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Insights on cryoprotectant toxicity from gene expression profiling of endothelial cells exposed to ethylene glycol

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ABSTRACT

Cryopreservation consists of preserving living cells or tissues generally at −80 °C or below and has many current applications in cell and tissue banking, and future potential for organ banking. Cryoprotective agents such as ethylene glycol (EG) are required for successful cryopreservation of most living systems, but have toxic side effects whose mechanisms remain largely unknown. In this work, we investigated the mechanisms of toxicity of ethylene glycol in human umbilical vein endothelial cells (HUVECs) as a model of the vascular endothelium in perfused organs. Exposing cells to 60% v/v EG for 2 h at 4 °C resulted in only a slight decrease in subsequent cell growth, suggesting only modest toxicity of EG for this cell type. Gene expression analysis with whole genome microarrays revealed signatures indicative of a generalized stress response at 24 h after EG exposure and a trend toward partial recovery at 72 h. The observed changes involved signalling pathways, glycoproteins, and genes involved in extracellular and transmembrane functions, the latter suggesting potential effects of ethylene glycol on membranes. These results continue to develop a new paradigm for understanding cryoprotectant toxicity and reveal molecular signatures helpful for future experiments in more completely elucidating the toxic effects of ethylene glycol in vascular endothelial cells and other cell types.

1. Introduction

Cryopreservation is a vanguard discipline that refers to the preservation of cells, tissues or organs at cryogenic temperatures, especially at −100 °C or below. For successful cryopreservation, cryoprotectants (also known as cryoprotective agents, or CPAs), which are chemicals used to protect biological systems from freezing damage, are usually required. For at least some organs, they are believed to be needed in concentrations high enough to prevent the formation of ice crystals during cooling [6] allowing instead preservation in a glassy state. This glassy state form of preservation, known as vitrification, is a challenge to achieve. Organs need to be able to survive not only “cooling injury” (injury caused by cooling per se) [10], and osmotic damage but also the direct biochemical effects of perfusion with the extremely high concentrations of CPAs that are required for the organs to be successfully vitrified [10]. Unfortunately, there is currently little information available regarding the toxicological and biochemical effects of these chemical agents [5,7].

An important factor in managing these challenges is the temperature of perfusion of CPA solutions. Although warm temperatures facilitate the diffusion of the CPA within tissues, lessening osmotic damage, it is generally believed that higher temperatures inherently increase the toxic effects of CPAs [11]. Previous research has resulted in a minimum toxicity, maximum stability combination of CPAs known as M22, which enables renal survival when perfused at −22 °C [10], even after perfusion sufficiently thorough to render the kidneys able to escape devitrification on warming [3]. M22 has even enabled life support function after transplantation of a previously vitrified rabbit kidney [8]. However, CPA toxicity continues to be a problem.

Ethylene glycol was chosen for the present study because, as apparently the least intrinsically toxic of all penetrating CPAs, very little is known about its mechanisms of toxicity, especially at the high concentrations needed for vitrification. In vivo toxic mechanisms, including conversion to toxic by-products by the liver, are presumably not operative outside the body at low temperatures and, therefore, do not provide guidance as to how toxicity may be avoided during organ vitrification.

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Human umbilical vein endothelial cells (HUVECs) are commonly used for physiological and pharmacological investigations and have been widely used as a model system for the study of endothelial cell function and have even been used to model the effects of vitrification solution exposure [4]. Endothelial cells play a pivotal role in a variety of physiological and pathophysiological processes such as angiogenesis, the perm-selectivity of the blood–brain barrier, arterial disease and cancer development. Most pertinently, endothelial cell integrity is required for the survival of all vascularized tissues that may require cryopreservation, including all natural or bioartificial organs, so the choice of this cell reflects its role as a strategic common denominator for many other systems of potential interest.

This study aims to discern mechanisms of cryopreservation-related injury, and specifically of the vasotoxic effects pertaining to EG, using gene expression profiling. Very few studies have profiled gene expression in the context of cryopreservation [12,15], and more data are needed to elucidate molecular mechanisms in more systems. In this study, HUVECs were exposed to high concentrations of EG, and resulting changes in gene expression were assessed through microarray analysis. Out of 70,523 detected transcripts, several distinct functional groups were found to be differentially expressed when compared to the controls. In most cases, these groups were differentially regulated at 24 h and, as time passed (at 72 h), this acute response was toned down.

