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Amphipathic polymer-mediated uptake of trehalose for dimethyl sulfoxide-free human cell cryopreservation ☆,☆☆

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ABSTRACT

For stem cell therapy to become a routine reality, one of the major challenges to overcome is their storage and transportation. Currently this is achieved by cryopreserving cells utilising the cryoprotectant dimethyl sulfoxide (Me_2SO). Me_2SO is toxic to cells, leads to loss of cell functionality, and can produce severe side effects in patients. Potentially, cells could be frozen using the cryoprotectant trehalose if it could be delivered into the cells at a sufficient concentration. The novel amphipathic membrane permeabilising agent PP-50 has previously been shown to enhance trehalose uptake by erythrocytes, resulting in increased cryosurvival. Here, this work was extended to the nucleated human cell line SAOS-2. Using the optimum PP-50 concentration and media osmolarity, cell viability post-thaw was 60 ± 2%. In addition, the number of metabolically active cells 24 h post-thaw, normalised to that before freezing, was found to be between 103 ± 4% and 91 ± 5%. This was found to be comparable to cells frozen using Me_2SO. Although reduced (by 22 ± 2%, $p = 0.09$), the doubling time was found not to be statistically different to the non-frozen control. This was in contrast to cells frozen using Me_2SO, where the doubling time was significantly reduced (by 41 ± 4%, $p = 0.004$). PP-50 mediated trehalose delivery into cells could represent an alternative cryopreservation protocol, suitable for research and therapeutic applications.

Introduction

In recent years, the developed world has seen an increase in demand for tissue replacement. While the number of donor organs and operations has remained relatively static, the number of patients on the transplant waiting list for kidney, pancreas, heart, lung, and liver has increased [31]. It is hoped that regenerative medicine, including stem cell-based therapies, could meet this need, as well as providing novel treatments for currently incurable conditions [39].

For stem cell-based therapies to be used routinely in a clinical setting, these cells must be stored and transported. Currently this need is met through cryopreservation, often using the cryoprotectant dimethyl sulfoxide (Me_2SO). However, the viability of both adult and embryonic stem cells has been found to be significantly decreased by cryopreservation using Me_2SO [20,42]. Perhaps more seriously, the functionality of cells can be adversely affected. For example, Katkov et al. [20] found that only 5–10% of human embryonic stem cells (hESCs) expressed the transcription factor Oct-4, a marker of pluripotency, following Me_2SO cryopreservation. This property of facilitating the loss of hESCs pluripotency has been utilised in hESC differentiation protocols [14]. Cryopreservation using Me_2SO may also have contributed to the failure of a phase III clinical trial using human mesenchymal stromal cells, due to loss of cell viability and functionality [17]. Indeed, it has been found that the genome-wide DNA methylation profiles of cells can be altered by Me_2SO [40].

In addition, patients may experience severe side effects from Me_2SO toxicity after cells preserved in this cryoprotectant are transplanted. These include cardiac arrest, severe respiratory arrest, severe neurotoxicity and epileptic seizures [12]. These side effects are thought to occur in one in 70 patients following autologous bone marrow transplantation [44]. Although this issue could be overcome by washing cells prior to implantation, this increases the complexity of the cell delivery method and could result in significant cell losses. Therefore there is a demand for Me_2SO-free cryopreservation techniques, utilising non-toxic cryoprotectants, which maintain cell viability and functionality.

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The non-permeating cryoprotectant trehalose may provide an alternative, however to provide maximum protection to the cells, the trehalose should be present on both sides of the cell membrane [15]. Recently, the amphipathic membrane permeabilising polymer PP-50 has been used to load human erythrocytes with trehalose, which led to a significant enhancement in cryosurvival [27]. PP-50, which can be removed from cell membranes by a small change in pH [26], is thought to be non-cytotoxic [11,22]. This is in stark contrast to previous studies using pore-forming bacterial toxins [1,6,15], where serious health concerns have been raised regarding the use of these proteins [32,41].

A number of alternative methods for the delivery of trehalose into cells have previously been employed [4,5]. These include the use of the ATP receptor channel $P2X_7$ [7,8], prolonged cell culture [19] or endocytosis [18,30,33]. Stimulation of the $P2X_7$ channel may lead to apoptosis, necrosis [2] or even neoplasia [3]. The latter two methods also have the disadvantage of requiring incubation times of 24 h or more. This is in contrast to the proposed method of PP-50 mediated trehalose delivery [27].

In the current study, the techniques for the cryopreservation of cells using trehalose and PP-50 developed by Lynch et al. [27] were extended to successfully preserve nucleated human cells. The Human osteosarcoma derived cell line SAOS-2 [16,35] was used as a model for nucleated, adherent human cells.

