



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 trans-membrane 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.

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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

discovery rate (FDR) values. Later more stringent filters were applied to exclude pseudo and uncharacterized genes, nucleolar, micro and ribosomal RNAs, as well as genes without official gene symbols since these are more difficult to interpret from a functional perspective (but as abovementioned, full results are available on-line). The different gene lists were then analysed separately using DAVID for functional enrichment annotation [14]. Enrichment scores in DAVID >1.3 are considered statistically significant [14].

2.6. qRT-PCR validation

8 μ L of total RNA was reverse transcribed using ThermoFisher Scientific's Superscript[®] III Reverse Transcriptase, with oligo (dT) primers for the 2-step qRT-PCR. Gene specific primer sets for selected genes (*ANGPT2* and *IL33*) and *GAPDH* (used to normalise the results) were chosen based on previous successful use in other articles and used on an ABI 7500 Fast using Sigma–Aldrich SYBR Green master mix. All samples and standards were run in triplicate and assays were run using the following protocol: 95 °C for 10 min followed by 30 s at 95 °C and 1 min at 60 °C for 40 cycles. Following amplification, the instrument software was used to set the baseline and threshold for each reaction. A cycle threshold (Ct) was assigned at the beginning of the logarithmic phase of the amplification and the difference in the Ct values of the negative control and the samples were used to determine the relative expression of the gene in each sample. Prior to quantitative analysis, a standard curve was constructed using serial dilutions of the RT products to verify that the efficiency of each of the primers was within a 90–110% window.

Relative expression levels between samples were then calculated as fold changes. Since 1 correlates to normal expression in control samples (as opposed to 0 in microarrays), values were corrected to allow for both techniques' values to be summarized in the same graph as both negative and positive fold changes.

3. Results

Our experimental system employs HUVECs and uses gradual addition of the CPA to cells to reduce osmotic stress. We have three conditions, each with three biological replicates (subcultured HUVECs with same passage number): Normal Group HUVECs, cells to which nothing was done and remained under their normal incubation conditions during the whole experiment; the Cold Control Group, basically followed the Experimental Group as closely as possible, except no CPA was added; the Experimental Group, where the normal medium was extracted from the HUVECs prior to the gradual increase of CPA concentration, until it reached 60%; the remaining 40% were LM5 and trehalose (see Materials and Methods). The Experimental Group and the Cold Control were then placed at 4 °C for 2 h and afterwards the CPA was gradually washed out with LM5/trehalose. Both the Experimental and Cold Control Groups were then placed in the incubator under their normal growth conditions for a period of time before the microarray was done. The two time points employed were 24 and 72 h.

3.1. Effects of cold and ethylene glycol exposure on cell recovery and growth

Cells in the Cold Control and the EG Group were reduced in numbers when compared to the Normal Group at 24 h (Fig. 1). The drop in cell numbers in the Cold Control and EG groups is to be expected given that the experiment (cold exposure, withdrawal of growth factors, etc.) is expected to be stressful to the cells. Between the Cold Control and the EG Group there is a slight decrease in cell numbers in the latter (Fig. 1), which could be attributed to the toxic effect of the EG. The same applies to the 72 h cell numbers (Fig. 2).

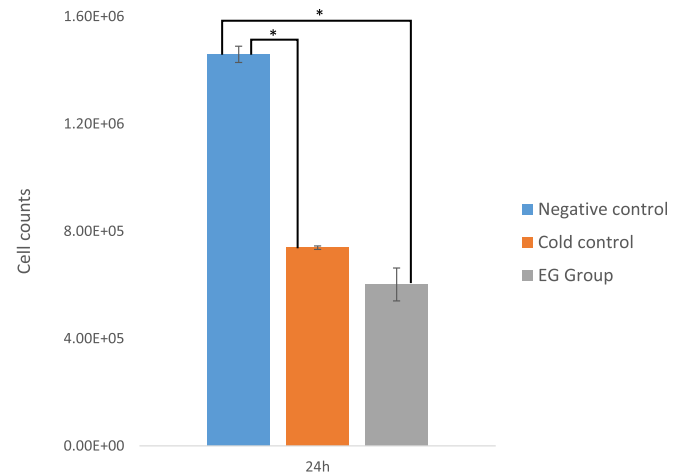


Fig. 1. Cell numbers in each group after the 24 h incubation period. * p-value < 0.01.

