Analysis of global gene expression following mouse blastocyst cryopreservation

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BACKGROUND: The aim of this study was to examine the effect of the cryopreservation procedure (slow freezing or vitrification) and cryoprotectants (1,2-propanediol or dimethylsulphoxide) on mouse blastocyst gene expression.

METHODS: Cultured mouse blastocysts were cryopreserved with different protocols. Following thawing/warming, total RNA from re-expanded blastocysts was isolated, amplified and then analyzed using mouse whole-genome microarrays.

RESULTS: Compared with non-cryopreserved control blastocysts, gene expression was only significantly altered by slow freezing. Slow freezing affected the expression of 115 genes (P, 0.05). Of these, 100 genes exhibited down-regulation and 15 genes were up-regulated. Gene ontology revealed that the majority of these genes are involved in protein metabolism, transcription, cell organization, signal transduction, intracellular transport, macromolecule biosynthesis and development. Neither of the vitrification treatment groups showed statistically different gene expression from the non-cryopreserved control embryos. Hierarchical cluster analysis, did however, reveal that vitrification using 1,2-propanediol could result in a gene expression profile closest to that of non-cryopreserved blastocysts.

CONCLUSIONS: Investigating the effects of cryopreservation on cellular biology, such as gene expression, is fundamental to improving techniques and protocols. This study demonstrates that of the cryopreservation regimens employed, slow freezing induced the most changes in gene expression compared with controls.

Key words: vitrification / slow freezing / microarray

Introduction

Embryo cryopreservation is considered a routine assisted reproductive technique and has many benefits. The benefits of successful cryopreservation include the ability to store supernumerary embryos following the initial transfer. This eliminates the negative aspects associated with further ovarian stimulation, including financial cost and ovarian hyper-stimulation syndrome and maximizing the cumulative success of a single IVF cycle. In Europe around 8000 children are born each year following frozen embryo transfer (Andersen et al., 2005). It is estimated that frozen embryo transfer results in a further 6000 births per year in the USA (Assisted Reproductive Technology Report by Centers for Disease Control and Prevention, 2007). A more recent application of embryo cryopreservation has been to permit successful comprehensive chromosome screening at the blastocyst stage. Due to the time taken (72 h) to perform genetic analysis on human blastocysts, cryopreservation is required to permit timely embryo transfer (Schoolcraft et al., 2010).

Although quantitative assessment of embryo viability through omics technologies, including metabolomics and proteomics, may become available in the future, at present the most proficient embryo selection technique remains morphological analysis (Kattera and Chen, 2004; Ciray et al., 2005; Sakkas and Gardner, 2005; Scott et al., 2007). Improvements in the ability to culture embryos to the blastocyst stage have provided not only a form of selection, whereby inherently poor quality embryos possess reduced developmental capacity, but also a point at which the subsequent supporting tissue (trophectoderm) and the embryo proper (inner cell mass) can be observed and independently graded (Gardner and Schoolcraft, 1999). A good example of how blastocyst culture translates to clinical success is through an oocyte donor model. By removing oocyte quality as a variable many aspects of the clinical procedure can be assessed, including

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in vitro culture and its ability to support viable embryo development. Blastocyst development rates of around 65% can be achieved. The transfer of two blastocysts together can result in an 80% clinical pregnancy rate (per retrieval) in an oocyte donor program (Gardner, 2008). The ability to perform extended blastocyst culture, which supports embryos with such a high developmental competence, means that single blastocyst transfer has the potential to become routine for a significant number of patients (Papanikolaou et al., 2005, 2006). Thus, the ability to efficiently cryopreserve surplus blastocysts is an absolute requirement.

Two cryopreservation methods are currently available: slow freezing and vitrification. The physical and practical differences, as well as the merits, of each technique have been discussed in detail elsewhere (Kuleshova and Lopata, 2002; Vajta and Nagy, 2006). The difficulty is how can cryopreservation protocols be subsequently improved and optimized? The majority of changes in cryopreservation protocols have been due to empirical measurements. Usually this consists of assessing survival rates and subsequent embryo development. Ultimately, implantation potential and resulting pregnancies provide a more insightful indicator of the success of a given protocol. However, to improve a technique the ability to investigate the impact of specific treatments on cell biology and gene expression holds greater potential (Coticchio et al., 2005; Gardner et al., 2007).

