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## *In vitro* viability of mouse 8-cell embryos vitrified in a simple solution of ethylene glycol

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### Abstract

A study was made to determine if ethylene glycol (EG) can be used in a simple solution to vitrify mouse 8-cell embryos and to determine the critical factors that affect its success. Mouse 8-cell embryos were vitrified after exposure to 2M and 7M EG prepared in Dulbecco's phosphate buffered saline (PBS) with 10% heat-inactivated calf serum (CS). Mouse 8-cell embryos exposed to 2M EG for 2, 5 and 10 min, and to 7M EG for 2 and 5 min had survival rates similar to the untreated controls (93.3-100%). No significant difference in their survival rates *in vitro* was observed. Higher room temperatures (>24°C) at exposure before cooling resulted in poor development rates to the blastocyst stage. The survival rates of embryos vitrified after 2 min in 7M EG at 18-22°C room temperature did not differ significantly from the control, but embryos vitrified after 5 min had significantly lower survival rates ( $p < 0.0001$ ).

In conclusion, effective vitrification of mouse 8-cell embryos can be achieved by initial exposure to 2M EG for 2-10 min followed by equilibration in 7M EG for 2 min at 18-22°C room temperature. This study has shown that 7M EG in PBS with 10% CS is sufficient to provide cryoprotection of vitrified mouse 8-cell embryos and that exposure of the embryos to the vitrification solution at temperatures over 24°C is critical to their subsequent development *in vitro*.

Key words : embryo, ethylene glycol, mouse, vitrification

### Introduction

When Rall and Fahy<sup>13)</sup> reported the successful vitrification of mouse 8-cell embryos for the first time, they probably had in mind the formulation of a universal vitrification solution (VS1) that would be effective in all stages of embryonic development and in a wide variety of animal species. However, Kasai et al.<sup>8)</sup> reported the efficacy of a new vitrification solution called EFS (Ethylene glycol-Ficoll-sucrose) that was of low toxicity and produced high viability rates of

mouse morulae after warming. As a result, EFS has been adopted as the vitrification solution that is effective in the vitrification of all stages of embryonic development in mice<sup>11,23)</sup> and in other domestic animal species<sup>5,9)</sup>. A key component of EFS is ethylene glycol (EG) which is a low (62.07) molecular weight cryoprotectant (CPA). The use of EG as a cryoprotectant was first reported by Miyamoto and Ishibashi<sup>12)</sup> in the freezing of mouse and rat embryos by the conventional freezing method.

The main components of a low toxicity

vitrification solution are CPA(s), macromolecules and sugars<sup>7</sup>). Each component has a key role in the cryobiology of cells. Cryoprotectants prevent the formation of intracellular ice during freezing and thawing<sup>10</sup>). Macromolecules assist in the formation of stable glasses<sup>7</sup>). Sugars contribute to the dehydration of the cells prior to permeation by the CPAs<sup>10,18</sup>), stabilization of the vitrification compound in and out of subzero temperatures<sup>10</sup>), and dilute the cryoprotective agent out of the cell<sup>20</sup>). In most cases, vitrification solutions with these components, hardly present the true nature of the action of cryoprotection by CPAs. Different CPAs have different molecular weights and permeability coefficients. Sugars further regulate these coefficients and solutions having more than one CPA obscure a better understanding of their singular cryoprotective action. To better understand their cryoprotective properties, CPAs must be used in solutions singly, without macromolecules or sugars. Furthermore, vitrification solutions with a single cryoprotectant, such as EG, should be less toxic and should minimize osmotic stresses during equilibration and dilution<sup>15</sup>).

How to achieve efficient cryoprotection with a single CPA is a challenge that should show a clear understanding of the critical factors affecting the success of such procedures. First, the minimum concentration of the CPA that would support vitrification should be determined. Second, critical exposure times for the CPA must be studied to understand the toxicities of these compounds. Third, effective techniques should be formulated to overcome the toxicities of these compounds which, at high concentrations, are likely to lead to osmotic stresses. Lastly, a vitrification procedure should be developed that would lead to high viability rates after warming.

