of external electric fields of sufficient strength and duration (Neumann and Rosenheck 1972; Kotnik et al. 2012; Rems and Miklavčič 2016). The technique has gained common acceptance because it is more controllable, reproducible, and efficient than other methods for intracellular delivery of xenomolecules (such as drugs, plasmids, and proteins) (Delteil et al. 2000; Miklavčič et al. 2000, 2014; Spiller et al. 1998; Yarmush et al. 2014; Haberl et al. 2013; Mahnič-Kalamiza et al. 2014; Golberg et al. 2016). Electroporation has also been used as a tool for introducing impermeable saccharides, however, mostly in plant cells (Shayanfan et al. 2013; Dymek et al. 2014; Phoon et al. 2008).

To avoid the use of toxic DMSO, we investigated the possibility of combining reversible electroporation with trehalose for cryopreservation of clinically relevant cell type—human adipose-derived stem cells (ASC). ASCs were electroporated in the presence of trehalose, cryopreserved, and recovered. The efficiency of our procedure was assessed by the evaluation of cell survival, viability, and differentiation potential in vitro. The results indicate that high number of functionally intact cells can be obtained by DMSO-free cryopreservation protocol. The new approach of cryopreservation might lead to safer stem cell therapies for patients.

Materials and Methods

Cell Culture

Adipose tissue of a single donor scheduled for elective lipoaspiration was obtained with informed consent and in accordance with the ethical guidelines of the National Medical Ethics Committee (code 21/09/07). ASCs were isolated as described previously (Zuk et al. 2001). Cells were cultured in DMEM/F-12 media with 10 % fetal bovine serum (FBS), 50 μg/mL gentamicin (all Gibco), and 1 ng/mL bFGF (Peprotech), in a humidified incubator at 37 °C and 5 % CO_2. Media were changed twice per week. When about 80 % confluency was obtained, cells were detached by 0.05 % trypsin-EDTA solution (Sigma Aldrich) and replated. For experiments, cells of the third (P3) or fourth passage (P4) were used.

Permeabilization Assay

Cell suspension was prepared at a density of 1×10^6 cells per 100 µL of low-conductive electroporation buffer (10 mM K_2HPO_4 (Merck), 10 mM KH_2PO_4, 1 mM $MgCl_2$ 250 mM trehalose (osmolality ~290 mOsm/kg), all obtained from Sigma Aldrich) containing 0.15 mM propidium iodide (PI) (Gibco). Cells were transferred to 2 mm electroporation cuvettes (Peqlab), and 8 pulses of 100 µs at 1 Hz were delivered at 0, 100, 200, 300, 400, 500, 600, and 680 V (which corresponds to 0–3.4 kV/cm electric filed), i.e., voltage divided with electrode distance in cuvette, with electroporator (Jouan GHT 1287 B). Pulses were measured with oscilloscope (Tektronix TDS 3012). After 2 min, cells were transferred to microcentrifuge tubes, and nonloaded PI was removed by centrifugation. ASC pellet was resuspended in DMEM/F-12, seeded in 96-well plate at a density of 260.000 cells/well, and PI fluorescence was measured at 530/615 nm using multilabel plate reader (Chameleon, Hidex). Experiment was performed in four independent repetitions.

Trehalose Uptake by Electroporation

Cell suspension was prepared at a cell density 1×10^6 cells per 100 µL of low-conductive electroporation buffer (10 mM K_2HPO_4, 10 mM KH_2PO_4, 1 mM $MgCl_2$) containing 250 mM (osmolality ~290 mOsm/kg) or 400 mM (osmolality ~410 mOsm/kg) trehalose (Sigma Aldrich). Osmolality was measured by freezing point depression with Knauer cryoscopic unit (model 7312400000, Knauer, Germany). Cell suspension was transferred to 2 mm electroporation cuvettes, and 8 pulses of 100 µs were delivered at 1 Hz of 0, 300 V amplitude (which represents 0 or 1.5 kV/cm electric field). After 2-min incubation at room temperature, 80 µL of cell suspensions was transferred to cryovials. Cells were incubated for additional 15 min at 37 °C to allow the cell membranes to reseal. As a positive control, standard cryopreservation protocol with 10 % DMSO (Wak Chemie) in 90 % FBS was used. Experiment was performed in three independent repetitions.

Cryopreservation and Thawing

Cryopreservation of cell suspension was initiated in a controlled rate container (freezing rate −1 °C/min) (Mr. Frosty, Nalgene) to achieve −80 °C. The next day cells were transferred into liquid nitrogen containers (−196 °C) for at least 1 week. Thawing was performed at 37 °C with 20 % FBS (standard protocol) or 250 mM trehalose in DMEM/F-12 for control and experimental protocol, respectively. Experiment was performed in three independent repetitions.

Cell Viability

Cell Survival and Recovery were assessed before and after Treatment by Trypan blue dye exclusion method and MTT Assay.

Trypan blue dye exclusion method: 1:1 dilution of the suspension was prepared using 0.4 % Trypan blue solution (Fluka), and staining was observed under inverted microscope (Nikon, Eclipse TS100). Viability was expressed as the percentage of viable unstained cells relative to the total number of cells counted in a hemocytometer counting chamber. Counts were done in duplicates.

