J Assist Reprod Genet (2013) 30:107–116
DOI 10.1007/s10815-012-9898-4

GAMETE BIOLOGY

# The presence of 1 mM glycine in vitrification solutions protects oocyte mitochondrial homeostasis and improves blastocyst development

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Received: 27 September 2012 / Accepted: 18 November 2012 / Published online: 18 December 2012


**Abstract**
*Purpose* Embryos generated from oocytes which have been vitrified have lower blastocyst development rates than embryos generated from fresh oocytes. This is indicative of a level of irreversible damage to the oocyte possibly due to exposure to high cryoprotectant levels and osmotic stress. This study aimed to assess the effects of vitrification on the mitochondria of mature mouse oocytes while also examining the ability of the osmolyte glycine, to maintain cell function after vitrification.
*Methods* Oocytes were cryopreserved via vitrification with or without 1 mM Glycine and compared to fresh oocyte controls. Oocytes were assessed for mitochondrial distribution and membrane potential as well as their ability to fertilise. Blastocyst development and gene expression was also examined.
*Results* Vitrification altered mitochondrial distribution and membrane potential, which did not recover after 2 h of culture. Addition of 1 mM glycine to the vitrification media prevented these perturbations. Furthermore, blastocyst development from oocytes that were vitrified with glycine was significantly higher compared to those vitrified without glycine (83.9 % vs. 76.5 % respectively; $p<0.05$) and blastocysts derived from oocytes that were vitrified without glycine had significantly decreased levels of *IGF2* and *Glut3* compared to control blastocysts however those derived from oocytes vitrified with glycine had comparable levels of these genes compared to fresh controls.
*Conclusion* Addition of 1 mM glycine to the vitrification solutions improved the ability of the oocyte to maintain its mitochondrial physiology and subsequent development and therefore could be considered for routine inclusion in cryopreservation solutions.

**Keywords** Glycine · Osmotic stress · Vitrification · Oocyte · Mitochondria

*Capsule* The beneficial effects of glycine in oocyte vitrification solutions on mitochondrial homeostasis and blastocyst development.

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## Introduction

The cryopreservation of supernumerary embryos is routine practise in clinical IVF; however there are many ethical issues that can arise regarding the subsequent fate of these embryos. One possible solution to these ethical concerns is to cryopreserve oocytes as the same ethical or moral objections to the cryopreservation of embryos do not apply to oocytes. Furthermore oocyte cryopreservation is of significant importance to oncology patients where their subsequent cancer treatment may render them subfertile.

Historically, slow freezing has been used for the cryostorage of mammalian oocytes however resultant thawing survival, fertilisation and pregnancy rates remain low. The alternative to slow-freezing cryopreservation is vitrification which involves the equilibration of cells in high concentrations of cryoprotectant solutions followed by cooling at rates of ≥1,500 °C/minute [11, 29, 33, 49, 51]. In animal models and the human, vitrification has resulted in improved oocyte survival and resultant embryo development compared to slow freezing as well as preservation of ultrastructure within the

oocyte and improved calcium signalling [25, 27, 29, 34, 40, 53, 64]. Due to this, the use of vitrification instead of slow freezing is becoming an increasingly popular in clinical IVF and a recent meta analysis has concluded that vitrification is the superior method for oocyte cryopreservation [11, 15, 30, 38, 59, 72].

Although oocyte vitrification has been demonstrated to result in significantly improved survival and blastocyst development compared to oocyte slow freezing and that recent studies have shown that blastocysts derived from vitrified oocytes result in similar pregnancy rates when compared to fresh oocytes [16, 22, 56], studies have demonstrated that vitrified oocyte still have significantly reduced fertilisation and blastocyst development rates compared to fresh oocytes [22]. In addition vitrification also decreases mitochondrial membrane potential and energy production in the oocyte immediately after warming, increases reactive oxygen species (ROS) generation and can also alter oocyte gene expression profiles [12, 39, 42, 74]. The reason for these changes is currently unknown however may be due to osmotic stress within the oocyte caused by exposure to high levels of cryoprotectants which are used to prevent ice crystal formation.

During the vitrification process, there is an extreme and very rapid increase in inorganic solute concentrations within the cells associated with the dehydration process due to the high concentrations of cryoprotectants used. It has been demonstrated that exposure to hyperosmotic solutions significantly increased the levels of abnormal spindle structure in human oocytes [43] and that blastocyst development was significantly reduced after exposure of bovine COC's to hyperosmotic solutions [2]. Furthermore, removal of sodium chloride from oocyte slow freezing solutions improves subsequent developmental outcomes in the mouse [60–62]. Embryo studies have found that osmolytes, particularly glycine, can prevent osmotic stress when embryos are cultured in hyperosmotic conditions and furthermore are also important for the maintenance of oocyte cell volume [5, 19, 55, 63, 69]; however the role of osmolytes in oocyte cryopreservation has received little attention.