This study was made to determine if ethylene glycol can be used in a simple solution to vitrify mouse 8-cell embryos and to determine some of the critical factors that affect its success.

## Materials and methods

Six-week old female ICR mice, purchased from a commercial breeder (Japan SLC Co., Tokyo, Japan), were induced to superovulate by intraperitoneal injection of 5 IU equine chorionic gonadotrophin (eCG, Serotropin, Teikoku Zoki, Tokyo, Japan) and 5 IU human chorionic gonadotrophin (hCG, Gonatrophin, Teikoku Zoki) given 48h apart. After hCG injection, they were paired with a male of the same strain and were inspected the following day for the presence of a vaginal plug. Eight-cell stage embryos were flushed from the oviduct at 66 h after hCG injection using Dulbecco's phosphate-buffered saline (PBS, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated calf serum (CS, Gibco Laboratories, Life Technology Inc., New York, USA). After collection, morphologically normal embryos were washed three times in PBS with 10% CS, then pooled and held at room temperature in sterile plastic Petri dishes (Nunc, Nunclon, Kamstrup, Denmark).

Before the exposure and vitrification experiments were made, different concentrations ranging from 6.0 to 9.0M of EG in PBS with 10% CS were loaded into 0.25ml French straws (I. M. V. L'Aigle, France) in 50 and 100  $\mu$ l columns and were cooled in liquid nitrogen (LN<sub>2</sub>) vapor on a styrofoam boat to determine the minimum concentration of EG which allowed vitrification. When the straws were cooled, the appearance of a transparent column indicated successful vitrification<sup>20</sup>).

Since most successful vitrification protocols show the advantage of a 2- or multiple step system of exposure and cooling<sup>9,19</sup>), a 2-step method was adopted in this study. Before the vitrification experiments were made, the effect of exposure to 2M and 7M EG, which was determined to be the final vitrification solution, at room temperature (18–22°C) on the survival of mouse 8-cell embryos without cooling was examined.

Six groups of 15 embryos were exposed to 100  $\mu$ l drops of 2 M EG for 2, 5 and 10 min for step 1, and to 50  $\mu$ l drops of 7M EG for 2 and 5 min for step 2. After the required exposure periods were attained, the embryos were transferred to 100  $\mu$ l drops of 1 M solution of sucrose in PBS with 10% CS for 5 min to dilute the CPA, were rehydrated in PBS with 10% CS for 5 min, were washed three times in Whitten's medium<sup>22)</sup>, and were cultured in microdrops of Whitten's medium at 37°C in 5% CO<sub>2</sub> in air for 48h. A group of 8-cell embryos was similarly cultured to serve as the control. Survival was assessed by the ability of the embryos to develop to expanded or hatching blastocysts after 48h of culture.

The vitrification method similarly consisted of two steps. Step 1 required the exposure of 10–15 embryos in 100  $\mu$ l drops of 2M EG in PBS with 10% CS for 2, 5 and 10 min, and step 2 required their equilibration in 50  $\mu$ l drops of 7M EG in PBS with 10% CS for 2 and 5 min. The treated embryos, together with the 7M EG solution were then aspirated into 0.25 ml straws, which were prefilled with 100  $\mu$ l of 1 M sucrose in PBS with 10% CS to dilute the CPA at the time of warming/dilution, a 0.5 cm bubble and 10  $\mu$ l of 7M EG. After aspirating the embryos together with the 7M solution, a 0.5 cm bubble was added to the column followed by a short column of 1M sucrose in PBS with 10% CS. The straws were then heat-sealed (Figure 1). After the required equilibration period of 2 and 5 min, the straws