Various forms of microscopy have been employed to assess the effects of cryopreservation on the ultra-structure of the oocyte, including cortical granules (Schalkoff et al., 1989), mitochondria (Jones et al., 2004) and microtubules (Larman et al., 2007). An excellent example of how analysis of the physiology can improve a technique is the use of polarized light microscopy to demonstrate that performance of the cryopreservation solutions at 37°C maintains the temperature-sensitive meiotic spindle of the vitrified oocyte (Larman et al., 2007; Ciotti et al., 2009). This means that only a minimal recovery period might be necessary before fertilization. A limited number of other techniques are also available for investigating the impact of cryopreservation on oocyte and embryo cellular biology. For example, analysis of the proteome demonstrated that the aberrant protein expression induced by slow freezing (Larman et al., 2007) is attributable to the protracted exposure to the cryoprotectant 1,2-propanediol (Katz-Jaffe et al., 2008). A clearer understanding of the impact of cryopreservation on embryo cellular biology may also be gleaned at the molecular level through monitoring changes in gene expression.

There are a limited number of publications investigating the effect of cryopreservation on oocyte and embryo gene expression (Boonkusol et al., 2006; Mamo et al., 2006; Park et al., 2006; Dhali et al., 2007; Succu et al., 2008). Most have been performed on mouse embryos using real-time PCR with primers to identify specific genes that may be affected by cryopreservation, for example, those related to oxidative stress, cold stress and apoptosis (Boonkusol et al., 2006; Dhali et al., 2007). One study on the mouse used high-density oligonucleotide microarrays to assess changes in almost 21,000 genes allowing a comparison between vitrified and non-vitrified control, in vivo derived, 8-cell embryos (Mamo et al., 2006). The advantage of microarray technology is that it allows comprehensive analysis of global transcript levels across different treatments. The ability to simultaneously compare changes in a vast number of genes permits a powerful insight into the cellular disruption caused by intrusive techniques such as cryopreservation, although, such an approach is not as sensitive as for a specific individual gene expression analysis. Therefore, the aim of this study was to investigate the effect of cryopreservation methods and cryoprotectants on mouse blastocyst gene expression using whole transcriptome microarray analysis.

Materials and Methods

Embryo collection and culture

Pronuclear oocytes were collected from 4- to 6-week old F1 (C57BL/6 × CBA/Ca) females (Jackson Laboratory, Bar Harbor, ME). Female mice were administered 5 IU of pregnant mare’s serum gonadotrophin and 48–50 h later 5 IU of human chorionic gonadotrophin (hCG). Following the administration of the hCG hormone, females were placed with males of the same strain and mating was confirmed by the presence of a vaginal plug the following morning. All steps of pronuclear oocyte collection and isolation were carried out at 37°C. Pronuclear oocytes were collected 21–22 h post-hCG and denuded by incubation in G-MOPS (Lane and Gardner, 2004) supplemented with 5 mg/ml human serum albumin, HSA) with 300 IU/ml hyaluronidase. Pronuclear oocytes were then washed three times through G-MOPS with HSA and subsequently cultured for a total of 96 h in groups of 10 in 20 μl drops of medium at 37°C, 6% CO2, 5% O2 and 79% N2 under a paraffin oil overlay (Ovoil; Vitrolife, Englewood, CO, USA). Embryos were placed into G1.3 with HSA (Vitrolife, Englewood, CO, USA) for the initial 48 h of culture and then moved to G2.3 with HSA for the remaining 48 h. All cultures were performed in 35 × 10 mm Petri dishes (Falcon 353001; BD, Franklin Lakes, NJ, USA). To avoid variation between females, embryos from each donor were pooled together and evenly distributed between treatments. Mouse in vitro cultured blastocysts were subjected to one of the four treatments: (1) non-cryopreserved (control), (2) slow frozen, (3) vitrified with dimethylsulphoxide (DMSO)/EG or (4) vitrified with 1,2-propanediol (PrOH)/EG. This work received prior approval from the Institutional Animal Care and Users Committee.