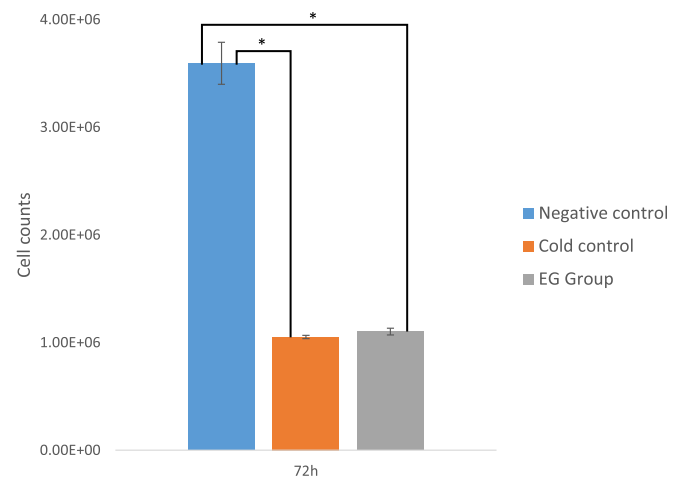


Fig. 2. Cell numbers in each group after the 72 h incubation period. * p-value < 0.015.

Whilst the Normal Group doubled (as expected), the Cold Control and EG Groups lagged behind (growth rate was 58–74% that of the Normal Group between 24 and 72 h).

3.2. Gene expression profiling

We employed microarray high-throughput gene expression profiling of all aforementioned conditions. Out of a total of 70,523 transcripts detected (44,699 of which are from coding genes) 5784 (8%) were differentially expressed compared to the untreated controls.

From the Principal Components Analysis (PCA) (Fig. 3) we can observe 3 main clusters of samples. All treated samples are quite distinct from all Normal Control Group samples at both time points. The Cold Control and EG Groups were similar after 72 h, but the 24 h EG Group showed additional changes seen in no other group.

Similar but additional insights can be obtained by examining the Pearson correlation heat map for the correlation between all the samples (Fig. 4). According to this map, gene expression in Normal Controls at 24 h correlates strongly with gene expression in 72 h Normal Controls but correlates weakly with the two experimental groups (EG and Cold Controls) at 24 h and at 72 h. The Cold Controls correlate most strongly with each other (i.e., 24 and 72 h) followed by the EG Groups, whereas the 24 h EG Group's expression profile

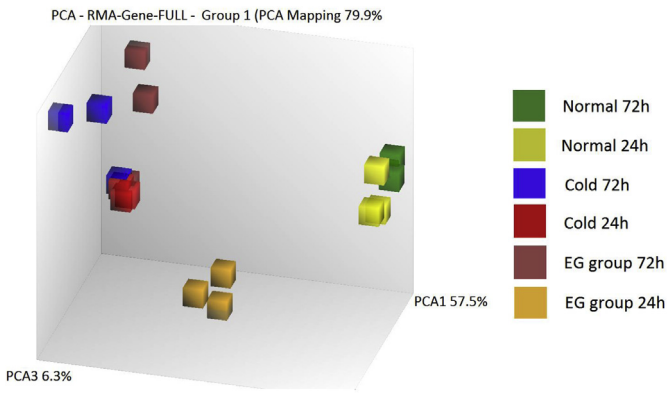


Fig. 3. Principal Component Analysis.

correlates weakly with all other groups, in line with the PCA (Fig. 3).