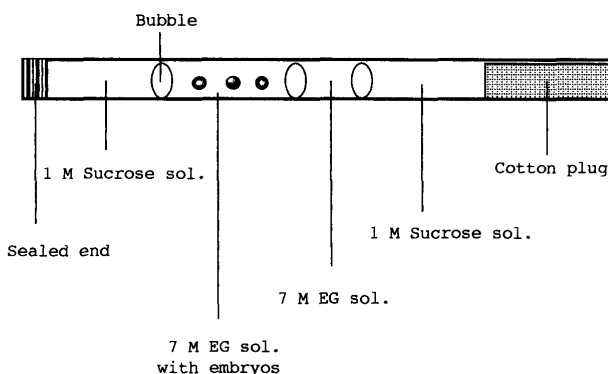


Fig. 1. Composition of the vitrified straw.

were laid horizontally on a styrofoam boat (140 mm  $\times$  60 mm  $\times$  5 mm) wrapped in stainless steel mesh (approx. 2 mm) and were cooled in LN<sub>2</sub> vapor<sup>16)</sup>. After at least 1 min in the LN<sub>2</sub> vapor, the straws were plunged into LN<sub>2</sub> and were stored for 1–120 days. The vitrification process was done under two room temperatures of 18–22°C and over 24°C. The experiments were replicated 5 times.

The straws were warmed by brief exposure to the air (approx. 10 sec) and by immersing in a water bath at 18–20°C for 15–20 sec. The straw ends were then cut, and the contents of the straw were expelled and were allowed to mix in a sterile petri dish to dilute the CPA. After 5 min, the embryos were pipetted into PBS with 10% CS and were allowed to rehydrate for 5 min. The embryos were washed three times and then they were cultured in microdrops of Whitten's medium under paraffin oil at 37°C for 48h in 5% CO<sub>2</sub> in air.

Freshly collected 8-cell embryos were cultured similarly in microdrops of Whitten's medium to serve as the control. Viability of vitrified-warmed and non-vitrified embryos was assessed by their ability to develop to the expanded or hatching blastocyst stage after 48h of culture. Experiments were replicated 5 times and the data were analyzed using ANOVA with Fisher's PLSD as a post-hoc test.

## Results

The minimum concentration of EG in PBS with 10% CS that vitrified on cooling in LN<sub>2</sub> vapor was 7M. Six and 6.5M EG solutions did not vitrify although Ali and Shelton<sup>1,2)</sup> reported that 6.5M EG vitrified on cooling but not on warming. True vitrification, which means the appearance of a transparent column of the vitrification solution on cooling and warming, occurred at 8M concentration which is in agreement with Ali and Shelton<sup>2)</sup> and Hotamisligil et al.<sup>6)</sup>. Table 1 shows the results of the cooling trials at different concentrations of EG in LN<sub>2</sub> vapor. Although

7M EG in PBS with 10% CS was not a true vitrification solution because it crystallized on warming, it was selected as the vitrification solution in this study due to presumably lower toxicity compared to 8 M EG<sup>6</sup>).

Table 2 shows the effects of exposure of mouse 8-cell embryos to 2M and 7M EG. Mouse 8-cell embryos exposed to 2M EG shrank during the first minute and floated in the drop, but slowly descended to the bottom as they slowly swelled back to their initial volume within 10 min. When these embryos were transferred to 7M EG, they initially floated in the drop but descended to the bottom after 2 min. When EG was diluted in 1M sucrose in PBS with 10% CS, the embryos immediately shrank and remained floating in the drop even after 5 min. When the exposed embryos were cultured in Whitten's

medium for 48 hours, very high proportions (93.3–100%) of mouse 8-cell embryos developed to the expanded or hatching blastocyst stage in culture. The survival rates were not significantly different from the untreated control.