Therefore, the aim of this study was to determine if the osmolyte glycine may play a protective role in cryopreservation procedures for mouse oocytes. In particular, the maintenance of cellular physiology was assessed by examining mitochondrial homeostasis as well as subsequent fertilisation, development and blastocyst cell numbers and gene expression.

## Materials and methods

### Oocyte collection

Female F1 (C57BL6×CBA) mice were superovulated by intraperitoneal injections of 5 IU pregnant mares' serum gonadotrophin (PMSG, Folligon Serum Gonadotrophin, Intervet Aust PTY LTD, Bendigo, Australia) and 5 IU human chorionic gonadotrophin (hCG, Pregnyl, N.V. Organon, Oss Holland) given 49 h apart. Mice were sacrificed 13 h after hCG injection by cervical dislocation and the oviducts were removed. Oocytes were excised from the oviducts directly into handling medium [24] containing 1 mg/mL hyaluronidase (Sigma Chemical Co., St. Louis, MO) to denude the oocytes of the surrounding cumulus cells. Once the oocytes were denuded, they were washed in handling medium and then either cryopreserved or examined as controls. All handling and culture media was supplemented with 5 % human serum albumin unless otherwise stated. All procedures were conducted in accordance with the NIH Guide [45] and had Institutional Animal Ethics approval.

### Oocyte vitrification

Oocyte vitrification was performed as described previously [32] where 1 mM glycine was included in all solutions for the glycine treatment group. Briefly, oocytes were pre-equilibrated in handling medium at 37 °C for a minimum of 5 min, then placed into handling medium containing 1.07 M (7.6 %) DMSO (Sigma Chemical Co.) and 1.36 M (7.6 %) ethylene glycol (Sigma Chemical Co.) at 37 °C for 1 min 45 s. Oocytes were then placed into handling medium containing 2.32 M (16.5 %) DMSO, 2.95 M (16.5 %) ethylene glycol, 0.67 M sucrose and 6.7 mg/mL ficoll (Sigma Chemical Co.) at 37 °C and picked up again immediately and placed onto a nylon loop (0.5–0.7 mm diameter) previously dipped in the final solution to create a film. The loop was then plunged into liquid nitrogen.

For warming, the loops were removed from the liquid nitrogen and placed directly into handling medium containing 0.33 M sucrose at 37 °C. The oocytes were incubated for 1 min, placed into handling medium containing 0.20 M sucrose for 2 min at 37 °C and finally placed into handling medium for 3 min at 37 °C and then placed into culture.

### Mitochondrial distribution

To assess the impact of vitrification on mitochondrial distribution, oocytes were collected and vitrified/warmed then stained using Mitotracker Green-FM (Molecular Probes, Eugene, OR). Mitotracker Green-FM probes contain a mildly thiol-reactive chloromethyl moiety for labeling mitochondria in live cells regardless of mitochondrial membrane potential. The stain passively diffuses across the plasma membrane and accumulates in active mitochondria which can then be assessed using confocal microscopy (ex: 490 nM, Em: 516 nM). Oocytes were incubated in 100 nM of Mitotracker Green-FM for 15 min at 37 °C in the dark. Oocytes were then washed in handling media without HSA

and were immediately imaged using confocal microscopy (Nikon C1 Confocal Scanning Head, Nikon TE2000F).

### Mitochondrial membrane potential

To assess the impact of vitrification on mitochondrial membrane potential, oocytes were collected and vitrified/warmed and stained using (JC-1 (5,5′6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolycarbocyanine iodide), Molecular Probes). JC-1 is a dual emission dye that exhibits potential-dependent accumulation within mitochondria. Increased mitochondrial membrane potential is indicated by a emission shift in fluorescence from green (529 nm) to red fluorescent J-aggregates (590 nm). Therefore a decrease in the red/green fluorescence ratio is indicative of mitochondrial depolarization. Oocytes were incubated with 1.5 mM JC-1 for 15 min at 37 °C in the dark. The ability of the staining procedure to detect differences in membrane potential was confirmed using a potassium diffusion procedure using the potassium ionophore valinomycin (data not shown, [52]).

### Assessment and quantification of mitochondrial staining

Mitochondrial distribution was analysed using ImageJ software (version 1.31v, National Institutes of Health, USA) and mitochondrial membrane potential was analysed using Adobe Photoshop (version 6.0.1, Adobe Systems Inc.) using a method adapted from Barnett et al that that has been previously described [8, 41]. For analysis of mitochondrial distribution mean fluorescent pixel intensity was obtained in three different areas within the oocyte by taking four different readings within the centre, intermediate and periphery region (Fig. 1). A template overlay was used on each image to eliminate bias. These were then expressed as a ratio between regions to demonstrate changes in distribution. For analysis of mitochondrial membrane potential a similar technique was used where mean fluorescent pixel intensity was obtained in three different areas within the oocyte by taking four different readings within the centre, intermediate and periphery region. The average membrane potential in each of the three areas was expressed as a ratio of red intensity to green intensity for each area.

