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Cytotoxicity effects of cryoprotectants as single-component and cocktail vitrification solutions ☆

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ABSTRACT

Cryoprotectant (CPA) cytotoxicity constitutes a challenge in developing cryopreservation protocols, specifically in vitrification where high CPA concentrations are necessary to achieve the ice-free, vitreous state. Few cytotoxicity studies have investigated vitrification-relevant concentrations of CPAs, and the benefits and disadvantages of cocktail solutions and of incorporating non-permeating solutes have not been fully evaluated. In this study, we address these issues by determining the cytotoxicity kinetics for dimethylsulfoxide (Me_2SO) and 1,2-propanediol (PD) on alginate-encapsulated βTC-tet mouse insulinomas for a range of concentrations and temperatures. Cytotoxicity kinetics were also determined for two cocktails, DPS (3 M Me_2SO + 3 M PD + 0.5 M sucrose) and PEG400 (1 M Me_2SO + 5 M PD + 0.34 M poly(ethylene)glycol with M.W. of 400). PD was found to be more cytotoxic than Me_2SO at higher concentrations and temperatures. This was reflected in PEG400 being more cytotoxic at room temperature than PEG400 at 4 °C or DPS at either temperature. Addition of non-permeating solutes increased the cytotoxicity of cocktails. Furthermore, results indicate that CPA cytotoxicity may not be additive and that combining CPAs may increase cytotoxicity synergistically. Finally, when comparing cytotoxic effects towards encapsulated HepG2 and βTC-tet cells, and towards βTC-tet cells in capsules and in monolayers, CPAs appear more cytotoxic towards cells with higher metabolic activity. The incorporation of these results in the rational design of CPA addition/removal processes in vitrification is discussed.

Introduction

Within the field of Cryobiology, much work has been done to determine the cytotoxic effects of the cryoprotectant agents (CPAs) necessary for preservation. The cytotoxicity of CPAs has been shown to increase with time, temperature and concentration [35]. Cytotoxicity is especially critical for vitrification, which requires much higher concentrations of CPAs. Recently, vitrification has been touted by some to be the most promising method of preservation for tissues [9] as well as tissue-engineered constructs [17,40] due to the need to minimize or eliminate ice formation during preservation. Very few of the cytotoxicity studies available achieve the high concentrations of cryoprotectants necessary for successful vitrification [35,38]. To improve the vitrification process, several investigators have chosen to use cocktail solutions combining CPAs to achieve the necessary concentrations. These cocktails have gained widespread use for two reasons. The combination of different permeating and non-permeating CPAs has been shown to decrease the total concentration necessary to achieve successful vitrification [33,23]. Also, the addition of non-permeating CPAs may improve the viability and function of the cells or tissues that are preserved [2,18,16]. Some studies have focused on determining the predictability of vitrification [28] and vitrification solution toxicity [9,7]. However, few of these studies have directly compared the cocktail solutions to their individual CPA components to determine if cytotoxicity may be additive or have synergistic effects to either reduce or increase cytotoxicity. Most that have investigated this have focused on the addition of additives that do not contribute to the overall glass-forming ability of the solution, such as amides [6,8].

Additional questions that remain on the use of CPAs in cryopreservation include variations on CPA cytotoxicity towards different cell types or even the same cell in different types of culture. Evidence for these differences can be seen in a review of studies which range from the preservation of embryos [16,19] to tissues [35,5] or cells [38,39]. To our knowledge, although cytotoxicity studies have been carried out for many of these, no studies have

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investigated differences in cytotoxicity towards cells cultured in monolayers and cells in tissue constructs or a tissue itself.

In this study, we address these critical issues concerning the cytotoxicity of CPAs. Alginate-encapsulated mouse insulinoma βTC-tet cells were chosen for the majority of the experiments due to their use as a pancreatic substitute [11,29]. Cytotoxicity measurements were performed in a systematic way so as to investigate the effects of temperature, concentration and exposure time. Initial studies focused on single-component CPAs applied at concentrations of 2–6 M in order to be relevant for both conventional freezing and vitrification. The cytotoxicity of cocktail CPAs with and without non-permeating solutes was compared to single-component CPAs. To address the effects of culturing method on cytotoxicity, the cytotoxic effects of CPAs towards βTC-tet cells in monolayers and in capsules were evaluated and compared. Lastly, variations of CPA cytotoxicity towards different cell types were studied by comparing CPA effects on encapsulated HepG2 cells and βTC-tet insulinomas. Conclusions regarding fundamental issues of CPA cytotoxicity and the use of such systematic studies in designing optimized cryopreservation protocols are discussed.

Materials and methods

Cell culture

Mouse insulinoma βTC-tet cells were obtained from Dr. Efrat, Albert Einstein College of Medicine, Bronx, NY [10]. Monolayer cultures were initiated from frozen stocks and propagated in T-flasks in complete growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Gemini Bioproducts, West Sacramento, CA), 1% L-glutamine (Mediatech, Inc., Manassas, VA) and 1% penicillin/streptomycin (Mediatech, Inc.). Monolayer human liver carcinoma HepG2 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM (Cellgro by Mediatech, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin as above. Cells were incubated in a humidified incubator at 37 °C and 5% CO_2 and were split at a ratio of 1:5 (βTC-tet) or 1:10 (HepG2) when 90% confluent. Experiments were performed with βTC-tet cells of passage 38–44 and with HepG2 cells of passage 18–23.