MTT assay was assessed as described by Mossman with some modifications (Mosmann 1983). ASCs were seeded in 96-well plates at 50.000 cells/well with addition of cell culturing medium (DMEM/F-12, 10 % FBS, 50 µg/mL gentamicin) and incubated overnight at 37 °C and 5 % CO_2. The next day, medium was removed from each well and replaced with MTT solution (final concentration 0.5 mg/ml), Sigma Aldrich). After at least 6 h of incubation at 37 °C, a purple-colored formazan product has been developed. MTT solution was removed, and formazan crystals were dissolved in acidified isopropanol (0.04 M HCl in isopropanol, both obtained from Sigma Aldrich). Afterward, absorbance at 570 nm was measured by microplate reader (Chameleon, Hidex). Experiment was performed in at least three independent repetitions.

Cell Differentiation Assays

To induce adipogenic differentiation, ASCs at 70–80 % confluence were treated with adipogenic medium: DMEM/F-12, 5 % FBS, 50 µg/mL gentamicin (all Gibco), 10 µg/mL insulin, 1 µM dexamethasone, 200 µM indomethacin, and 500 µM 3-isobutyl-1-methylxanthine (all obtained from Sigma Aldrich), for 2 weeks. Medium changes were carried out twice per week. The presence of intracellular lipid droplets, indicative of adipocyte differentiation, was assessed by Oil Red O (Sigma Aldrich) staining.

To induce osteogenic differentiation, ASCs at 70–80 % confluence were cultured in osteogenic medium: Advanced DMEM/F-12, 5 % FBS, 50 µg/mL gentamicin, 200 mM GlutaMAX (all Gibco), 0.1 mM ascorbic acid, 0.1 µM dexamethasone, and 10 mM β-glycerophosphate (all obtained from Sigma Aldrich), for 4 weeks. The medium was changed twice per week. The osteogenic differentiation was evaluated by quantification of Ca^{2+} deposits by Calcium LiquiColor test (StanBio Laboratory).

Statistical Analysis

The results are represented as mean ± standard error (SE) of the mean. The statistical significance of differences

between the groups was evaluated by one-way, parametric ANOVA test (*NS* not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$). Statistical analysis was performed using GraphPad Prism, version 6.01 (GraphPad Software, Inc).

Results and Discussion

Selection of Optimal Electroporation Parameters for Cell Permeability and Recovery

In the first set of experiments, we evaluated the uptake of propidium iodide (PI) into human adipose-derived stem cells (ASC). We also evaluated the influence of electroporation on ASCs' viability. Therefore, in parallel, we tested the recovery of ASC after exposure to increasing electric field in the presence of PI (Fig. 1). To avoid osmotic stress, experiments were performed in isotonic media. With increasing electric field, fluorescence intensity was increasing, indicating higher PI uptake. The highest uptake was detected between 0.5 and 1.5 kV/cm. At about 2 kV/cm permeability of PI reached a plateau, meaning that by increasing electric field above this value, no more PI could penetrate into cells. On the contrary, by augmenting electric field, the recovery of cells was decreasing. At electric field between 1 and 1.5 kV/cm (i.e. 200 and 300 V), fluorescence of PI uptake and MTT absorbance measurement intercepted, and thus, this value was considered an optimal value between cell recovery and PI uptake for all further experiments. Despite trehalose and PI vary greatly in charge and structure, they have similar molecular weight (Mohr et al. 2006).

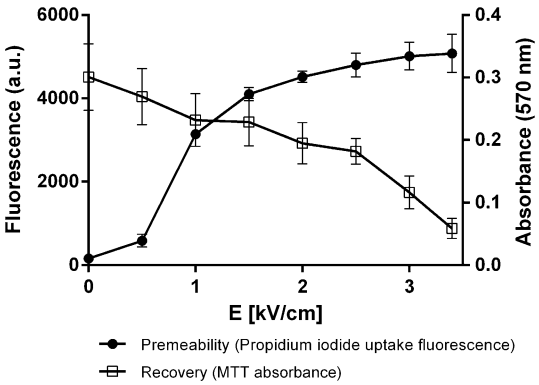

Fig. 1 PI uptake and recovery of ASC after electroporation at 100 µs, 1 Hz, and 8 pulses. Permeabilization of PI increased with higher voltages and reached a plateau at about 2 kV/cm. Cell recovery was decreasing with increasing electric field. Mean values with SE of four independent experiments are presented

Cryopreservation by Electroporation Combined with Trehalose

Various approaches have been studied for trehalose delivery into mammalian cells; however, these methods were not efficient or were demanding to perform (Beattie et al. 1997; Wolkers et al. 2001; Eroglu et al. 2000; Guo et al. 2000). However, Shirakashi et al. (2002) have shown that by electroporation, comparable amounts of trehalose can be loaded into murine cell line. In human cells, combination of electroporation and trehalose was already tested for freeze-drying of red blood cells (Zhou et al. 2010). To date, we could not find any reports describing cryopreservation of human stem cells by electroporation-mediated trehalose loading. Cryopreservation of human umbilical cord mesenchymal stem cells intended for humans' therapy without the use of toxic DMSO was recently proposed (Dovgan et al. 2015).