Methods and materials

Materials

Unless otherwise stated, all reagents were purchased from Sigma–Aldrich (UK). Materials for the PP-50 polymer synthesis were sourced as previously described [25]. Foetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin were purchased from Invitrogen (UK). Dulbecco's Phosphate-Buffered Saline (DPBS), 10 × DPBS and trypsin–EDTA were purchased from Life Technologies™ (UK). The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (UK). The SAOS-2 cells were purchased from the European Collection of Cell Cultures. The Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (UK).

PP-50 synthesis and characterisation

The synthesis and characterisation of the PP-50 polymer were as previously described by Lynch et al. [25].

Cell culture

SAOS-2 cells were grown in tissue culture flasks containing "growth media": Dulbecco's Modified Eagle's Medium – high glucose (DMEM), supplemented with 10% (v/v) FBS, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). At approximately 70% confluency, the cells were subcultured with trypsin (0.05% w/v) and EDTA (0.02% w/v), and were subsequently split at a ratio of 1:6. The cells were maintained in a humidified incubator at 37 °C with 5% CO_2. The cells were used between passages 4 and 20.

Calcein and propidium iodide fluorescence assay

Calcein, which is membrane impermeable, was used as a tracer for hydrophilic species delivery into the cells. The viability of the cells was assessed using propidium iodide (PI) staining.

SAOS-2 cells were seeded into 35 mm glass bottom culture dishes (PAA, UK) at 2×10^5 cells/dish, in growth media. After 48 h of incubation in a humidified incubator at 37 °C with 5% CO_2, a positive control for PI staining was prepared by fixation with paraformaldehyde solution (4% w/v, in DPBS) for 10 min, followed by washing (×3) with DPBS.

For the remaining dishes, the cells were washed twice with DPBS. Afterwards, the cells were incubated for 4 h in serum-free media supplemented with 0.2 M trehalose, 2 mM calcein, and with or without PP-50 (200 μg/ml), at pH 7.05. The cells were washed twice with DPBS, and incubated with growth media containing Hoechst 33342 (2 μg/ml) and PI (2 μg/ml) for 15 min. Following three washes with DPBS, the cells were imaged using a TCS SP5 inverted laser scanning confocal microscope (Leica, Germany).

PP-50 toxicity

SAOS-2 cells were seeded into 96-well tissue culture plastic plates (Corning, UK) at 5000 cells/well. After 24 h, the cells were washed twice with DPBS at either pH 7.4 or pH 7.05. The cells were incubated (37 °C with 5% CO_2) in serum-free growth media containing different PP-50 concentrations (0–1000 μg/ml) at pH 7.4 or pH 7.05, for 2 or 24 h. The cells incubated for 2 h were subsequently washed with DPBS and incubated for 22 h in growth media. An MTS assay was performed at the 24 h mark according to the manufacturer's instructions.

Cryopreservation and reconstitution

SAOS-2 cells were seeded into 6-well tissue culture plastic plates (Corning, UK) at 10^5 cells/well. After 24 h, the cells were washed with DPBS (pH 7.4), then DPBS (pH 7.05), and were then incubated (37 °C with 5% CO_2) in "incubation media": serum-free media with 0.2 M trehalose, with or without PP-50 at different concentrations, and water (18.2 MΩ.cm, Milli-Q® filtered, Millipore, USA), at pH 7.05. Following incubation, the osmolarity of all solutions was adjusted to that of the incubation media using 10 × DPBS (PAA, UK) and/or water unless otherwise stated.

After 2 h of incubation (37 °C with 5% CO_2), the cells were washed twice with DPBS, and trypsin/EDTA was added at 200 μl/well. After 15 min of incubation (37 °C with 5% CO_2), 500 μl/well growth media was added and the cells were centrifuged at 350*g* for 5 min and resuspended in 150 μl of 0.2 M trehalose in FBS. Controls using un-incubated cells were also prepared and resuspended in FBS (90%) and Me_2SO (10%). All samples were transferred into cryovials (Greiner, UK), and transferred into an isopropanol freezing container (Nalgene, USA), then passively cooled in a −80 °C freezer overnight, before storage in vapour-phase liquid nitrogen for at least 48 h.

The cells were subsequently thawed by immersing the cryovials in a 37 °C water-bath, after which 850 μl/cryovial of growth media were slowly added. After centrifugation, the cells were resuspended in growth media, and added to the wells of 96-well plates (100 μl/well). Non-frozen SAOS-2 cells were seeded into the plates at 5000 cells/well. After 4 h of incubation (37 °C with 5% CO_2), the media was changed to growth media of normal osmolarity. MTS assays were subsequently performed at 24, 48 and 72 h, according to the manufacturer's instructions.

$$t_d = (t_2 - t_1)\frac{ln2}{ln\frac{N(t_2)}{N(t_1)}} \quad (1)$$

$$N(0) = \frac{N(24)}{2^{\frac{24}{t_d}}} \quad (2)$$

The number of metabolically active cells was found using a standard curve. The doubling times, t_d, were calculated using Eq. (1), where t_1 and t_2 represent the time at time-points 1 and 2, respectively, and $N(t_1)$ and $N(t_2)$ represent the number of cells at