To validate our high throughput microarray platform, qRT-PCR was performed on two selected genes (one upregulated, *ANGPT2*, and one down regulated, *IL33*). As expected, differential gene expression changes were comparable between qRT-PCR and microarray (Fig. 5), validating our microarray approach.

3.3. Gene expression signatures of EG

The gene expression profiles of HUVECs exposed to EG were used to obtain signatures of EG exposure in endothelial cells to gain insights on potential mechanisms of toxicity.

3.4. 24 h EG vs Cold Control

Applying a cut-off ANOVA p-value of <0.05 and fold change >1.5× and <−1.5× and further filtering (see Materials and Methods) to exclude nucleolar RNAs, microRNAs and uncharacterized, ribosomal and pseudo genes (full results are available

online, however), we found 118 up-regulated and 257 down-regulated genes in the EG Group at 24 h post-stress when compared to the Cold Control Group. The most up-regulated gene, with a fold change of 2.86× (FDR < 0.01), is *ANGPT2* (Table 1), a gene expressed at sites of vascular remodelling.

CXCL10 is not only the most down-regulated – out of the 257 genes (Table 2) – but in fact, in absolute terms, *CXCL10* is the gene that registers the greatest fold change of all the lists (FDR = 0.06). Interestingly, in the Top 20 most down-regulated list, we can find 5 Taste Receptor genes *TAS2R14*, *TAS2R19*, *TAS2R30*, *TAS2R31*, *TAS2R45*, with fold changes between −3.15 and −2.9 (FDR 0.009–0.051). Presumably these play a different role in HUVECs, but may involve transduction of signals across the cell membrane.

It is also crucial in gene expression analysis to study genes as part of the processes and pathways they participate in [2]. A functional enrichment analysis of the up-regulated genes (Table 3) shows several processes with high enrichment scores. The top three scores were identified as “transmembrane” (3.31), “extra-cellular” (3.40), and “cytoplasmic” (3.31). Overall, since glycoproteins are frequently membrane proteins, and “transmembrane” and “extracellular” both involve the plasma membrane and its extracellular attachments, there is a strong representation of membrane-associated changes in the most affected pathways.

Amongst the down-regulated genes, “mammalian taste receptor” and “sensory perception of chemical stimulus” related genes were within the annotation cluster with the highest enrichment score (4.4). The second annotation cluster showed groups related to GTPase and GTP binding roles, with an enrichment score of 2.79 while the third annotation cluster indicated a less cohesive group of terms, with “ATPase”, “Transport” and “Defence mechanism” all with an enrichment score of 2.73 (Table 4). Most of these categories, once again, point to a membrane response to EG.

3.5. 72 h EG vs Cold Control

In total, 107 genes were differently expressed – 18 up-regulated and 89 down-regulated – at 72 h (EG/Cold). Both lists have most