In the first set of experiments, the electric field of 1.5 kV/cm was determined as an optimum between efficient cell permeabilization and still high cell viability (Fig. 1). Therefore, before cryopreservation cells were electroporated at 1.5 kV/cm in the presence of trehalose at two different concentrations, 250 and 400 mM (Fig. 2). 250 mM trehalose in electroporation buffer ($\sim$290 mOsm/kg) was chosen to prevent osmotic shock in cells. For a comparison hypertonic buffer with 400 mM trehalose ($\sim$410 mOsm/kg) was used. To see the effect of electroporation on trehalose loading into the cells, nonelectroporated samples (denoted in Fig. 2 as 0 kV/cm) in the presence of trehalose were cryopreserved following exactly the same protocol. As a positive control, standard cryopreservation protocol containing 10 % DMSO in FBS was used.

Figure 2a presents cells' survival rate before and after cryopreservation. Before cryopreservation almost all cells were alive, having a survival rate around 100 % at all measured parameters, control (before adding DMSO), and 250 and 400 mM with and without electroporation. Electroporation did not have any negative effect on cell survival. After thawing, survival rates of about 66 % were observed in nonelectroporated cells in the presence of trehalose at both concentrations. Electroporation in combination with trehalose yielded higher survival rates, 83.8 ± 1.8 and 78.4 ± 1.5 % with 250 and 400 mM trehalose, respectively. Using electroporation in the presence of trehalose allowed us to achieve high survival rate approaching the values obtained with standard DMSO protocol (91.5 ± 1.6 %). No statistically significant difference between DMSO and 250 mM trehalose treated with electroporation was observed, with only slight difference ($*p < 0.05$) between DMSO and 400 mM trehalose with electroporation. Using higher trehalose

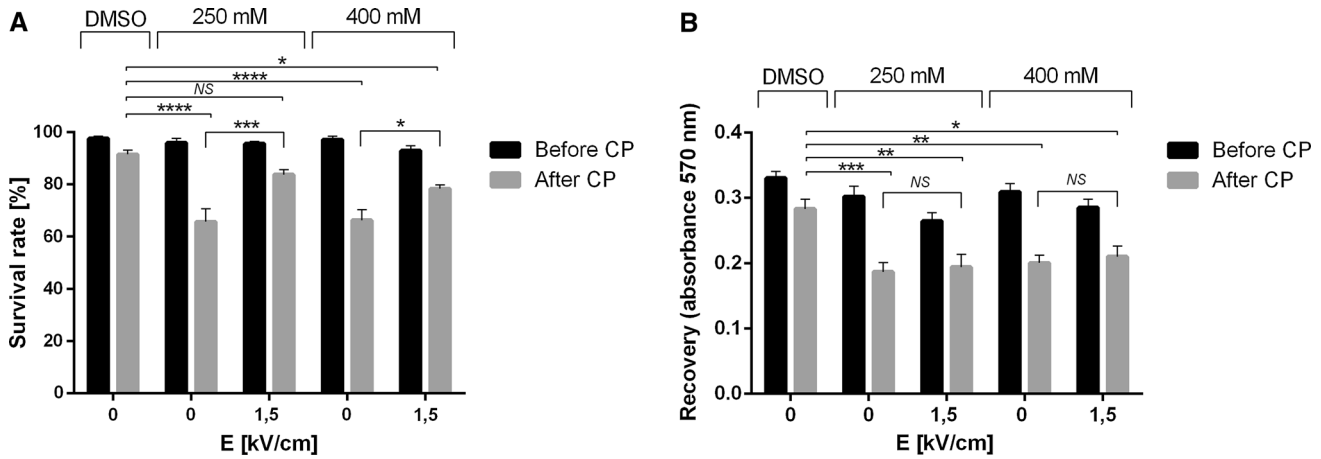

Fig. 2 **a** Survival rate before and after cryopreservation was measured with Trypan blue exclusion method. Before cryopreservation the survival rate is around 100 %, almost all cells were alive. After thawing, cells' survival rate was lower in nonelectroporated cells in comparison to control protocol using DMSO. Using electroporation, the survival rate was almost as high as in the DMSO. *p* values: *NS* control versus 250 mM trehalose; $p < 0.0001$ control versus 400 mM trehalose. Similar results were obtained with 250 mM and 400 mM trehalose. Mean values with SE of three independent experiments are presented. Here *NS* not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. **b** Recovery rate before and after cryopreservation was measured with MTT assay. Before cryopreservation the recovery rate is comparable to control (prior addition of DMSO), almost all cells were alive. After thawing, no difference between electroporated and nonelectroporated cells is seen; however, the values are lower than DMSO. Mean values with SE of three independent experiments are presented. Here *NS* not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$