### Spindle analysis

Oocytes were collected and vitrified/warmed then fixed in 4 % paraformaldehyde either immediately after vitrification/warming (0 h) or after 2 h of culture in 20 μl drops of G1.2 medium [24] under oil at 37 °C in an atmosphere of 6%$CO_2$, 5%$O_2$ and 89%$N_2$. Again, fresh control oocytes were examined at 0 h. Following fixation, oocytes were washed in PBS and placed into PBS containing 0.1 % Triton-X100 (Sigma Chemical Co.) for 40 min at room temperature (RT). Oocytes were then placed directly into a blocking solution containing 150 mM Glycine and 3 mg/ml BSA in PBS and left for 30 min at RT. After rinsing in PBS, oocytes were placed into anti-β-tubulin monoclonal antibody (Sigma Chemical Co.) diluted 1:100 in PBS for 1 h at 37 °C. After another rinse in PBS, oocytes were placed into a FITC conjugated goat-anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) diluted 1:100 in PBS for 1 h at 37 °C. Oocytes were then rinsed in PBS and incubated in 20 μg/ml propidium iodide (Sigma Chemical Co.) for 5 min at 37 °C to stain chromatin. Oocytes were then loaded in handling medium and imaged using confocal microscopy. Images were visually analysed and spindles and chromosomes were categorised as normal (barrel shaped spindles with lined up chromosomes), or abnormal including either normal spindles with abnormal chromosomes (barrel shaped spindles with non-lined up chromosomes), normal chromosomes with abnormal spindles (lined up chromosomes with non-barrel shaped spindles) or completely abnormal (both spindles and chromosomes abnormal) [47, 48].

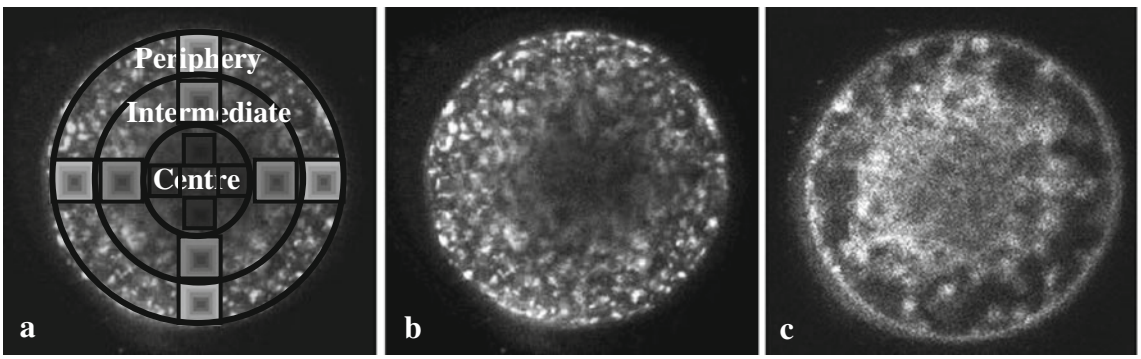


**Fig. 1** **a** Example of template overlay indicating three regions measured where each box indicates the region where the actual measurement was taken **b** image of a control oocyte with punctuate distribution throughout the periphery and intermediate **c** image of a vitrified oocyte (without 1 mM glycine) with an altered mitochondrial distribution ratio (increased location in the intermediate and centre and decreased presence in the periphery)

## Fertilisation and embryo culture

Sperm was collected from male C57BL/6×CBA F1 mice and placed into 1 ml of in vitro fertilisation (IVF) medium [24] under oil at 37 °C in an atmosphere of 6%$CO_2$, 5%$O_2$ and 89%$N_2$ to disperse and capacitate for 1/2-1 h before insemination. Oocytes were collected and then vitrified/warmed as described above. Using a surgical laser (Fertilase), a 5 μM hole was made in the zona pellucida to facilitate sperm penetration in all treatment groups. Insemination was carried out in 100 μl drops of IVF medium under oil at 37 °C in an atmosphere of 6%$CO_2$, 5%$O_2$ and 89%$N_2$. Oocytes and sperm were co-incubated for 4 h, after which oocytes were placed into 20 μl drops of G1.2 medium to support cleavage stage development [24] under oil at 37 °C in an atmosphere of 6%$CO_2$, 5%$O_2$ and 89%$N_2$. After 26 h (from the time of insemination) the number of 2-cells was recorded and these embryos were placed into fresh drops of G1.2 medium under oil at 37 °C in an atmosphere of 6%$CO_2$, 5%$O_2$ and 89%$N_2$ for a further 24 h. Embryos were placed into 20 μl drops of G2.2 medium to support development to the blastocyst stage [24] under oil at 37 °C in an atmosphere of 6%$CO_2$, 5%$O_2$ and 89%$N_2$. After a further 24 and 49 h of culture development was assessed. At the end of the culture period, resultant blastocysts were placed in RLT buffer (Qiagen, Doncaster, Victoria) and stored at −80 °C for subsequent gene expression analysis or were immediately stained to assess cell number and allocation.