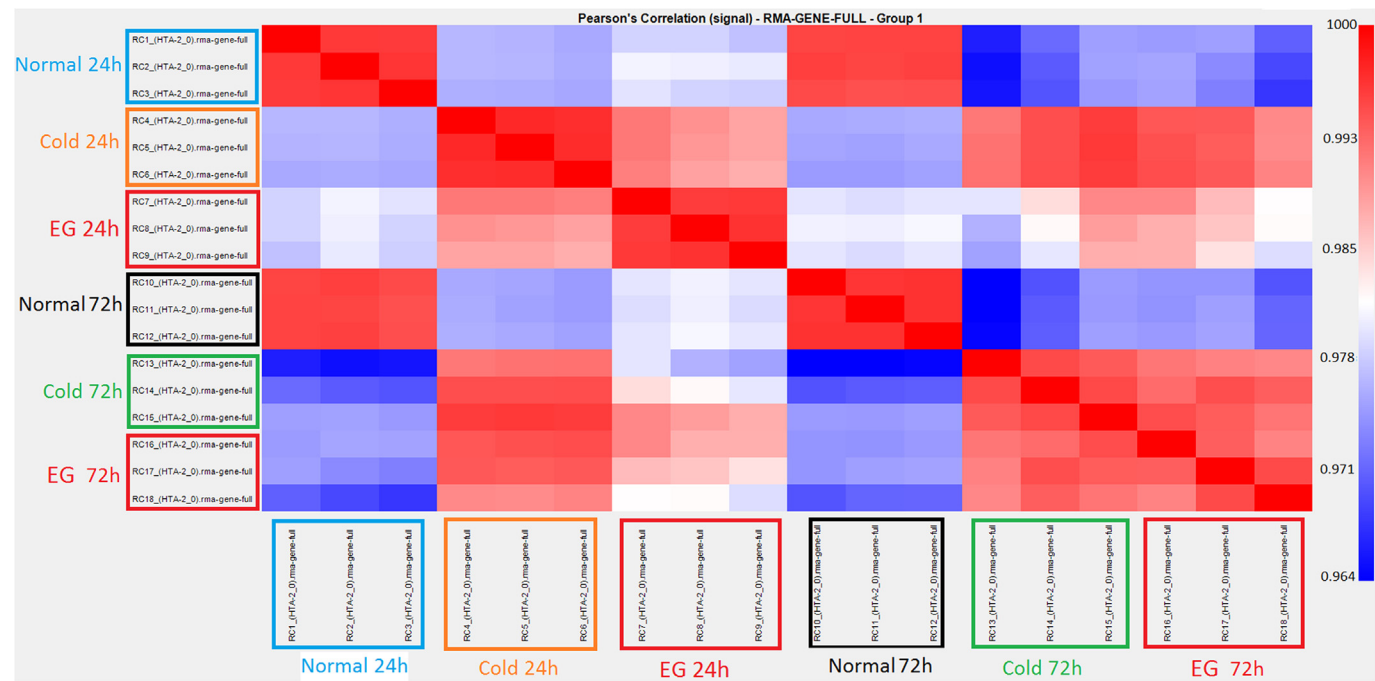


Fig. 4. Pearson's correlation of the signal values between samples, obtained from the Affymetrix Expression Console. Red = stronger correlation; blue = weaker correlation. Each of the groups is represented by three biological samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

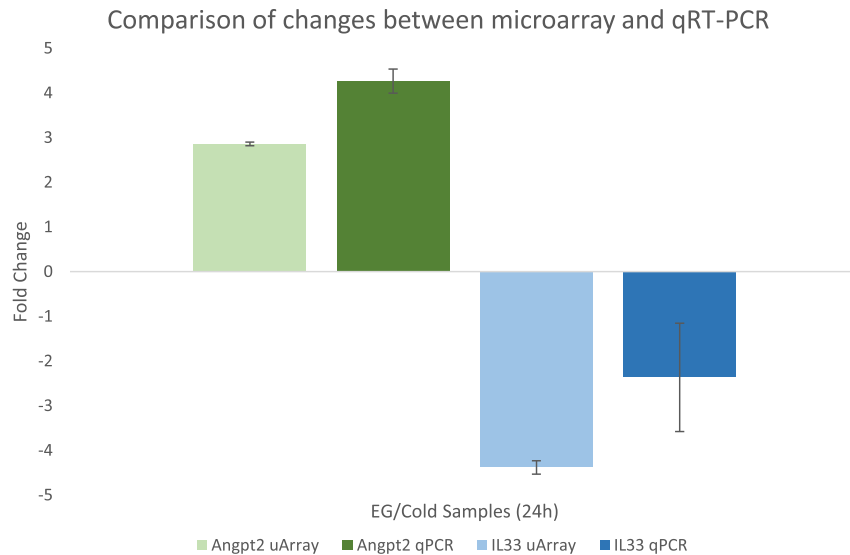


Fig. 5. Comparison of differential expression between qRT-PCR and microarrays for two selected genes (one up- and one down-regulated) in the EG vs Cold Control at 24 h.

Table 1

Top 20 EG-up-regulated genes at 24 h, compared to the Cold Control Group.