concentration in electroporation medium did not increase postthaw recovery, probably due to hypertonic conditions during electroporation and freeze/thaw procedure. Determination of intracellular trehalose was not in the scope of this study; however, in the study of Zhou et al. (2010), a positive correlation between higher extracellular and intracellular trehalose on recovery rate was observed. Yet, in Zhou's study, red blood cells were used. Ergolu et al. (2000) also reported that at least 200 mM intracellular trehalose concentration is necessary for high postthaw survival (70–80 %) of frozen human fibroblasts and keratinocytes. It appears that membranes of adipose-derived stem cells enable for some loading of trehalose into cytosol (Fig. 2a), probably by fluid-phase endocytotic mechanism as already observed in human mesenchymal stem cells (Oliver et al. 2004) and mouse fibroblasts (Zhang et al. 2016). Using electroporation, we achieved additional 15 % increase in survival rate. As a logical step following this study, we planned to determine intracellular trehalose concentration in electroporated cells and their counter controls. It needs to be noted though that when cells were cryopreserved without cryoprotectant, without trehalose and without electroporation, the survival after freeze/thaw was about 5 % (data not shown).

Figure 2b presents recovery rate measured with MTT assay. Before cryopreservation the MTT absorbance value was comparable to control (prior addition of DMSO) at all parameters, almost all cells were alive. After thawing, there was however no difference between electroporated and nonelectroporated cells; even more, both treatments resulted in similar decrease of recovery rate in comparison to DMSO. These results are not correlated with results obtained by Trypan blue exclusion method. We are assuming that MTT assay is not the most adequate method to assess these differences. MTT assay was performed just after cell morphology pictures were taken in the same seeding plate (Fig. 3). In Fig. 3, obvious differences between nonelectroporated and electroporated cells after thawing exist; at nonelectroporated cells (Fig. 3e) after thawing, more round, dead cells appeared in comparison to electroporated cells (Fig. 3f) and also in comparison to control DMSO (Fig. 3d) can be observed. However, MTT assay did not confirm these observations. When performing MTT assay on individual biological samples some differences were observed (data not shown), but when we pooled the results for statistical analysis, the differences were not evident. The reason for this inconsistency in results might be in low seeding densities which lead to inaccurate cell numbers. Another possible explanation is also that MTT assay which is based on metabolic cell activity is not directly related to cell viability. It was previously suggested by Jakštys et al. (2015), MTT is not the most suitable assay for determining cell viability after electroporation. Also in our study, we have not observed any differences, thus confirming unsuitability of MTT assay for evaluation of viability after combination of electroporation and cryopreservation. For future experiments, other viability assays will be tested.

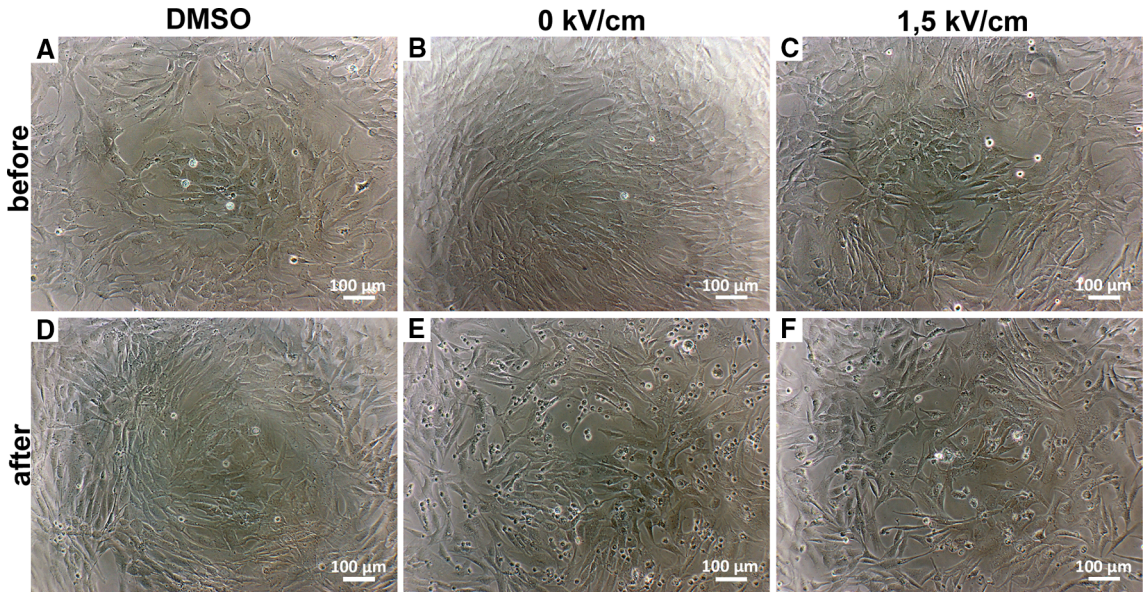

Fig. 3 Cell morphology before (**a-c**) and after (**d-f**) cryopreservation. Samples of cells were electroporated or nonelectroporated in the presence of 400 mM trehalose. After the treatment, half of the cells were seeded into 96-well plate, and the other half were cryopreserved. Before cryopreservation, all treated cells have the same morphology as the control cells (prior addition of DMSO). After thawing there are more round (dead) cells in electroporated (**f**) sample in comparison to control cells cryopreserved using DMSO (**d**). Nonelectroporated (**e**) sample has more dead cells than electroporated (**f**) sample. (Photographs were taken with the use of inverted microscope Nikon; scale bar represents 100 µm)