Slow freezing and thawing

All slow freezing procedures were performed at room temperature and the base medium for all freezing solutions was G-MOPS supplemented with 5 mg/ml HSA. For slow freezing, blastocysts were first exposed to G-MOPS containing 5% (v/v) glycerol and 0.1 mol/l sucrose for 10 min, followed by G-MOPS with 10% glycerol and 0.2 mol/l sucrose for 10 min. Blastocysts were loaded into 25 cc straws (Institute Medicine Veterinaire, Biced, L’Aigle, France) and placed into a freezing machine (Freeze Control CL2000; CryoLogic, Napa, CA, USA). The starting temperature of the freezing procedure was ~−6.0°C (Gardner et al., 2001). Blastocysts were held for 2 min and then seeded before being held for a further 10 min. Blastocysts were then cooled at the rate of 0.5°C/min to −32.0°C and then plunged into liquid nitrogen. Blastocysts were stored in liquid nitrogen for a minimum of 24 h before thawing.

Blastocysts were thawed in air for 30 s before being placed in a 30°C water bath for 10 s. The base medium for thawing was G-MOPS supplemented with 5 mg/ml HSA. Blastocysts were thawed at room temperature. Blastocysts were expelled into 10% glycerol with 0.2 mol/l sucrose before being immediately moved to 5% glycerol with 0.1 mol/l sucrose for 5 min. Finally, blastocysts were washed in G-MOPS for 5 min before being moved to G2.3.
Microarray results were validated by quantitative real-time PCR on the Roche Biosciences LightCycler® using the LightCycler® FastStart DNA Master SYBR Green I with 2.5 μl of amplified RNA template. After a 10 min incubation at 95°C, the following thermal cycling profile was performed for 45 cycles of amplification: denaturation at 95°C for 10 s, annealing for 5 s at primer-dependent temperatures and extension at 72°C for 2 s varying times depending on the size of the amplicon. The quantification of three genes were analyzed: Fatty acid-binding protein 5 (Fabp5) (Fwd: ATGACAATGCACACCTGTC; Rev: AGGATGACGAGGAAGGCC), which is associated with a water permeability barrier; fructose-1,6-bisphosphate 1 (Fbp1) (Fwd: TTATGTCGACAGGGGACG; Rev: CAGGGTCGAAGTTGCTTGG), a critical enzyme in the gluconeogenesis pathway and T-box 20 (Tbx20) (Fwd: CGGGGAATTCCTCGACAGTAG; Rev: GGCACTGACATGCTGTCATGG), a transcriptional regulator. Quantification was performed relative to the transcription in every sample of the housekeeping gene, Gapdh (Fwd: TGGACCACTCTGTTAGG; Rev: GCATGACATGCTGTCATGG). Standard curves were generated using serial dilutions for each gene with the slope of the standard curve illustrating the PCR reaction efficiency (3.32 ± 0.01%). The correlation coefficient, a measure of how accurate the individual measurements are in defining the curve was calculated >0.99. Melt curve analysis was also performed for each reaction to confirm specificity and integrity. Statistical analysis was performed with REST-2009® software using the pairwise fixed reallocation randomization test. Gene expression fold differences with P < 0.05 were considered significant.

Results
Survival of the blastocysts following thawing/warming was assessed by the re-expansion of the blastocoel cavity within 6 h. To gain further insight into the degree of absolute survival, those blastocysts that did not re-expand within 6 h were cultured overnight and assessed for blastocoel cavity re-expansion the following morning. Figure 1 shows the percentage of blastocysts with re-expanded blastocoel cavities following recovery periods of 6 and 24 h. Six hours post-recovery, the percentage of blastocysts displaying blastocoel re-expansion was not significantly different (P = 0.60) between treatments 2, 3 and 4 (61.3, 58.1 and 56.2%, respectively). This trend was maintained for those blastocysts that were cultured overnight. After 24 h, no significant differences (P = 0.98) were observed between treatments 2, 3 and 4 (93.4, 95.1 and 93.4%, respectively). Those blastocysts that presented re-expanded blastocoel cavities after 6 h underwent total RNA isolation, amplification and whole transcriptome analysis. On comparison with non-cryopreserved blastocysts, only the slow-frozen blastocysts exhibited any statistically significant differences in gene expression (P < 0.05). The majority of gene expression changes were observed to be decreased expression compared with controls (n = 100 transcripts). Chromosome mapping of these 115 differentially expressed genes revealed locations on most mouse chromosomes except chromosome 13 and the Y chromosome.