Gene symbol	Fold change	ANOVA p-value
ANGPT2	2.86	5.00E-06
EVI2B	2.7	2.10E-03
STC1	2.64	7.40E-05
PTPN22	2.5	5.40E-05
MPP4	2.37	9.10E-05
PLAT	2.27	1.40E-05
C15orf54	2.23	7.78E-04
AK4	2.13	3.61E-04
BCL2A1	2.11	3.78E-04
TNFSF15	2.09	1.57E-04
OR1L8	2.05	2.39E-03
APLN	2.04	1.79E-04
SERPIND1	2.03	8.37E-04
P4HA1	2.02	3.40E-05
MME	2	7.00E-06
NSAP11	1.99	1.64E-03
CXADR	1.99	2.49E-04
MMP10	1.97	3.57E-03
PTPLAD1	1.95	1.61E-03
ERO1L	1.94	3.10E-05

Table 2

Top 20 down-regulated genes at 24 h, comparing the EG and the Cold Control Groups.

Gene symbol	Fold change	ANOVA p-value
CXCL10	-12.68	9.66E-03
VCAM1	-4.86	1.08E-03
FABP4	-4.85	1.49E-04
TAS2R30	-4.69	1.28E-04
IL33	-4.38	1.64E-04
A2M	-3.85	1.26E-04
HERC5	-3.8	5.00E-06
TAS2R31, TAS2R30, TAS2R45	-3.15	3.30E-05
WINK1	-3.09	5.00E-06
TAS2R19	-3.09	6.62E-03
TNFSF10	-3.05	2.08E-04
ABCG2	-3.05	1.49E-04
CXCL11	-3.01	6.86E-04
TAS2R14	-2.9	2.73E-03
RSF1-IT2	-2.89	3.76E-04
RSAD2	-2.86	7.40E-05
PDK4	-2.86	3.80E-05
OASL	-2.83	8.60E-05
GBP4	-2.75	4.19E-04
LINC00597	-2.75	3.01E-04

genes in common with the results at 24 h: 17 up-regulated and 50 down-regulated. Comparing changes at 24 h to those at 72 h, the list of the top 20 most up-regulated genes at 72 h has six genes that are also in the top 20 most up-regulated genes at 24 h, namely, *MPP4*, *SERPIND1*, *BCL2A1*, *PTPN22*, *CD44* and *STC1* (Table 5). These genes are of particular interest because reversible changes in gene expression may be expected to be less consequential for longer term survival and function than irreversible changes, even though, in this case, upregulation is slightly less extreme at 72 h than at 24 h. The same persistence of altered expression can be seen by comparing the 72 h top 20 most down-regulated list to its 24 h equivalent: *IL33* (now the most down-regulated), *A2M*, *HERC5*, *TNFSF10* and *PDK4* all had higher negative fold changes at 24 h, yet negative fold changes persisted at 72 h (cf. Tables 2 and 6).

Looking at the up-regulated genes' functional enrichment (Table 7), the first annotation cluster with an enrichment score of 2.72 shows groups related to wound healing and response to wounding. The next group with an enrichment score of 2.50 indicates genes related to response to nutrient and organic substance

Table 3

Top 3 annotation clusters with the highest enrichment score of the 24 h up-regulated genes.

Up 24 h EG/cold		
Cluster	Enrichment score	Number of genes
Glycoprotein	5.83	53
Signal peptide		40
Disulphide bond		33
Extracellular region	3.40	29
Transmembrane	3.31	50
Intrinsic to membrane		52

sensing. Such categories are indicative of a generalized stress response.

For the same period (72 h), when looking at the functional enrichment of the down-regulated genes (Table 8) we find the same 3 first annotation clusters observed amongst up-regulated genes at 24 h (Cluster 1: Glycoprotein/disulphide bond; Cluster 2:

Table 4

Top 3 annotation clusters with the highest enrichment score of the 24 h down-regulated genes.