## Differential staining

Blastocysts were stained for cell allocation to either the inner cell mass (ICM) or trophectoderm (TE) using a differential staining protocol [23, 73]. Briefly, the blastocyst zona was removed by incubation in 0.5 % pronase for 2–5 min followed by incubation in 2,4,6-Trinitrobenzenesulfonic acid (Sigma Chemical Co.) at 4 °C for 10 min. Blastocysts were then incubated in 0.1 mg/ml anti-dinitrophenyl-BSA (Sigma Chemical Co.) at 37 °C for 10 min and then incubated in guinea pig serum and 10 μg/ml propidium iodide at 37 °C for 5 min. Blastocysts were counterstained in 6 μg/ml hoescht (Sigma Chemical Co.) in 100 % ethanol overnight at 4 °C. Blastocysts were then washed in 100 % ethanol and placed into drops of glycerol on and imaged using fluorescent microscopy.

## RNA extraction, reverse transcription and real time — Polymerase Chain Reaction (PCR)

Blastocysts were extracted in groups of 30 using the RNeasy Micro kit according to the manufacturer's protocol (Qiagen). The extracted RNA was then placed into RNase-free eppendorf tubes in 6 μl aliquots and stored at −80 °C. Reverse transcription was carried out using the Sensiscript Reverse Transcription kit according to the manufacturers protocol (Qiagen). The resultant cDNA was stored at −20 °C.

Real-Time Polymerase Chain Reaction (RT-PCR) was carried out with a master mix consisting of 10 μl SYBR Green Mix (Applied Biosystems, Foster City, California), 1 μl of both forward and reverse primers (Table 1) and 6 μl water with 2 μl cDNA diluted 1:2 (0.75 embryo equivalents), as determined by a preliminary dilution experiment (data not shown). The RT-PCR reaction was carried out on a GeneAmp 5700 Sequence Detection System PCR machine with a thermal cycling program of 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All oligonucleotide primers (Table 1) were designed using Primer Express (Applied Biosystems, Victoria, Australia) and synthesized by Geneworks (Adelaide, Australia). The genes examined were *Glut1, Glut3, Citrin, Aralar* and *IGF2* with *18 s* used to normalise the results and were expressed as a fold change using a previously described method [37].

**Table 1** Details of primers used for analysis of gene expression in blastocysts

| Name | Primer | Sequence | Amplicon size (bp) | GenBank accession |
|---|---|---|---|---|
| *18 s* | 5′ | AGA AAC GGC TAC CAC ATC CAA | 92 | AF176811 |
| | 3′ | CCT GTA TTG TTA TTT TTC GTC ACT ACC T | | |
| Slc2a1 (*Glut1*) | 5′ | CCA GCT GGG AAT CGT CGT T | 77 | M23384 |
| | 3′ | CAA GTC TGC ATT GCC CAT GAT | | |
| Slc2a3 (*Glut3*) | 5′ | CTC TTC AGG TCA CCC AAC TAC GT | 121 | X61093 |
| | 3′ | CCG CGT CCT TGA AGA TTC C | | |
| *IGF2* | 5′ | AAG AGT TCA GAG AGG CCA AAC G | 103 | U71085 |
| | 3′ | CAC TGA TGG TTG CTG GAC ATC T | | |
| Slc25a12 (*Aralar*) | 5′ | TGC CAA CCC CGA TCA CA | 101 | NM_172436 |
| | 3′ | TTT CAA TGC CAG CGA AAG TG | | |
| *Slc25a13 (Citrin)* | 5′ | TTC ACT CAG TTT TTG TTG GAA ATA CAG | 101 | NM_015829 |
| | 3′ | TTG GCA TTG TCT CGT TGC A | | |

### Statistical analysis

Differences in mitochondrial distribution, spindle characteristics and cell numbers were analysed using a univariate general linear model within SPSS (version 12.0.1; SPSS Inc, Chicago, IL), with a Least Significant Difference (LSD) test for multiple comparisons. Mitochondrial membrane potential was first examined by a repeated measures general linear model with LSD within SPSS to determine if regional differences existed within treatment groups. Differences between treatment groups were then analysed using a univariate general linear model with LSD within SPSS. Differences in survival and development data were assessed using the log likelihood statistic within GLIM (version 4.0, National Algorithm Group, Oxford, UK). Gene expression values were normalised against the average of the embryos derived from fresh, non-cryopreserved oocytes (control) and differences between treatment groups were analysed using a Students paired *t*-test. For all analyses a p value of <0.05 was considered significant.