Microarray data were validated using quantitative real-time PCR for the following randomly selected transcripts with decreased expression, relative to the house-keeping gene, Gapdh: Fatty acid-binding protein 5 (Fabp5) which is associated with a water permeability barrier; Fbp1, a critical enzyme in the gluconeogenesis pathway and T-box 20 (Tbx20) a transcriptional regulator. The real-
Slow freezing negatively impacts gene expression

Time PCR results showed that all three transcripts were down-regulated significantly following slow freezing compared with non-cryopreserved controls (P < 0.05) (Fig. 2).

GO annotation was performed for the 115 transcripts that were observed to be significantly altered following slow freezing compared with the non-cryopreserved controls. Only 50% of the differentially expressed transcripts had a known annotation (Table I). Although a >1.5-fold change in gene expression was stipulated as a cut off for statistical analysis, most genes that were significantly different exhibited a >2-fold change. Figure 3 shows a pie chart displaying the known biological processes; metabolism, in particular protein metabolism, accounted for the highest proportion of altered genes (16%). Genes were also involved in transcription (15%), cell organization (13%), signal transduction (13%), intracellular transport (13%), macromolecule biosynthesis (11%) and development (9%). It is important to note that some genes have more than one function and thus could be represented in more than one category.

With regard to GO molecular function, the majority of differentially expressed genes were involved in binding (60%), in particular nucleic acid binding, or hydrolase (13%), transport (11%) or transcription factor activity (13%). For cellular localization, 70% of the annotated differentially expressed genes were identified as intracellular. Further analysis of these intracellular located genes revealed 50% to be membrane bound.

Unsupervised hierarchical analysis of all microarray samples revealed two main branches clearly separating the non-cryopreserved controls and the slow-frozen blastocysts (Fig. 4). Alongside the non-cryopreserved controls branched two of the three vitrification with PrOH/EG microarray samples and one of the three vitrification with DMSO/EG microarray samples. Thus, the remaining vitrification with PrOH/EG microarray sample and the two vitrification with DMSO/EG microarray samples branched alongside all three of the slow-frozen microarray samples.

Discussion

The ability to cryopreserve gametes and embryos efficiently is of great value in a number of fields. Cryostorage of unique strains of transgenic mice, commercially valuable domestic animals and conservation of endangered species are examples of how cryopreservation can act as a form of biosecurity, protecting the germplasm of invaluable animals. Cryopreservation also offers many advantages during clinical IVF and is considered a routine procedure. Despite the ubiquitous nature of cryopreservation in biosecurity and assisted reproduction, there is still a substantial lack of knowledge of how cryopreservation impacts cellular biology. Undoubtedly, these techniques and protocols will ultimately be improved through a better understanding of how cryopreservation affects gametes and embryos at the cellular level (Coticchio et al., 2005; Gardner et al., 2007).

Only one other study has investigated the impact of cryopreservation on blastocyst gene expression. Park et al., 2006 vitrified bovine blastocysts (in straws) in different volumes of a vitrification solution containing a single cryoprotectant (ethylene glycol) and compared apoptosis-related gene expression (survivin, Fas, Hsp 70 and caspase-3) using real-time quantitative RT–PCR. Regardless of the vitrification method, the expression of the four apoptosis-related genes was significantly increased compared with the non-cryopreserved control. Expression of such genes is indicative of cells that are responding to significant stress and may result in compromised embryo development. Interestingly, despite the fact that more blastocysts survived the minimal volume technique (<2 µl), gene expression of the integral cell membrane glycoprotein, Fas and heat shock protein, Hsp 70, were significantly increased compared with

**Figure 1** Blastocyst survival following thawing/warming. Blastocysts were cryopreserved using three treatments: slow freezing with glycerol (SF; n = 137), vitrification with DMSO/EG (n = 129) or PrOH/EG (n = 162). Blastocyst survival was determined through the re-expansion of the blastocoel cavity at 6 and 24 h after thawing/warming. Those blastocysts that had re-expanded within 6 h were used for gene expression analysis. Five replicates were performed.