When mouse 8-cell embryos were vitrified in LN<sub>2</sub> vapor at a room temperature of more than 24°C, the embryos hardly survived the warming process or had very low development rates to the blastocyst stage. Only a few embryos cooled after 2 min exposure at step 2 survived the warming process and developed to the blastocyst stage. The highest survival rate (32.7%) was observed for embryos exposed at 2 min each in steps 1 and 2. No survival after warming was observed for the embryos vitrified after 5 min in step 2 (Table 3).

Table 4 shows the effects of vitrification on the post-warming survival rate of mouse 8-cell embryos cooled in LN<sub>2</sub> at a room temperature range of 18–22°C. No significant difference in viability rates to the blastocyst or hatching blastocyst stages was found between the controls and embryos vitrified in 7M EG after 2 min in step 2, but significant differences were found between the control and those vitrified after 5 min in step 2. Likewise, significant differences were found

Table 1. Vitrification properties of different concentrations of ethylene glycol in PBS with 10% calf serum

| Concentration (M) | Vitrification |         |
|-------------------|---------------|---------|
|                   | Cooling       | Warming |
| 6.0               | —             | —       |
| 6.5               | —             | —       |
| 7.0               | +             | —       |
| 7.5               | +             | —       |
| 8.0               | +             | +       |
| 8.5               | +             | +       |
| 9.0               | +             | +       |

Table 2. Viability of 8-cell embryos exposed to 2 and 7 M ethylene glycol in PBS with 10% calf serum

|          | Step 1 Exposure to 2M EG (min) | Step 2 Exposure to 7M EG (min) | Number exposed | Number cultured | Developed to blastocyst (%) |
|----------|--------------------------------|--------------------------------|----------------|-----------------|-----------------------------|
| Control  | —                              | —                              | —              | 48              | 48(100%)                    |
| Treated: |                                |                                |                |                 |                             |
|          | 2                              | 2                              | 30             | 30              | 30(100%)                    |
|          | 5                              | 2                              | 30             | 30              | 29(96.7%)                   |
|          | 10                             | 2                              | 30             | 30              | 28(93.3%)                   |
|          | 2                              | 5                              | 30             | 30              | 28(93.3%)                   |
|          | 5                              | 5                              | 30             | 30              | 29(96.7%)                   |
|          | 10                             | 5                              | 30             | 30              | 28(93.3%)                   |

Data were pooled from 2 replicates

Table 3. Viability of 8-cell embryos vitrified in ethylene glycol with 10% calf serum at room temperature over 24°C

|          | Step 1 2M EG (min) | Step 2 7M EG (min) | Vitrified /Warmed | Cultured | Developed to blastocyst (Mean ± SEM) |
|----------|--------------------|--------------------|-------------------|----------|--------------------------------------|
| Control  | —                  | —                  | —                 | 50       | 100 ± 0.0 <sup>a</sup>               |
| Treated: |                    |                    |                   |          |                                      |
|          | 2                  | 2                  | 50                | 50       | 32.0 ± 14.6 <sup>b</sup>             |
|          | 5                  | 2                  | 50                | 50       | 30.0 ± 12.2 <sup>b</sup>             |
|          | 10                 | 2                  | 50                | 50       | 18.0 ± 9.7 <sup>bc</sup>             |
|          | 2                  | 5                  | 50                | 50       | 0.0 ± 0.0 <sup>c</sup>               |
|          | 5                  | 5                  | 50                | 50       | 0.0 ± 0.0 <sup>c</sup>               |
|          | 10                 | 5                  | 50                | 50       | 0.0 ± 0.0 <sup>c</sup>               |

Values are mean percentages of 5 replicates.