## Results

### Effect of vitrification (+/−1 mM glycine) on oocyte survival and mitochondrial distribution

The addition of 1 mM glycine to the vitrification solutions did not significantly alter oocyte survival rates compared to survival after vitrification without glycine (77.4 % $n$=746 oocytes vs. 75.5 % $n$=521 respectively).

For all of the oocytes examined, it was apparent that there were regional differences in mitochondrial distribution within the oocyte therefore as a result different areas were examined separately and differences between areas are expressed as a ratio between areas (Fig. 1). Immediately after collection (0 h), mitochondria in fresh, non-cryopreserved oocytes (control) were distributed throughout the oocyte and were most abundant around the periphery as compared to the centre (Fig. 1, Table 2). Oocytes vitrified in the absence of glycine and analysed immediately after warming had a significantly reduced ratio for periphery: centre, as compared to controls (Table 2; $p$<0.01) indicating movement of the mitochondria away from the periphery and into the intermediate and central regions. Analysis of these oocytes following 2 h of culture revealed that this abnormal mitochondrial distribution did not recover. Oocytes vitrified in the presence of glycine analysed immediately after warming showed similar mitochondrial distribution compared to control oocytes (Table 2).

### Effect of vitrification (+/−1 mM glycine) on mitochondrial membrane potential

In addition to differences in mitochondrial distribution, there were also regional differences in mitochondrial membrane potential within the oocyte with the peripheral region being hyperpolarised compared to mitochondria that were more centrally located. As a result, the different areas were also examined separately. Oocytes vitrified in the absence of glycine had significantly higher mitochondrial membrane potential in both the centre and intermediate compared to fresh oocytes and glycine-vitrified oocytes ($p$<0.05, Table 3). After 2 h of culture these differences were no longer evident indicating the oocytes recovered after 2 h of culture (Table 3).

### Effect of vitrification (+/−1 mM glycine) on spindle and chromosome normality

Analysis of spindle and chromosomal configurations at 0 h revealed that vitrified oocytes had a significantly higher percentage of abnormal spindle and chromosome configurations compared to control oocytes ($p$<0.05; Fig. 2) however after a 2 h incubation period the percentage of abnormal spindle and chromosomes was equivocal between all three treatment groups indicating recovery of this parameter (Fig. 2).

### Effect of vitrification (+/−1 mM glycine) on oocyte fertilisation and blastocyst development

Vitrification with or without 1 mM glycine did not alter fertilisation rate (70.9 % $n$=320 and 74.0 % $n$=265 respectively) compared to fresh control oocytes (72.4 % $n$=638). Blastocyst development, as assessed by expanded or hatching blastocyst

**Table 2** Effect of vitrification (+/-1 mM glycine) on mitochondrial distribution within the central, intermediate and periphery of the oocyte

| | Intermediate : Central | Periphery : Central | | |
|---|---|---|---|---|
| Control | 1.66±0.10 [a] | 2.34±0.24 [b] | | |
| | 0 h | | 2 h | |
| | Intermediate : Central | Periphery : Central | Intermediate : Central | Periphery : Central |
| Vitrification | 1.73±0.15[a] | 1.58±0.20[a,c] | 1.48±0.13[a] | 1.20±0.12[a] |
| Glycine Vitrification | 1.87±0.16[a,d] | 1.97±0.16[b,d] | 1.84±0.11[a,d] | 2.00±0.26[b,d] |

Data presented as mean ± SEM, $n$≥20 per treatment per time point.

Values with different superscripts (a-d) indicate significant difference between treatment groups and regions within the oocyte ($p$<0.05)

**Table 3** Effect of vitrification (+/-1 mM glycine) on mitochondrial membrane potential within the central, intermediate and periphery of the oocyte

| | Centre | Intermediate | Periphery | | | |
|---|---|---|---|---|---|---|
| Control | 0.52±0.01[a] | 0.53±0.02 [a] | 0.60±0.03 [b] | | | |
| | 0 h | | | 2 h | | |
| | Centre | Intermediate | Periphery | Centre | Intermediate | Periphery |
| Vitrification | 0.56±0.01[c] | 0.57±0.01[c] | 0.62±0.02 [b] | 0.55±0.01 [a] | 0.56±0.02 [a] | 0.59±0.02 [b] |
| Glycine Vitrification | 0.53±0.01[a] | 0.55±0.01 [a,c] | 0.63±0.02 [b] | 0.53±0.01 [a] | 0.54±0.01 [a] | 0.62±0.02 [b] |

Data presented as mean ± SEM, $n \geq 20$ per treatment at each time point. Values with different superscripts (a-c) indicate significant difference between treatment groups and regions within the oocyte ($p<0.05$)

formation as a percentage of 2-cells was significantly reduced in the vitrification group compared to control (76.5 % $n=196$ vs. 84.1 % $n=451$ respectively; $p<0.05$). Glycine vitrification resulted in blastocyst formation similar to control (83.9 % $n=227$ vs. 84.1 % $n=451$ respectively) (Table 4).