Cell Morphology and Multilineage Differentiation Capacity After Electroporation and Cryopreservation

Morphology of ASC before and after cryopreservation is presented in Fig. 3. Samples of cells were electroporated (c, f) or nonelectroporated (b, e) in the presence of 400 mM trehalose and seeded into tissue culture wells. As hypertonic media (400 mM trehalose) are more critical for cell survival and proliferation, only these results are presented in Fig. 3. Cells' morphology is comparable to nontreated control cells (prior addition of DMSO) (a, d). After cryopreservation and thawing (d–f), control cells cryopreserved with DMSO exhibit the same morphology as before cryopreservation (a–c). In electroporated sample (f), more round, dead cells were observed after thawing. In nonelectroporated sample (e), the highest amount of round, dead cells was observed in comparison to electroporated (f) and DMSO (d) cryopreserved cells. Similar results were obtained using 250 mM trehalose (data not shown). These results are in agreement with cell survival before and after cryopreservation presented in Fig. 2a.

Differentiation of adipose-derived stem cells under appropriate culture conditions into osteoblasts, adipocytes, and chondrocytes has been described before (Ryden et al. 2003; Wagner et al. 2005). Since trehalose is not a common molecule in human cells' metabolism (Campbell and Brockbank 2012, Crowe and Crowe 2000), we assessed its potential influence on ASCs' differentiation capacity. It was also published that electroporation induces stress in human cells, which results in the expression of stress response mechanism (Mlakar et al. 2009; Todorovic et al. 2011). Therefore, by differentiation studies, we also evaluated the effect of electroporation on cells' functionality.

Before and after electroporation and cryopreservation cells were seeded for induction of adipogenic and osteogenic differentiation. Figure 4 presents adipogenic differentiation after cryopreservation for electroporated and nonelectroporated samples in the presence of hypertonic media (400 mM trehalose). The first row (a–c) shows nondifferentiated cells cultured in noninduction control medium. In the second row (d–f), cells were cultivated in adipogenic medium. All tested samples were capable of adipogenesis, as seen from red-colored lipid vacuoles. Similar results were obtained in samples before cryopreservation and also when 250 mM trehalose was used (data not shown).

Figure 5 presents osteogenic differentiation after cryopreservation for electroporated and nonelectroporated samples in the presence of 400 mM trehalose. Noninduced cells did not differentiate. Cells, cultivated in osteogenic medium, produced high amount of Ca^{2+} deposits, indicating efficient osteogenic differentiation. If cells would lose differential ability, they would not produce any Ca^{2+} and measured Ca^{2+} concentration would be around noninduced DMSO. At the end of cultivation, Ca^{2+}

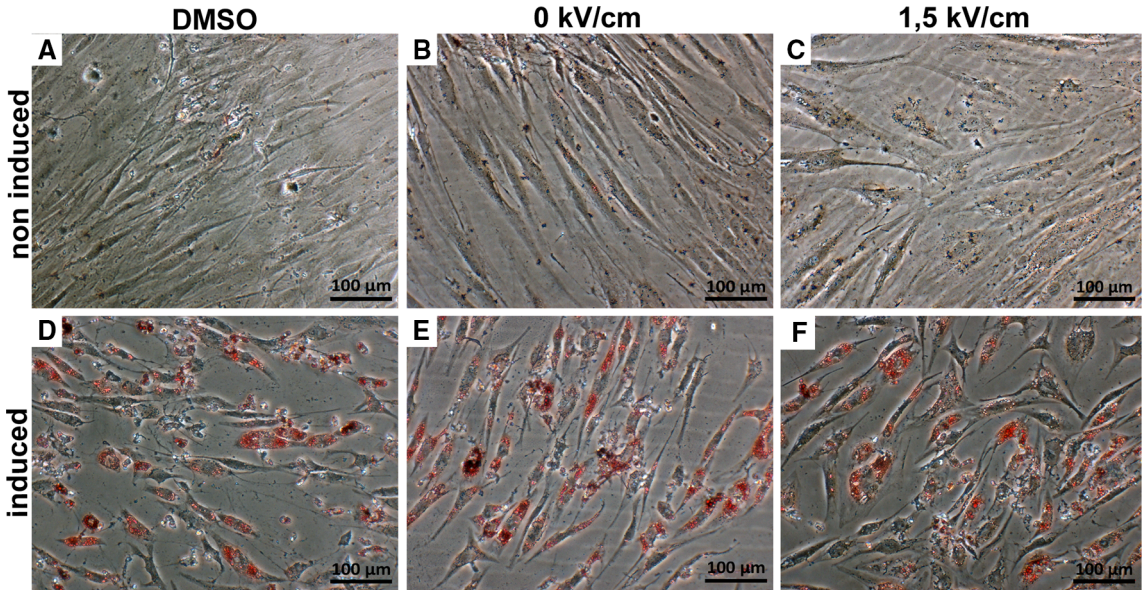

Fig. 4 Adipogenic differentiation of ASC after cryopreservation. Before cryopreservation cells were treated with and without electroporation in the presence of 400 mM trehalose. In a control sample, cells were cryopreserved using DMSO. After thawing cells were seeded into culture wells for adipogenic differentiation. **a–c** noninduced cells, **d–f** induced cells in adipogenic medium. After 10 days, cells were fixed and stained with oil red O. Red colorization presents lipid vacuoles of adipogenically differentiated cells. (Photographs were taken with the use of inverted microscope Nikon; scale bar represents 100 µm)