<sup>a,b</sup>Values with different supercripts differ significantly  $p < 0.05$

Table 4. Viability of 8-cell embryos vitrified in 7 M ethylene glycol in PBS with 10% calf serum at 18–22°C room temperature

|          | Step 1<br>2M EG<br>(min) | Step 2<br>7M EG<br>(min) | Number<br>vitrified<br>/Warmed | Number<br>cultured | Developed to<br>blastocyst<br>(Mean% ± SEM) |
|----------|--------------------------|--------------------------|--------------------------------|--------------------|---|
| Control  | —                        | —                        | —                              | 30                 | 100 ± 0.0 <sup>a</sup>                      |
| Treated: |                          |                          |                                |                    |   |
|          | 2                        | 2                        | 50                             | 50                 | 94.4 ± 4.0 <sup>a</sup>                     |
|          | 5                        | 2                        | 50                             | 50                 | 88.0 ± 4.9 <sup>a</sup>                     |
|          | 10                       | 2                        | 50                             | 50                 | 84.0 ± 7.5 <sup>a</sup>                     |
|          | 2                        | 5                        | 50                             | 50                 | 24.0 ± 2.4 <sup>b</sup>                     |
|          | 5                        | 5                        | 50                             | 50                 | 20.0 ± 8.9 <sup>b</sup>                     |
|          | 10                       | 5                        | 50                             | 50                 | 14.0 ± 7.5 <sup>b</sup>                     |

\* Values are mean percentages of 5 replicates.

<sup>a,b</sup> Values with different superscripts differ significantly  $p < 0.0001$

between the embryos vitrified after 2 min in step 2 and those vitrified after 5 min in step 2 ( $p < 0.0001$ ). The highest overall survival rate (94.4%) was obtained after 2 min exposure each in steps 1 and 2.

### Discussion

Ethylene glycol may be less toxic than the other CPAs<sup>8,16</sup> and an 8M concentration is needed when EG alone is used in the vitrification solution<sup>2</sup>. Seven moles (7M = 39.1% v/v) EG in PBS with 10% CS is (approximately) similar to 40% (v/v) EG, which has been shown to vitrify on cooling but not on warming<sup>8,18</sup>, although Darvelid et al.<sup>4</sup> reports that a solution with 40% (v/v) EG in PBS allowed vitrification. Compared to other CPAs, EG requires a higher concentration to vitrify (8M vs. 6.5M glycerol<sup>15</sup> and 6M DMSO<sup>3</sup>).

In the exposure trials without cooling to 2M and 7M EG, prolonged exposure could have shown deleterious effects. As the results have proven, mouse 8-cell embryos can tolerate exposure to these concentrations of ethylene glycol at room temperature without appreciable loss of viability *in vitro*, although Ali and Shelton<sup>2</sup> reported that 7M EG is toxic after 5 min

exposure.

When mouse 8-cell embryos were initially exposed to 2M EG solution, they shrank immediately because the osmotic pressure of the solution exceeded isotonicity, thus, water leaves the cell, but as EG permeates the cell, water re-enters the embryo to maintain osmotic equilibrium<sup>10</sup>. Hotamisligil et al.<sup>6</sup> studied the volumetric response of mouse oocytes in 2M EG and observed that when oocytes were exposed in 1 step, the oocytes first rapidly shrank and then they swelled back to their initial volume with maximum shrinkage (55.5%) within the first minute. This partial dehydration of the cell before freezing is essential because intracellular ice formation is reduced<sup>10</sup>. When the exposed embryos are transferred to a higher (7M) EG concentration, an increase in the intracellular concentration of EG alone would occur, because the rapid efflux of water would not need EG transport<sup>6</sup> and the resulting greatly increased concentration of EG and the endogenous macromolecules contribute to the vitrification of the cytoplasm<sup>14</sup> on cooling in LN<sub>2</sub> vapor.