Down 24 h EG/cold		
Cluster	Enrichment score	Number of genes
Taste receptor	4.43	11
Sensory transduction		13
Cognition		17
GTP binding	2.79	11
ATPase activity	2.73	10
Defense mechanism		3

Table 5

Top 10 most up-regulated genes at 72 h when comparing the EG group with the Cold Control Group.

Gene symbol	Fold change	ANOVA p-value
MPP4	1.93	6.84E-04
SERPINB10, SERPINB2	1.92	1.82E-02
CDCP1	1.88	1.48E-02
SERPIND1	1.84	7.14E-04
NEK10	1.8	1.82E-03
LPXN	1.79	1.80E-03
BCL2A1	1.79	2.74E-02
EIF2B2	1.68	4.66E-02
PTPN22	1.67	3.10E-02
CD44	1.64	5.79E-03

Table 6

Top 10 most down-regulated genes at 72 h when comparing the EG group with the Cold Control group.

Gene symbol	Fold change	ANOVA p-value
IL33	−3.87	4.48E-04
A2M	−3.02	1.58E-03
MGP	−2.69	1.82E-03
IL3RA	−2.4	2.30E-03
IL3RA	−2.37	2.15E-03
ATP8A1	−2.27	3.90E-05
HERC5	−2.21	3.72E-03
CD34	−2.1	1.34E-03
TNFSF10	−2.08	5.12E-03
PDK4	−1.99	1.78E-02

Extracellular; Cluster 3: Transmembrane/cytoplasmic – [Table 3](#)) further indicating the significant persistence of gene expression abnormalities, even though the sign of gene expression changes may reverse.

3.6. EG exposure at 72 h vs 24 h

Whereas at 24 h nearly 70% of differentially expressed genes were being down-regulated in response to EG (compared to Cold Control), after 72 h the genes are converging towards their normal expression levels ([Fig. 6](#)). This is consistent with what was to be expected from the recovery process from a response to toxic stress. One open question, however, is whether cells stressed at 24 h are

Table 8

Top 3 annotation clusters with the highest enrichment score of the 72 h down-regulated genes.

Down 72 h EG/Cold		
Cluster	Enrichment score	Number of genes
Glycoprotein	5.52	41
Disulphide bond		30
Signal peptide	3.47	30
Extracellular region		16
ATPase activity	2.73	10

recovering at 72 h or whether the cells stressed at 24 h stop proliferating (or even die) and at 72 h are outnumbered by less stressed cells.

The functional enrichment terms amongst up-regulated genes by EG at 24 h (glycoprotein, disulphide bond and extracellular) show up as down-regulated at 72 h while wound healing and coagulation are up-regulated ([Table 7](#)). Although there is an overlap between functions upregulated at 24 h and those down regulated at 72 h, there is little overlap between the specific genes involved, suggesting that this could also reflect a reorganization of signalling pathways.

The pattern in [Fig. 6](#) is seen in all the samples that suffered some sort of stress. Looking back at the PCA ([Fig. 3](#)) this is also quite clear. While the 24 h EG group remains isolated from the rest – probably due to the acute response to the high concentration of EG – the similarity between the Cold Control Group and the EG Group at 72 h is stronger. One possible explanation is that the 24 h Cold Control Group starts exhibiting healing patterns similar to those shown by the 72 h EG group, but with the toxic effects of EG being more severe than the simple exposure to cold temperatures, as would be expected.

3.7. Effects of cold stress

When looking at functional enrichment of the controls (24 h Cold Control versus the Negative Control) there seems to be an up-regulation ([Supplementary Material Table 1](#)) of genes related to cell motion, leukocyte migration and inflammatory response, and a strong down-regulation ([Supplementary Material Table 2](#)) of cell cycle, proliferation genes and DNA replication genes that is still happening in the same comparison at 72 h (easily seen by the Gene Ontology enrichment in [Supplementary Material Tables 3 and 4](#) and in [Supplementary Material Figs. 1–4](#)). These results are consistent with our observations of a reduced cell growth in the Cold Control and EG Groups ([Figs. 1 and 2](#)), and of a stress response (e.g. inflammation) from cold exposure (plus removal of growth factors, etc.), as would be expected.