**Figure 2** Quantitative real-time PCR validation of randomly selected differentially expressed genes from the microarray analyses. Quantitative real-time PCR was performed to validate the microarray data. Randomly selected differentially expressed genes: fatty acid-binding protein 5 (Fabp5), fructose-1,6-bisphosphate 1 (Fbp1) and T-box 20 (Tbx20) were analyzed relative to the housekeeping gene, Gapdh. All three genes were down-regulated significantly following slow freezing compared with controls in both the microarray and real-time PCR results (P < 0.05).
### Table 1 Annotation of known transcripts differentially expressed between slow frozen and control blastocysts.

<table>
<thead>
<tr>
<th>Description of 58 genes</th>
<th>GO biological process</th>
<th>GenBank accession number</th>
<th>Fold change&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-Co-enzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Co-enzyme A thiolase)</td>
<td>Fatty acid metabolism</td>
<td>NM_177470</td>
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<tr>
<td>Aldo-keto reductase family 1, member B8</td>
<td></td>
<td>NM_008012</td>
<td>2.3</td>
</tr>
<tr>
<td>Anaphase promoting complex subunit 13</td>
<td>Biological process unknown</td>
<td>NM_181394</td>
<td>2.8</td>
</tr>
<tr>
<td>Ankyrin 3, epithelial</td>
<td></td>
<td>AK046981</td>
<td>2.5</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>Regulation of cholesterol absorption</td>
<td>NM_009692</td>
<td>4.0</td>
</tr>
<tr>
<td>ATPase, H&lt;sup&gt;+&lt;/sup&gt; transporting, V1 subunit G isoform 1</td>
<td>ATP biosynthesis; proton transport</td>
<td>NM_024173</td>
<td>2.9</td>
</tr>
<tr>
<td>B-cell receptor-associated protein 31</td>
<td>Apoptosis; immune response; intracellular protein transport</td>
<td>NM_012060</td>
<td>2.5</td>
</tr>
<tr>
<td>Chromobox homolog 8 (Drosophilia Pc class)</td>
<td>Chromatin assembly or disassembly; chromatin modification; chromatin silencing; regulation of transcription, DNA-dependent</td>
<td>NM_013926</td>
<td>2.5</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)</td>
<td>Cell cycle; negative regulation of cell cycle; perception of sound; regulation of transcription, DNA dependent</td>
<td>NM_009878</td>
<td>2.5</td>
</tr>
<tr>
<td>COMM domain containing 3</td>
<td>Two-component signal transduction system (phosphorelay)</td>
<td>NM_147778</td>
<td>2.8</td>
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<tr>
<td>Crystallin, gamma F</td>
<td></td>
<td>W78478</td>
<td>3.1</td>
</tr>
<tr>
<td>Dedicator of cytokinesis 7</td>
<td></td>
<td>BC006868</td>
<td>2.6</td>
</tr>
<tr>
<td>Erythroblast membrane-associated protein</td>
<td>Biological process unknown</td>
<td>NM_013848</td>
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<td>Fatty acid-binding protein 5</td>
<td>water permeability barrier</td>
<td>NC_000069</td>
<td>2.0</td>
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<tr>
<td>Follicular lymphoma variant translocation 1</td>
<td>Cell growth and/or maintenance</td>
<td>BC023820</td>
<td>2.5</td>
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<tr>
<td>Fructose-1,6-bisphosphate 1</td>
<td>critical enzyme in the gluconeogenesis pathway</td>
<td>NC_000079</td>
<td>1.5</td>
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<td>Gamma-aminobutyric acid (GABA-A) receptor-associated protein-like 2</td>
<td>Intra-Golgi transport; intracellular protein transport</td>
<td>NM_026693</td>
<td>3.0</td>
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<tr>