2. Materials and methods

2.1. Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Walkersville, MD, USA) and were grown in EGM-2 (Lonza, NH, USA) at 37 °C. According to the supplier, cells were pooled from multiple donors and isolated in the absence of defining growth factors. Early passage cells (<PD9) were used for all experiments. Cells were grown in T25 tissue culture flasks with phenolic-style screw caps for gas exchange (Corning, NY, USA). Lonza EGM-2 Bulletkit is an optimized medium for umbilical vein endothelial cell growth in a low-serum environment and includes bovine brain extract (BBE) with heparin; human epidermal growth factor (hEGF); hydrocortisone; gentamicin; amphotericin B and foetal bovine serum (FBS). Growth medium was changed the day after seeding and then every other day. Cells were trypsinized at 70–80% confluency for subculturing.

Cell solutions were divided in equal parts so as to seed every T25 flask with the same number of cells. Cells were counted the day after being seeded by a Coulter Counter Model Z1 (Coulter Electronics Ltd., England) to ensure the cell numbers were equalized between all the flasks, and were again counted prior to RNA extraction to ascertain the effects of the various procedures.

2.2. HUVEC exposure to ethylene glycol

After seeding the HUVECs in T25 flasks under their normal growth conditions (NGC), the cells were incubated for 24 h, roughly doubling their numbers and allowing them to attach to the surface of the flask. A total of 18 flasks – all of which were seeded at the same time and with the same cell concentration – were used. Two time points were examined after EG treatment, 24 h and 72 h (the period the cells remained in the incubator under their NGC after the experiment to recover from the induced stress). These 2 groups were each further divided into 3 others: the Normal Group (a control group that remained inside the incubator at 37 °C under NGC); the Cold Control Group (another control group that followed the protocol of the CPA Group as closely as possible except for the addition and removal of EG); and the EG Group.

To decrease toxic effects, we employed LM5 as a carrier solution. LM5 is an established carrier solution for vitrification solutions [9] and was shown in preliminary experiments (data not shown) to reduce hypothermic injury in HUVECs. LM5 was obtained from 21st Century Medicine, Inc. (Fontana, California).

Prior to EG addition, the HUVEC medium was replaced with 950 μL of 0.3 M trehalose in PBS and the flasks were moved to a refrigerator at 4 °C. After 5 min, the solution was aspirated and 650 μL of LM5 with trehalose (0.3 M) was added, followed by 40 μL of EG to result in a first EG step of 5.8% v/v. 10 min later, the EG concentration was doubled using a similar procedure, and this step was repeated several times until reaching 60% EG. This concentration was selected after preliminary experiments showed lower concentrations to be too non-toxic to be meaningful. For the Cold Control Group, the same quantities of LM5/trehalose (0.3 M) were added, with no EG. After EG addition was complete, the cells were held at 4 °C for 2 h after which they were washed, using the inverse procedure - diluting the EG in LM5/trehalose (0.3 M). The final step was aspirating the total volume of liquid in the flask and adding a solution containing 0.045 M trehalose in LM5. This solution was left in contact with the cells for 5 min at room temperature after which the solution was replaced with normal HUVEC culture medium. The flasks were then placed under NGC for either 24 or 72 h, before the cells were trypsinized and the RNA was extracted for microarray analysis.

2.3. RNA extraction

The cells were trypsinized and extracted from the flask into a 1.5 mL Eppendorf tube and centrifuged at 165 g for 5 min to form a pellet. The pellet was frozen with RLT buffer or used immediately to extract RNA following Qiagen's RNeasy protocol (Qiagen RNeasy Micro-kit, Qiagen, Valencia, CA). A Nanodrop was used to quantify the RNA sample's purity, after which it was frozen at −80 °C for further downstream applications.

2.4. Microarray

A GeneChip® WT Plus Reagent kit and protocol from Affymetrix were used to label the samples for the Human HTA_2.0 array. The arrays were washed and stained using Fluidics script Fs450_0001. A total of 250 ng of RNA was used per microarray.

The GeneChip® Human Transcriptome Array 2.0 is a high-resolution array that contains >6 million distinct probes (approximately 109 per gene) covering coding and non-coding transcripts (70% cover exons for coding transcripts and the remaining 30% cover exon–exon splice junctions and non-coding transcripts). Microarray analyses were performed at the Liverpool Centre for Genomic Research (http://www.liv.ac.uk/genomic-research/). Results are available online (http://cryopreservation.org.uk/), and full microarray data have been submitted to GEO (GSE67511).

2.5. Bioinformatics analysis

The microarray analysis was performed using Affymetrix's software. The Expression Console software was used for quality control of the data so it could be used with the Transcriptome Analysis Console (TAC), and also to create the Principal Component Analysis (PCA) graphs. In the TAC software, filters were applied to generate the gene lists provided in results: a fold change cut-off of >1.5× and <−1.5× as well as an ANOVA p-value of <0.05. (Note: for purposes of facilitating graphical representation of data, downward fold changes are designated with negative numbers.) Multiple hypothesis testing correction was obtained from the software as false

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