3.8. Effects of cold stress plus EG stress

Since the fate of the cell in vivo depends on all stresses previously encountered in vitro, both cold stress and EG stress in this case, it is critical as well to compare the EG Groups to the Normal

Table 7

Top 3 annotation clusters with the highest enrichment score of the 72 h up-regulated genes.

Up 72 h EG/cold		
Cluster	Enrichment score	Number of genes
Wound healing	2.72	6
Coagulation		3
Response to nutrient levels and response to organic substance	2.50	4
Protease inhibitor	2.47	3

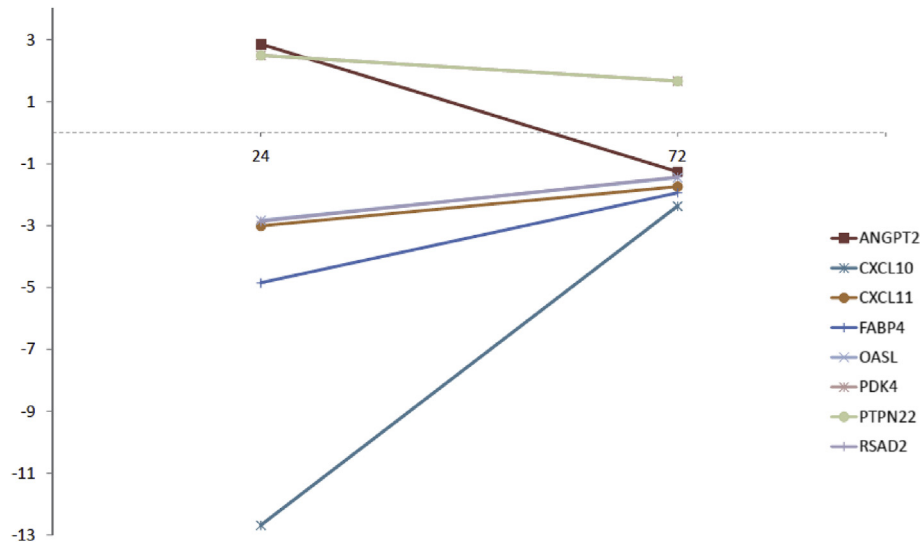


Fig. 6. Gene expression levels of some of the most recurrent top differentially expressed genes due to EG across time. The Y-axis shows the fold change when comparing EG vs Cold Control and the X-axis indicates the time-points 24 h and 72 h.

Control group (Supplementary Material Tables 5–8). This comparison shows genes responsible for angiogenesis and damage repair are being consistently up-regulated at both 24 and 72 h, while genes related to mitosis are being down-regulated, also at both time points.

4. Discussion

Our work aimed to study CPA toxicity in a cell model of the vascular endothelium. Based on the minimal cell loss induced by exposure to a high EG concentration, it seems that EG is only modestly toxic in HUVECs. We employed microarrays to survey gene expression levels and investigate potential mechanisms. To our knowledge, this is the first high-throughput gene expression profiling of cryoprotectant toxicity in a human cell model. We observed that EG induces changes at various levels, including stress response and changes in signalling pathways.

Wusteman et al. compared EG, DMSO, propylene glycol (PG), and 2,3-butanediol (BD), all at concentrations roughly approximating the concentrations needed to vitrify for each agent, on an immortalized endothelial cell line and found that PG and BD were the least toxic agents and EG and DMSO were the most toxic agents, but these results were obtained using methods of addition and washout that were not described, and that were admitted to be divorced from permeability data that were obtained only after the toxicity evaluations had been done. Since DMSO and EG were found to be the least permeable agents of those studied, it may be that the results reflected osmotic injury more than intrinsic toxicity [23]. In contrast, our studies were done using sufficiently small and lengthy concentration steps, and with the use of an osmolyte to reduce osmotic swelling during cryoprotectant washout, to minimize osmotic damage. In support of our findings, Pollack et al. [20] reported that for cultured porcine endothelial cells at 10 °C, EG was less toxic than PG, DMSO, and glycerol.