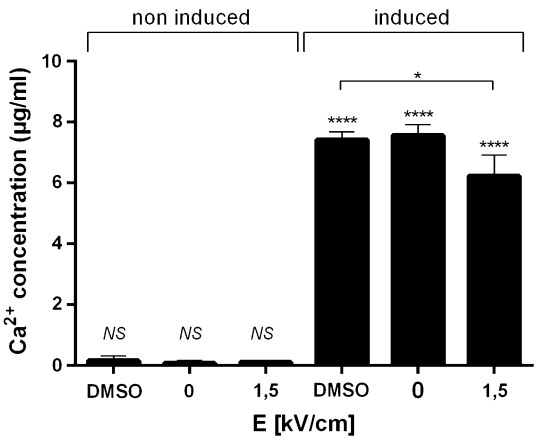

Fig. 5 Osteogenic differentiation of ASC after cryopreservation. Before cryopreservation cells were treated with and without electroporation in the presence of 400 mM trehalose. In a control sample, cells were cryopreserved using DMSO. After thawing cells were seeded into culture wells for osteogenic differentiation. Noninduced cells (in nonosteogenic medium) and induced cells (in osteogenic medium) were sampled after 4 weeks and Ca^{2+} concentration was measured. In nonosteogenic medium, cell did not produce Ca^{2+}; however, in osteogenic medium, all cells produced Ca^{2+} in high amounts. Mean values with SE of three technical repetition are presented. All p values are compared to noninduced DMSO, excluding comparison between induced DMSO and electroporated cells. Here *NS* not significant; $*p < 0.05$; $****p < 0.0001$

concentration was measured. For the three tested cryopreservation protocols, i.e., DMSO, 400 mM trehalose with electroporation and 400 mM trehalose without electroporation, no significant biological differences were observed, although induced electroporated cells produced slightly lower Ca^{2+} concentration. Similar results were obtained before cryopreservation and also using 250 mM trehalose (data not shown).

According to our results, electroporation in combination of 250 or 400 mM trehalose does not impair the functionality of adipose-derived stem cells.

Conclusion

In certain clinical therapies, cell products (e.g., cord blood and bone marrow) are used directly after thawing, without removing DMSO (Syme et al. 2004). Cytotoxicity of DMSO in clinical settings is associated with the occurrence of serious or fatal side effects (Zenhausern et al. 2000, Grigg et al. 2000; Higman et al. 2000; Sharp et al. 2013). In our study, we propose an approach for cryopreservation of clinically applicable cell type while avoiding the use of toxic DMSO. As by electroporation cell membrane permeability is temporary increased, we assume we loaded trehalose into adipose-derived stem cells, although we did not measure intracellular trehalose concentration. Optimized parameters of electroporation protocol ensured resealing of the membrane, thus preserving high cell viability. After cryopreservation and thawing, we were able to achieve high yield of viable and functionally intact cells using the nontoxic cryopreservant.

To conclude, our pilot study shows that by electroporation and trehalose, cryopreservation of clinically relevant cell types in the absence of toxic DMSO is feasible.

Acknowledgments The authors would like to thank Prof. Dr. Marija Bešter Rogač and Anton Kelbl from the Faculty of Chemistry and Chemical Technology, the University of Ljubljana and Janja Dermol from Faculty of Electrical Engineering, and the University of Ljubljana for their help with the osmolality measurements. Research was supported with Slovenian Research Agency through Research Programme, Electroporation-Based Technologies and Treatments (P2-0249), and Infrastructure Programme, Network of research infrastructure centres at University of Ljubljana (IP-0510). The work was performed in the scope of LEA EBAM.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

Beattie GM, Crowe JH, Lopez AD, Crulli V, Ricordi C, Hayek A (1997) Trehalose: a cryoprotectant that enhances recovery and preserves function of human pancreatic islets after long-term storage. Diabetes 46:519–523

Brockbank KGM, Campbell LH, Greene ED, Brockbank MCG, Duman JG (2010) Lessons from nature for preservation of mammalian cells, tissues, and organs. Vitro Cell Dev Biol Anim 47(3):210–217

Campbell LH, Brockbank KG (2012) Culturing with trehalose produces viable endothelial cells after cryopreservation. Cryobiology 64(3):240–244

Cox MA, Kastrup J, Hrubiško M (2012) Historical perspectives and the future of adverse reactions associated with haemopoietic stem cells cryopreserved with dimethyl sulfoxide. Cell Tissue Bank 13(2):203–215

Crowe JH, Crowe LM (2000) Preservation of mammalian cells-learning nature's tricks. Nat Biotechnol 18(2):145–146