### Effect of vitrification (+/−1 mM glycine) blastocyst cell number and allocation

The blastocysts that were derived from oocytes that were vitrified either with and without 1 mM glycine had similar total blastocyst cell numbers compared to blastocysts derived from fresh oocytes however both treatment groups had significantly decreased numbers of inner cell mass (ICM) cells ($p<0.05$; Fig. 3). There was also a significant change for both groups in regards to the proportion of ICM: trophectoderm cells ($p<0.05$; Fig. 3).

### Effect of vitrification (+/−1 mM glycine) blastocyst gene expression

Blastocysts resulting from oocytes vitrified without glycine had significantly less *IGF2* and *Glut3* mRNA compared to fresh controls ($p<0.05$, Fig. 4). Oocytes vitrified with 1 mM glycine resulted in blastocysts with similar gene expression of *IGF2* and *Glut3* compared to fresh control oocytes. Levels of the calcium binding mitochondrial transporters and *Aralar* and *Citrin* were also examined as levels of these transporters control glucose fate in a cell via the malate-aspartate shuttle. There was a significant increase in *Aralar* in embryos that were vitrified without glycine and significantly increased *Citrin* expression in the blastocysts derived from glycine-vitrified oocytes compared to control ($p<0.05$, Fig. 4). There were no significant differences found for levels of *Glut1* in blastocysts resulting from oocytes vitrified with or without glycine compared to controls (Fig. 4).

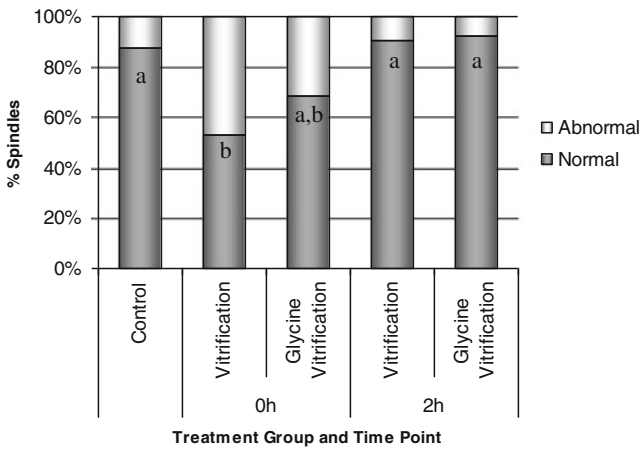


**Fig. 2** Abnormalities in spindle and chromosome configuration following vitrification with or without glycine either immediately after warming and after a 2 h incubation period. $N \geq 20$ per treatment at each time point Different superscripts between columns are significantly different ($P<0.05$)

## Discussion

The introduction of vitrification into clinical IVF has revolutionised oocyte and embryo cryopreservation and is now considered the superior cryopreservation technique compared to slow freezing. Although recent studies have shown that blastocysts generated from vitrified oocytes can result in comparative pregnancy rates compared to those generated from fresh oocytes, the reported decreases in fertilisation rates, blastocyst development as well as alterations to mitochondrial membrane potential, ATP levels, ROS production and gene expression still indicate that vitrification still requires further optimisation [3, 12, 22, 36, 39, 42]. In addition many of the studies that demonstrate comparable pregnancy rates between fresh and vitrified oocytes are undertaken using young oocyte donors and it is likely that oocyte quality can play a role susceptibility to damage during cryopreservation [14]. Studies using animal models have demonstrated that the negative impact of vitrification on the oocyte increases with maternal age so it is likely that the impact of vitrification in older women will be far greater than that of young donors however this has not been investigated [71].

In lieu of the impact that vitrification can have on oocyte viability this study investigated the ability of the osmolyte glycine to act as a protective agent during the vitrification process. We have demonstrated that vitrification alters oocyte physiology as measured by changes to spindle and

**Table 4** Effect of vitrification (+/−1 mM glycine) on oocyte fertilization and development

| | 19 h culture | 96 h of culture | | | |
|---|---|---|---|---|---|
| | % Fertilisation | % Cleavage Arrest | % Morula | % Blastocyst | % Hatching Blastocyst |
| Control | 72.4 | 3.5[a] | 12.4 | 84.1[a] | 54.7[a] |
| Vitrification | 74.0 | 13.8[b] | 9.7 | 76.5 [b] | 66.8[b] |
| Glycine Vitrification | 70.9 | 5.8[a] | 12.7 | 83.9 [a,b] | 66.5[b] |