In general, high-throughput gene expression profiling has been used very little in the context of vitrification, and then primarily in reproductive cells (oocytes, blastocysts, embryos) [1,16–18], where described changes have typically been minimal and, not surprisingly, different from the changes observed in the present study. There is no prior study on endothelial cells to our knowledge, the closest study being that of Guan et al., which was focused on

chilling injury rather than primarily on cryoprotectant toxicity in precision-cut rat liver slices [12]. This study showed few similarities to our results in the patterns of gene expression alterations reported, presumably reflecting both the physiological differences between liver tissue and endothelial cells per se and the different effects of different cryoprotectant formulations, and supporting the need to examine different tissues of interest separately. Similarly, although gene expression profiling has been done on whole hypothermic organs [19,21,22], these were done in different tissues and using different conditions from the present study. The closest study to ours was done by Yang et al. [24] by exposing HUVECs to mild hypothermia (33 °C). Interestingly, Yang et al. also observed changes in genes related to cell cycle, cell adhesion and inflammation but in opposite directions to our results when comparing cold to negative controls.

A major component of the endothelial cell response to EG is a membrane response. Normally, one would tend to expect cryoprotectant toxicity to affect cellular “housekeeping” systems required for cell viability, but in this case, the changes may be more functional than vital. This might relate to the fact that the major role of the endothelial cell is membrane-related: adherence to the pericapillary basement membrane, maintenance of a non-thrombogenic interface with blood, etc. Our results raise the interesting possibility that sublethal changes in endothelial cells might result in changes in the reperfusion of organs treated with EG, to the detriment of the latter. The persistence of several of the changes in gene expression may also be significant, and reminiscent of changes in urinary plasminogen activator lasting for as long as a month in transplanted rabbit kidneys following perfusion with cryoprotectants [8]. The upregulation of membrane proteins is an interesting clue to the nature of the interaction between the membrane and EG, and may be related to membrane effects observed for dimethylsulfoxide [13].

To summarize, our results show that: 1) HUVECs exhibit only slight toxicity to EG at 60% v/v for 2 h and therefore are not particularly sensitive to EG. This observation supports the general reputation of EG as a particularly low-toxicity cryoprotectant. 2) Gene expression profiles of these cells exposed to EG revealed a generalized stress response at 24 h including changes in signalling peptides, glycoproteins and genes with extracellular and trans-membrane functions, all of which could be indicative of EG's effects

on membranes. 3) Recovery of expression is observed at 72 h post stress in genes that were differentially expressed at 24 h, and in addition, an activation of processes related to healing is also observed at 72 h. However, recovery remains incomplete even 72 h after EG treatment. Finally, 4) a generalized stress response involving inflammatory changes and reduced cell growth was also seen in the cold controls. The basis of these changes is unclear, but many are similar to changes seen in the EG group, and may relate to effects of low temperature, growth factor deprivation, trehalose exposure, osmotic excursions, etc.

As noted above, there is a lack of agreement between the results obtained in HUVECs and prior work [12] in precision-cut liver slices exposed to variants of M22 rather than to EG, which is interesting and informative in several ways and suggests that mechanisms of injury in one system do not necessarily convey to other systems. Apart from suggesting a potential effect of EG on membranes, our results do not yet allow us to pinpoint precise toxic mechanisms of EG, yet they provide a new paradigm and a starting point for more detailed studies in endothelial and other cells. Our full results are available online (<http://cryopreservation.org.uk/>), and our work sets the stage for further high throughput studies of cryopreservation and cryoprotectant toxicity.

Conflict of interest

The authors declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.cryobiol.2015.10.142>.

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