Crowe JH, Crowe LM, Carpenter JF, Rudolph AS, Wistrom CA, Spargo BJ, Anchordoguy TJ (1988) Interactions of sugars with membranes. Biochim Biophys Acta 947(2):367–384

Crowe JH, Crowe LM, Hoekstra FA (1989) Phase transitions and permeability changes in dry membranes during rehydration. J Bioenerg Biomembr 1:77–91

Delteil C, Teissié J, Rols MP (2000) Effect of serum on in vitro electrically mediated gene delivery and expression in mammalian cells. Biochim Biophys Acta 1467(2):362–368

Dovgan B, Dermol J, Barlič A, Knežević M, Miklavčič D (2015) Cryopreservation of Human Umbilical Stem Cells in Combination with Trehalose and Reversible Electroporation. 1st World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine and Food & Environmental Technologies. IFMBE Proc 53:307–310

Dymek K, Dejmek P, Galindo FG (2014) Influence of Pulsed Electric Field Protocols on the Reversible Permeabilization of Rucola Leaves. Food Bioprocess Tech 7:761–773

Eroglu A, Russo MJ, Bieganski R, Fowler A, Cheley S, Bayley H, Toner M (2000) Intracellular trehalose improves the survival of cryopreserved mammalian cells. Nat Biotechnol 18(2):163–167

Fang B, Song Y, Lin Q, Zhang Y, Cao Y, Zhao RC, Ma Y (2007) Human adipose tissue-derived mesenchymal stromal cells as salvage therapy for treatment of severe refractory acute graft-vs.-host disease in two children. Pediatr Transpl 11(7):814–817

Golberg A, Sack M, Teissie J, Pataro G, Pliquett U, Saulis G, Töpfl S, Miklavčič D, Vorobiev E, Frey W (2016) Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development. Biotechnol Biofuels 9:94

Grigg A, Hoyt R, Szer J (2000) Neurological events associated with the infusion of cryopreserved bone marrow and/or periph-eral blood progenitor cells. Bone Marrow Transpl 25:1285–1287

Guo N, Puhlev I, Brown DR, Mansbridge J, Levine F (2000) Trehalose expression confers desiccation tolerance on human cells. Nat Biotechnol 18(2):168–171

Gurtovenko AA, Anwar J (2007) Modulating the Structure and Properties of Cell Membranes: the Molecular Mechanism of Action of Dimethyl Sulfoxide. J Phys Chem B 111:10453–10460

Haberl S, Miklavčič D, Serša G, Frey W, Rubinsky B (2013) Cell Membrane Electroporation—Part 2: the Applications. IEEE Electr Insul M 29(1):29–37

Higman MA, Port JD, Beauchamp NJ, Chen AR Jr (2000) Re-versible leukoencephalopathy associated with re-infusion of DMSO preserved stem cells. Bone Marrow Transpl 26:797–800

Jakštys B, Ruzgys P, Tamošiūnas M, Šatkauskas S (2015) Different Cell Viability Assays Reveal Inconsistent Results After Bleomycin Electrotransfer In Vitro. J Membr Biol 248(5):857–863

Kanias T, Acker JP (2006) Mammalian cell desiccation: facing the challenges. Cell Presev Technol 4(4):253–277

Karlsson OMJ, Toner M (1996) Long-term storage of tissues by cryopreservation: critical issues. Biomaterials 17(3):243–256

Katkov II, Kim MS, Bajpai R, Altman YS, Mercola M, Loring JF, Terskikh AV, Snyder EY, Levine F (2006) Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells. Cryobiology 53(2):194–205

Konov KB, Leonov DV, Isaev NP, Fedotov KY, Voronkova VK, Dzuba SA (2015) Membrane-Sugar Interactions Probed by Pulsed Electron Paramagnetic Resonance of Spin Labels. J Phys Chem B 119(32):10261–10266

Kotnik T, Kramar P, Pucihar G, Miklavčič D, Tarek M (2012) Cell Membrane Electroporation—Part 1: the Phenomenon. IEEE Electr Insul M 28(5):14–23

Larson DJ, Middle L, Vu H, Zhang W, Serianni AS, Duman J, Barnes BM (2014) Wood frog adaptations to overwintering in Alaska: new limits to freezing tolerance. J Exp Biol 217(Pt 12):2193–2200

Lendeckel S, Jödicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, Hedrick MH, Berthold L, Howaldt HP (2004) Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. J Craniomaxillofac Surg 32(6):370–373

Mahnič-Kalamiza S, Vorobiev E, Miklavčič D (2014) Electroporation in food processing and biorefinery. J Membr Biol 247(12):1279–1304

Mazur P (1984) Freezing of living cells: mechanisms and implications. Am J Physiol 247(3 Pt 1):C125–C142

Miklavčič D, Semrov D, Mekid H, Mir LM (2000) A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy. Biochim Biophys Acta 1523(1):73–83

Miklavčič D, Mali B, Kos B, Heller R, Serša G (2014) Electrochemotherapy: from the drawing board into medical practice. Biomed Eng Online 13(1):29

Mlakar V, Todorovic V, Cemazar M, Glavac D, Sersa G (2009) Electric pulses used in electrochemotherapy and electrogene therapy do not significantly change the expression profile of genes involved in the development of cancer in malignant melanoma cells. BMC Cancer 9:299