For monolayer studies, βTC-tet cells were plated at a density of 500,000 cells/well in 24-well cell-culture treated plates (Corning Inc., Corning, NY). The cells were cultured as above for two days before cytotoxicity studies were performed.

Alginate encapsulation

Encapsulation of βTC-tet and HepG2 cells was carried out using previously published protocols [31], briefly as follows. Cells were detached from monolayer cultures by trypsinization (0.25% Trypsin–EDTA, Mediatech Cellgro) and suspended at a density of 3.0×10^7 cells/ml of 2% sodium alginate (Pronova Ultra Pure LVM alginate NovaMatrix of FMC BioPolymer AS, Norway). An electrostatic droplet generator (Nisco Engineering AG, Zurich, Switzerland) was used to generate droplets which fell into a well-stirred 1.1% $CaCl_2$ bath, forming beads of gel containing entrapped cells. Complete growth medium was used to wash and store the beads. Beads were cultured overnight in a non-tissue culture treated T-flask on a rocker plate in a 37 °C and 5% CO_2, humidified incubator. For cytotoxicity studies, beads were transferred to a 100 μm cell strainer (Becton–Dickinson, Franklin Lakes, NJ) and exposed to CPA solutions in non-treated 6-well plates (Corning). Beads were agitated throughout CPA addition and removal except for the final addition step where they were agitated for 4 min regardless of incubation time.

CPA solutions

Solutions for cytotoxicity studies were prepared using a concentrated and modified version of the EuroCollins carrier solution containing 174.76 g/L dextrose, 5.6 g/L KCl, 4.2 g/L $NaHCO_3$ and 8.2 g/L NaCl. This concentrated EuroCollins solution was diluted in the final solution volume at a ratio of 1:5. The cocktail solutions were DP6 (3 M Me_2SO + 3 M PD), DPS (3 M Me_2SO + 3 M PD + 0.5 M Sucrose), PEG400 (1 M Me_2SO + 5 M PD + 0.34 M polyethylene glycol with M.W. 400) and 5/1 (1 M Me_2SO + 5 M PD). All chemicals were purchased from Sigma–Aldrich except sucrose and $NaHCO_3$ (Fisher). DP6 was compared to the complete solution, DPS, and 5/1 compared to the complete solution, PEG400, in order to investigate the effect of the non-permeating solutes. CPAs were added in a stepwise fashion. Protocols for addition and removal were designed using a previously established model [21]. Cells were incubated in the final solution for different times to determine the kinetics of cytotoxicity. Incubation times for other addition and removal steps remained constant for all solutions. The addition/removal protocols for cocktails and high concentration single-component CPA solutions are shown in Table 1. All addition steps were carried out at the indicated temperature (4 °C or room temperature) and all removal steps were carried out at room temperature. Room temperature was 25 °C ± 1 °C, while 4 °C was achieved by keeping solutions in an ice/water bath.

Metabolic activity and viability

To determine the metabolic activity of cells, 100 μL alginate beads or a cell monolayer were incubated with a solution of alamarBlue™ consisting of 100 μL alamarBlue™ and 1 mL complete growth medium in a 12 well plate for 3 h (encapsulated βTC-tet cells), 4 h (monolayer βTC-tet cells) or 1.5 h (encapsulated HepG2 cells) in a 37 °C and 5% CO_2, humidified incubator. Incubation times

Table 1
Addition and removal protocols for 6 M PD, 6 M Me_2SO, DPS, PEG400, DP6 and 5/1. Sucrose is denoted as S. Lower concentration single component CPAs were added and removed in the same manner: 2 M was added in one step (A2) and removed in one step (R4) and 4 M was added in two steps (A2 and A3) and removed in two steps (R3 and R4) for corresponding CPA.

Solution (M)	6 M PD PD/S	6 M Me_2SO Me_2SO/S	DPS Me_2SO/PD/S	PEG400 Me_2SO/PD/PEG	DP6 Me_2SO/PD/S	5/1 Me_2SO/PD/S	Time (min)
A1	–	–	–	0.25/1/0	–	–	4
A2	2/0	2/0	1/1/0.15	0.5/2/0.1	1/1/0	0.33/1.67/0	4
A3	4/0	4/0	2/2/0.3	0.75/3.5/0.2	2/2/0	0.67/3.33/0	4
A4	6/0	6/0	3/3/0.5	1/5/0.3384	3/3/0	1/5/0	15*
R1	–	–	2.25/2.25/0.3	0.75/4/0.2	–	–	2
R2	4/0.5	4/0.6	1.5/1.5/0.2	0.5/2/0.2	2/2/0.5	0.67/3.33/0.5	2
R3	2/0.25	2/0.35	0.75/0.75/0.1	0.25/1/0	1/1/0.25	0.33/1.67/0.3	2
R4	0/0	0/0	0/0/0	0/0/0	0/0/0	0/0/0	4

* Incubation in final addition step was changed to determine cytotoxicity kinetics.