<td>Glucocorticoid modular element-binding protein 1</td>
<td></td>
<td>NM_020273</td>
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<td>G protein-coupled receptor 125</td>
<td>G-protein coupled receptor protein signaling pathway; neuropeptide signaling pathway</td>
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<td>Histidine triad nucleotide-binding protein 3</td>
<td>DNA packaging; regulation of transcription, DNA-dependent; tRNA aminoacylation for protein translation</td>
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<tr>
<td>High-mobility group 20 B</td>
<td></td>
<td>NM_010440</td>
<td>3.2</td>
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<tr>
<td>Heme oxygenase (decycling) 2</td>
<td></td>
<td>AK012397</td>
<td>2.4</td>
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<tr>
<td>Heterogeneous nuclear ribonucleoprotein methyltransferase-like 3 (S. cerevisiae)</td>
<td></td>
<td>AK082136</td>
<td>2.5</td>
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<tr>
<td>Heat shock protein, alpha-crystallin-related, B9</td>
<td></td>
<td>Z49229</td>
<td>2.3</td>
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<tr>
<td>Heat shock protein 1, beta</td>
<td>Protein folding; response to heat; response to unfolded protein</td>
<td>NM_008302</td>
<td>2.3</td>
</tr>
<tr>
<td>5-hydroxytryptamine (serotonin) receptor 1A</td>
<td>G-protein coupled receptor protein signaling pathway</td>
<td>U39391</td>
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</tr>
<tr>
<td>Potassium inwardly rectifying channel, subfamily J, member 14</td>
<td>Ion transport; potassium ion transport</td>
<td>NM_145963</td>
<td>2.4</td>
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<td>Lymphoid enhancer-binding factor 1</td>
<td>Wnt receptor signaling pathway; odontogenesis (sensu Vertebrata); positive regulation of transcription from Pol II promoter; regulation of transcription, DNA dependent</td>
<td>AK028246</td>
<td>2.3</td>
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<tr>
<td>Microtubule-associated protein, RP/EB family, member 3</td>
<td></td>
<td>NM_133350</td>
<td>2.4</td>
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<tr>
<td>Muscle and microspikes RAS</td>
<td>Small GTPase-mediated signal transduction</td>
<td>NM_008624</td>
<td>2.4</td>
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<tr>
<td>Muscle and microspikes RAS</td>
<td>Small GTPase-mediated signal transduction</td>
<td>AF043581</td>
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<tr>
<td>Mitochondrial ribosomal protein L30</td>
<td>Ribosome biogenesis</td>
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<td>Mitochondrial ribosomal protein S18C</td>
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<td>Microtubule-associated protein 1 B</td>
<td></td>
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<td>2.6</td>
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<tr>
<td>5, 10-methylenetetrahydrofolate synthetase</td>
<td>Metabolism</td>
<td>NM_026829</td>
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Continued
the conventional cooling volume (∼50 µl). This indicates that empirical assessment of survival is not necessarily a reflection of cellular normality. The percentage of cryoprotectant (ethylene glycol) in the vitrification solutions for each volume were, however, different making it difficult to ascertain if the effect was that of the cryopreservation solution or the volume per se.