Fertilisation is expressed as number of 2-cells per oocyte inseminated and blastocyst development is expressed as number per fertilised oocyte
$n \geq 196$ oocytes fertilised per treatment. Values with different superscripts (a–b) within a column indicate significant difference between groups ($P<0.05$)

chromosome configuration, mitochondrial distribution and membrane potential as well as altering gene expression in the resultant blastocysts. Furthermore, this study has demonstrated the novel finding that the addition of 1 mM glycine to the vitrification solutions results in maintenance of oocyte mitochondrial function and subsequent improvements in blastocyst development such that the glycine vitrified oocytes were similar to that of fresh control. We propose that glycine is acting as an osmolyte and/or cell volume regulator and therefore it is possible that the aberrations to oocyte physiology that were found in this study in oocytes that were vitrified without glycine may result from osmotic stress caused by an increase in concentration of inorganic solutes within the oocyte during the vitrification process.

The oocyte is completely dependent on mitochondrial metabolism as its sole source of energy production, with a reduction in mitochondrial ATP production being associated with developmental delay followed by embryo arrest [6, 9]. Mitochondria move throughout the cell along a network of microtubules [26, 65] and it has been previously hypothesised that rearrangement of mitochondrial distribution may result in a redistribution of ATP throughout the cell [6], a process that has been found to occur during mouse oocyte maturation and early embryo cleavage [65]. It is thought that

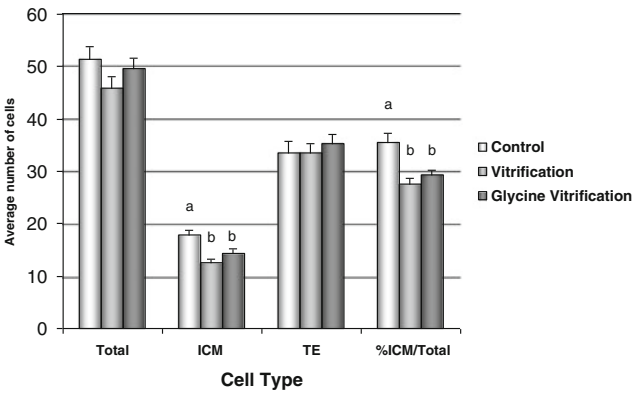


**Fig. 3** Effect of vitrification with or without 1 mM glycine on blastocyst cell numbers and differentiation. Values are expressed as mean ± SEM, $n \geq 30$ blastocysts/group a–b indicates significant differences between groups ($P<0.05$)

this redistribution allows increased levels of ATP to be produced in different areas of the cell when higher energy requirements are needed [7, 8, 17, 65] and disruptions to mitochondrial distribution is associated with developmental arrest [8]. In addition a lack of microtubules within the oocytes is related to low competence through an inability of mitochondria to relocate within the cell [9]. The alteration to mitochondrial distribution in oocytes vitrified in the absence of glycine may be a result of the dehydration process and indicates that the microtubular arrays within the oocyte may be altered therefore preventing the mitochondria from returning to a normal distribution following rehydration. This notion is supported by previous studies showing that cryopreservation can disrupt microtubules formation within the oocyte [21, 54, 70]. The inability of the mitochondria to return to control distribution patterns suggests that these oocytes may be less competent as altered ATP distribution may impact on vital processes during fertilisation and development. In addition there is increasing evidence in other tissues that osmotic stress can induce bundling and perturbed structure of microtubules [44]. The more normal mitochondrial distribution that we observed in this study after vitrification with glycine indicates that a similar sensitivity of microtubules to osmotic stress may occur in oocytes and thus osmolytes are of benefit in vitrification solutions.

The inner mitochondrial membrane potential (MMP) of oocytes has been the focus of several studies [28, 67, 68] as it has been suggested to be a marker of oocyte competence [66, 68]. It has been reported that high and low-polarized mitochondria occur in different regions throughout the oocyte, such that high-polarized mitochondria occur in the periphery region, a phenomenon that was also found in this study [67]. In this study vitrification did not alter the pattern of hyper-polarization, with all groups having hyperpolarised mitochondrial in the periphery however; the level of hyper-polarization in mitochondria in the centre and intermediate regions of the oocyte was significantly increased in vitrified oocytes however this was not seen when oocytes were vitrified in the presence of 1 mM glycine. It has been suggested that an increase in hyper-polarized mitochondria