Equilibration of the embryos in the freezing or vitrification medium, therefore, is of critical importance for embryo survival<sup>18,21</sup>. Sufficient permeation of the CPA depends on the optimal time of exposure, which depends on the concentration of EG, the temperature, the developmental stage and species of the embryo<sup>7</sup>. When 8-cell embryos were exposed for 2 min to the final vitrification solution (7M) at 18–22°C, very high (84–94.4%) survival rates not significantly different from the controls were obtained. However, when the embryos were exposed to the final vitrification solution at room temperatures over 24°C, very low (18–32%) survival rates were obtained, indicating that the optimum exposure temperature for 8-cell embryos vitrified in 7M EG falls within 18–22°C. This is in agreement with the result of Kasai et al.<sup>8</sup> who exposed mouse morulae and with the result of Miyake et

al.<sup>11)</sup> who exposed all developmental stages in EFS solution containing 40% EG at  $20 \pm 0.5^\circ\text{C}$ . The significant difference between the survival rates of embryos exposed to 2M and 7M EG at  $18\text{--}22^\circ\text{C}$  and those exposed over  $24^\circ\text{C}$ , however, may not be totally due to the effect of permeation of EG as influenced by the temperature because the CPA was diluted out of the embryos with 1M sucrose in PBS. Szell and Shelton<sup>18)</sup> have indicated that embryos may be sensitive to high concentrations of sucrose at increased temperatures and that the chances of survival were improved by removing the CPA (glycerol) at  $20^\circ\text{C}$ . The vitrification solution used in this study did not contain sucrose and that the dilution of EG with sucrose at increased room temperature ( $>24^\circ\text{C}$ ) may have led to osmotic shock<sup>15)</sup>. Apparently, a lower temperature of the solution allows the efflux of water from the cell causing shrinkage, followed by a slow influx of the vitrification solution<sup>21)</sup>, and the partial dehydration favors intracellular vitrification<sup>20)</sup>.

Although a high concentration of CPA is essential in the vitrification process to eliminate ice formation altogether both in the embryos and the surrounding medium<sup>10)</sup>, 7M EG in PBS with 10% CS seems adequate to provide efficient cryoprotection of vitrified mouse 8-cell embryos. The anticipated effect of ice formation by devitrification during warming did not result in decreased survival rates, as indicated by the results of the embryos vitrified after 2 min in step 2 at  $18\text{--}22^\circ\text{C}$ , which did not differ from the controls. This is in agreement with the result of Rall and Wood<sup>14)</sup> who claimed that crystallization of the embryo suspension was not inconsistent with high survival after thawing. On the other hand, the significant difference ( $p < 0.0001$ ) between the survival rates of the embryos vitrified after 2 min in step 2 and the embryos vitrified after 5 min in step 2 at  $18\text{--}22^\circ\text{C}$  can only be traced to the chemical toxicity of EG. Other studies have shown that prolonged exposure to high concen-

trations of EG results in low survival rates<sup>4,8,17)</sup> after warming. Hotamisligil et al.<sup>6)</sup> observed that when oocytes exposed to 2M EG for 15 minutes were transferred to 6 and 8M EG, bleb formation occurred after 5 min in 6M and as early as 4 min in 8M. Although the number and size of blebs per oocyte increased and coalesced during 30 min exposure, the oocytes did not lyse during EG treatment. Whether bleb formation was a sign of chemical toxicity of EG was not clear, but Hotamisligil et al.<sup>6)</sup> chose an equilibration time of less than 5 min in 4–8M EG to preserve membrane integrity of the oocytes. Furthermore, bleb formation may not be noticed in mouse 8-cell embryos because of the smaller size of their blastomeres, but exposure for 5 min in 7M EG may be sufficient cause for damaged membrane integrity of the cells to affect their survival at vitrification temperatures.

In conclusion, effective vitrification of mouse 8-cell embryos can be achieved by initial exposure to a lower concentration (2M) of EG for 2–10 min, followed by equilibration in 7M EG for another 2 min at  $18\text{--}22^\circ\text{C}$  room temperature. This study has shown that 7M EG in PBS with 10% CS is sufficient to provide cryoprotection of vitrified mouse 8-cell embryos and that exposure of the embryos to the vitrification solution at increased temperatures over  $24^\circ\text{C}$  is critical to their subsequent development *in vitro*.

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