Analysis of stress-related gene expression using more comparable vitrification solutions was performed by Boonkusol et al. (2006). As with Park and colleagues, a single cryoprotectant (ethylene glycol) was used in the vitrification solutions and two vitrification methods that employed different volumes were compared with non-cryopreserved embryos. The minimal volume method was achieved by vitrifying the embryos in drops of 1–2 µl on a liquid nitrogen-cooled metal surface. A larger vitrification volume was produced by loading the embryos into a 0.25 ml straw. Pronuclear and 8-cell stage mouse embryos were vitrified and the gene expression of Hsp70, MnSOD, CuSOD, CirpB, Rbm3 and Trp53 was assessed using RT–PCR after 3 and 10 hours post-warming. Although all 6 genes exhibited increased expression with both vitrification techniques, the pronucleate oocytes that were vitrified in the larger...
volume (in-straw technique) had a significantly higher fold increase compared with those vitrified in a minimal volume (2−33 and 0.2−2-fold, respectively). Only those pronuclear oocytes vitrified with the in-straw technique resulted in statistically significant changes in gene expression for Hsp70 and CirpB, which were subsequently rectified 10 h post-warming.

As with the pronucleate stage, 8-cell stage embryos also showed an increase in expression in all 6 genes following the in-straw technique (3 h post-warming), with MnSOD and CirpB reaching statistical significance. Although gene expression was also assessed 10 h post-warming, embryos had undergone compaction and cell divisions making inferences from the gene expression profiles difficult. However the up-regulation of stress response genes reported by Boonkusol et al. appeared to be transient since the gene expression profile was not significantly different from non-cryopreserved controls after 10 h. This suggests that aberrant gene expression can be rectified over time. This conclusion, however, is in contrast to a study by Dhali et al. (2007) where the resulting blastocysts from vitrified pronucleate oocytes possessed significantly down-regulated apoptosis-related genes: Bax, Bcl2 and p53. Bax induces apoptosis, whereas Bcl2 is an anti-apoptotic and promotes cell survival. Therefore, the ratio of these two genes may indicate the stress levels incurred by the embryo. The altered genes that were observed by Park et al. (2006), Boonkusol et al. (2006) and Dhali et al. (2007) were not found within the group of differentially expressed genes reported in this study.

Only one other study has used microarrays to investigate the effect of cryopreservation on embryo gene expression. Mamo et al. (2006) compared control and vitrified in vivo derived 8-cell mouse embryos. Out of the 20 800 genes analyzed, 183 showed a statistically significant change in expression. No statistically significant differences in gene expression were observed following vitrification in the present study. Mamo et al. used a different vitrification protocol that involved ethylene glycol as the cryoprotectant and a non-contact method to vitrify the embryos held in a 1−2 µl volume. It is likely that the difference in embryonic stage and vitrification protocol explains the contrasting results, which highlights the difficulty in comparing studies.

Unsupervised hierarchical clustering of the four different treatments in this present study indicates that slow freezing with glycerol generates the most divergent gene expression profile from the non-cryopreserved control. This observation is similar to data from the protein expression profile of mouse oocytes that had been slow frozen or vitrified, whereby oocytes that had been slow frozen were grouped separately from the control and those that had been vitrified, following unsupervised hierarchical cluster analysis (Larman et al., 2007). Isolation of the steps of the slow-freezing protocol indicated that the aberrant protein expression was a result of the protracted exposure to the PrOH cryoprotectant (Katz-Jaffe et al., 2008).

Although higher concentrations of cryoprotectants are used during vitrification, the exposure times are generally an order of magnitude less. Thus, it is plausible that the prolonged cryoprotectant exposure during slow freezing also contributes to altered gene expression observed in this present study. Hierarchical cluster analysis revealed that two of the three vitrification replicates with PrOH/EG possessed gene expression profiles similar to that of the control. Therefore, it appears that the blastocyst gene expression profile may be altered more significantly by exposure to DMSO than that of PrOH. DMSO
has been shown to cause an intracellular calcium release in mouse oocytes (Larman et al., 2006) and it appears that a DMSO-induced calcium release is responsible for cell differentiation and apoptosis that have been observed in other cell types (Morley and Whitfield, 1993). It may also be the cause of toxicity observed from frozen bone marrow and stem cell transplants (Hanslick et al., 2009). Further studies are, however, required to determine if PrOH is indeed preferential over DMSO for vitrification.

Recent reviews of clinical outcome following cryopreservation of human embryos are encouraging (Loutradi et al., 2008; Wannerholm et al., 2009). This study supports the recent findings from the meta-analysis performed by Loutradi et al. (2008) that observed a trend to better pregnancy rates following vitrification of blastocysts, compared with slow freezing. It is also reassuring that the largest study of blastocyst vitrification, to date, observed that the rates of pregnancy, implantation, abortion and congenital birth defects were similar to that of fresh blastocyst transfers (Takahashi et al., 2005). The incomplete and inadequate reporting of clinical and long-term results, however, makes comparisons between protocols and techniques extremely difficult, if not impossible. Thus, analysis of cellular biology will go some way to understanding and improving gamete and embryo cryopreservation.

## Authors’ roles

M.L. co-designed the study, performed embryo culture and cryopreservation, performed data analysis and co-wrote manuscript. J.F. performed embryo culture and cryopreservation and compiled data. D.K.G. obtained the funding, co-designed the study and co-wrote the manuscript. M.G.K.G. co-designed the study, co-wrote the manuscript, generated protocol, performed data analysis and interpreted the results. B.R.M. performed microarray and real-time PCR experiments, designed primers and analysed real-time PCR data.

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## References


Gardner DK. Dissection of culture media for embryos: the most important and less important components and characteristics. Reprod Fertil Dev 2008;20:9–18.


Papanikolaou EG, Camus M, Kolibianakis EM, Van Landuyt L, Van Steirteghem A, Devroey P. In vitro fertilization with single...