Mohr JC, de Pablo JJ (2006) Palecek SP (2006) Electroporation of Human Embryonic Stem Cells: small and macromolecule Loading and DNA Transfection. Biotechnol Prog 22:825–834

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1–2):55–63

Neumann E, Rosenheck K (1972) Permeability changes induced by electric impulses in vesicular membranes. J Membr Biol 10(3):279–290

Oliver AE, Jamil K, Crowe JH, Tablin F (2004) Loading Human Mesenchymal Stem Cells with Trehalose by Fluid-Phase Endocytosis. Cell Preserv Technol 2(1):35–49

Petrenko YA, Rogulska OY, Mutsenko VV, Petrenko AY (2014) A sugar pretreatment as a new approach to the Me2SO- and xeno-free cryopreservation of human mesenchymal stromal cells. Cryo Letters 35(3):239–246

Phoon PY, Galindo FG, Vicente A, Dejmek P (2008) Pulsed electric field in combination with vacuum impregnation with trehalose improves the freezing tolerance of spinach leaves. J Food Eng 88(1):144–148

Polge C, Smith AU, Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164(4172):666

Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, Taureau C, Cousin B, Abbal M, Laharrague P, Penicaud L, Casteilla L, Blancher A (2005) Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br J Haematol 129(1):118–129

Rada T, Reis RL, Gomes ME (2009) Adipose tissue-derived stem cells and their application in bone and cartilage tissue engineering. Tissue Eng 15:113–125

Rems L, Miklavčič D (2016) Tutorial: electroporation of cells in complex materials and tissue. J Appl Phys 119:201101

Ryden M, Dicker A, Gotherstrom C, Aström G, Tammik C, Arner P, Le Blanc K (2003) Functional characterization of human mesenchymal stem cell–derived adipocytes. Biochem Biophys Res Commun 311:391–397

Sharp DMC, Picken A, Morris TJ, Hewitt CJ, Coopman K, Slater NKH (2013) Amphipathic polymer-mediated uptake of trehalose for dimethyl sulfoxide-free human cell cryopreservation. Cryobiology 67:305–311

Shayanfan S, Chauhan O, Toepfl S, Heinz V (2013) The interaction of pulsed electric fields and texturizing - antifreezing agents in quality retention of defrosted potato strips. Int J Food Sci Tech 48:1289–1295

Shirakashi R, Köstner CM, Müller KJ, Kürschner M, Zimmernann U, Sukhorukov VL (2002) Intracellular delivery of trehalose into mammalian cells by electropermeabilization. J Membrane Biol 189:45–54

Spiller DGl, Giles RV, Grzybowski J, Tidd DM, Clark RE (1998) Improving the intracellular delivery and molecular efficacy of antisense oligonucleotides in chronic myeloid leukemia cells: a comparison of streptolysin-O permeabilization, electroporation, and lipophilic conjugation. Blood 91(12):4738–4746

Syed BA, Evans JB (2013) Stem cell therapy market. Nat Rev Drug Discov 12(3):185–186

Syme R, Bewick M, Stewart D, Porter K, Chadderton T, Glück S (2004) The role of depletion of dimethyl sulfoxide before autografting: on hematologic recovery, side effects, and toxicity. Biol Blood Marrow Transplant 10(2):135–141

Todorovic V, Sersa G, Mlakar V, Glavac D, Flisar K, Cemazar M (2011) Metastatic potential of melanoma cells is not affected by electrochemotherapy. Melanoma Res 21(3):196–205

Tsujimoto M, Imura S, Kanda H (2016) Recovery and reproduction of an Antarctic tardigrade retrieved from a moss sample frozen for over 30 years. Cryobiology 72(1):78–81

Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, Blake J, Schwager C, Eckstein V, Ansorge W, Ho AD (2005) Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 33:1402–1416

Wang HY, Lun ZR, Lu SS (2011) Cryopreservation of umbilical cord blood-derived mesenchymal stem cells without dimethyl sulfoxide. Cryo Letters 32(1):81–88

Wolkers WF, Walker NJ, Tablin F, Crowe JH (2001) Human platelets loaded with trehalose survive freeze-drying. Cryobiology 42(2):79–87

Yarmush ML, Golberg A, Serša G, Kotnik T, Miklavčič D (2014) Electroporation-based technologies for medicine: principles, applications, and challenges. Annu Rev Biomed Eng 16:295–320

Zenhausern R, Tobler A, Leoncini L, Hess OM, Ferrari P (2000) Fatal cardiac arrhythmia after infusion of dimethyl sulfoxide-cryopreserved hematopoietic stem cells in a patient with severe primary cardiac amyloidosis and end-stage renal failure. Ann Hematol 79:523–526

Zhang M, Oldenhof H, Sieme H (2016) Wolkers WF (2016) Freezing-induced uptake of trehalose into mammalian cells facilitates cryopreservation. Biochim Biophys Acta 1858(6):1400–1409

Zhou X, Yuan J, Liu J, Liu B (2010) Loading trehalose into red blood cells by electroporation and its application in freeze-drying. Cryo Letters 31(2):147–156

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228