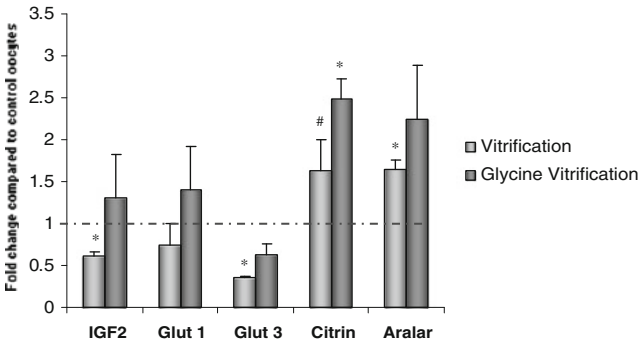


**Fig. 4** Fold change in gene expression compared to control oocytes. Results for each gene were normalised to the calibrator (control) which was given the arbitrary value of 1 (as indicated by the *dashed line*). Values are mean ± SEM based on four replicates with 30 pooled blastocysts per replicate. *Asterisk* indicates significant difference from control ($P<0.05$), # indicates a trending difference from control ($P=0.09$). No significant differences were found between vitrification and glycine vitrification

is related to an increase in fragmentation during the cleavage of human embryos, and in mouse embryos an increase was associated with cleavage arrest possibly due to loss of metabolic regulation [1]. Interestingly this increase in membrane potential did not completely recover after 2 h of incubation and therefore may be involved in the increased rates of cleavage arrest seen in embryos derived from oocytes vitrified without glycine however this remains to be investigated.

Interestingly this study also demonstrated that oocyte vitrification +/− glycine resulted in blastocysts with significantly lower inner cell mass (ICM) cell number compared to control which then impacted on the ICM: trophectoderm ratio. Previous studies have demonstrated that alterations to this ratio can be correlated to implantation and fetal development [31] and it is possible that this decreased may result in blastocysts that have a reduced ability to implant and form a fetus of normal weight however this remains to be investigated and if this is the case may be indicative that although glycine is improving vitrification outcome, further optimisations may be required.

Analysis of the gene expression determined an overall trend for reduced levels of gene expression for blastocysts derived from oocytes vitrified without glycine with the most marked differences evident were in the metabolic genes. *Glut3*, expressed on the apical membranes of trophectoderm cells, has been shown to be responsible for blastocyst glucose uptake from the environment [46]. A decrease in *Glut3* expression may lead to a decrease in glucose uptake, which has been shown to increase apoptosis at the blastocyst stage and cause impaired development following implantation [13]. In addition IGF2 levels were also significantly reduced. Studies using gene knockout have shown that placental size is influenced by *IGF2*, with under-expression leading to significantly reduced fetal size [10, 20]. It is possible therefore that the lower levels of *IGF2* as well as *Glut3* may lead to alterations in fetal weight however this remains to be investigated. Interestingly, there was an increase in the levels of *Aralar* and *Citrin* in the blastocysts derived from vitrified oocytes. This is an interesting observation as these genes are isoforms of the aspartate glutamate carrier [58] that form a vital component of the malate-aspartate shuttle and can therefore direct the metabolic fate of glucose towards oxidation [57]. Production of ATP from glucose oxidation is an important energy generating pathway in the mouse blastocyst [35]. The observed increase in the expression of these genes may reflect a mechanism for the blastocyst to increase glucose oxidation and therefore ATP production to possibly compensate for the reduced levels of glucose uptake as a result of the reduced levels of glucose transporters. This would be an essential adaptation to preserve ATP production and therefore viability. The increase in the genes for these mitochondrial carriers was greater and the decrease in *Glut3* levels were smaller in the blastocysts derived from vitrification with glycine compared to those vitrified without glycine suggesting that preservation of their metabolic function may be enhanced.

In summary, this study has demonstrated that the addition of glycine to the vitrification media can be advantageous for a number of parameters important for embryo viability. This implies that glycine is likely acting as an osmolyte during the vitrification procedure, protecting the oocyte against osmotic stress. Glycine has been shown have a protective role when embryos cultured in anisosmotic conditions ([4, 18, 19, 55, 63, 69]), however this is the first time that is has been shown to act as an osmolyte during oocyte cryopreservation procedures. The numerous disruptions seen as a result of oocyte cryopreservation have previously been suggested to be caused by osmotic stress is supported by other studies that demonstrated a beneficial effect of replacing sodium with choline in the slow freezing media for mouse oocytes [60–62]. Despite this the blastocyst development rate was still low and subsequently no increase in pregnancy rates for human oocytes was attributed to this approach [50]. Whether glycine can have the same beneficial effects on the human oocyte and embryo, as those observed in this study, is a promising concept and warrants further investigation. In conclusion, this study advocates the addition of glycine to vitrification solutions as it assists in maintaining mitochondrial homeostasis and results in increased blastocyst development rates compared to those vitrified without it.

**Acknowledgements** The authors wish to thank Dr. Karen Kind for her assistance with the gene expression experiments, Dr. Jeremy Thompson assistance with experimental design and David Froiland for his assistance with confocal microscopy. The authors acknowledge the support of the NHMRC and Channel 7 Children's Research Foundation.

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