

## The Comparison of One and Two Steps Equilibration in Vitrification Process on The Morphology and Viability of Mouse Blastocysts

(PERBANDINGAN EKUILIBRASI SATU DAN DUA TAHAP PADA PROSES VITRIFIKASI  
TERHADAP MORFOLOGI DAN VIABILITAS BLASTOSIS MENCIT)

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### ABSTRACT

A study was conducted to compare the effect of one and two steps equilibration method of vitrification on the morphology and viability of mouse blastocysts. Blastocysts were firstly exposed to modified Phosphate Buffered saline (mPBS) containing 1% Bovine Serum Albumin (BSA) proceeded by exposure in mPBS respectively containing 0.25M sucrose (S) for 2 minutes. Blastocysts were then exposed for 2 minutes respectively to mPBS+0.5M S (one step method) or in mPBS+0.5M S+10% ethylene glycol (EG) (two step method). Blastocysts were then exposed in mPBS+0.5M S+30% EG for 60 second, loaded into 0.25 ml plastic straw, and exposed immediately in vapor of liquid nitrogen for 10 second before they were and then plunged into liquid nitrogen. The blastocysts were reconstituted by diluting with mPBS+0.5M S followed by mPBS+0.25M S for each 3 min and washed in mPBS without sucrose. The viability of cells was assessed by fluorescent vital staining, by re-expansion for 24 hours *in vitro* culture, and by implantation into the recipient oviduct. The percentages of morphologically normal blastocysts following recovery from vitrification were higher ( $p < 0.05$ ) in one step equilibration than in those of two steps methods (89.6% vs 82.6%). The viability of blastocysts examined under light microscope after staining with bis-benzimidazole-propidium iodine and 24 hours *in vitro* culture in one step methods (64.0%; 57.8%) were higher ( $p < 0.05$ ) compared with two steps methods (40.0%; 35.6%), respectively. The implantation rate of vitrified blastocysts (23.1%) was not significantly different to that of fresh blastocysts (33.4%). These results showed that the one and two step equilibration methods are effective for vitrification and maintaining the viability of the mouse blastocysts.

Key words: Morphology, viability, vitrification, blastocysts, mouse

### INTRODUCTION

Cryopreservation is a way of preserving gamete or embryo in liquid nitrogen for long term purposes. Such cryopreservation provides tools to preserve germplasm and to rescue genetic resources which are important in the conservation of the wild animals and in maintaining rare or unusual strains of laboratory animals (Moore and Bonilla, 2006). Embryo cryopreservation has been applied in the preservation of genetic variants of laboratory animals, in breeding of livestock, and in assisting reproduction in humans. As the ability of each embryo to develop into an individual is valuable, it is important to minimize the damage caused by the cryopreservation (Kasai *et al.*, 2002).

Currently, two major cryopreservation methods have widely used in storing embryos in liquid nitrogen, the conventional freezing and vitrification methods. However, vitrification which is defined as solidification of liquid by dehydration and an extreme elevation in viscosity during very rapid cooling (Rall and Fahy, 1985; Liebermann *et al.*, 2003) is preferred for its simplicity and rapidity without requiring expensive equipment (Vajta, 1997).

In vitrification process, the rapid solidification of liquid can have potential detrimental effect on extracellular and intracellular components of embryos. In the transition state of solid formation, the components of vitrification solution is important (Kasai and Mukaida, 2004). The presence of a

high concentration of cryoprotectant in vitrification solution is required to improve the survival rate of embryos. However, the cryoprotectant itself can damage the embryo caused by the osmotic stress and/ or chemical toxicity (Rall and Fahy, 1985). To prevent such cell injury, the toxic effects and osmotic stress caused cryoprotectant must be reduced.

An attempt to reduce the cryoprotectant toxicity was conducted by using ethylene glycol which is considered less toxic than the others (Valdez et al, 1992). Ethylene glycol as a single intracellular cryoprotectant was combined with sucrose as the extracellular cryoprotectant. Vitrification was achieved by stepwise dehydration in the solution containing increasing concentration of sucrose and two steps equilibration in the vitrification solution. The effect of one and two steps equilibration in the vitrification solution on the morphology and viability of the mouse blastocysts was examined.

## METHODS

### Blastocysts Collection

Female Balb/c mice (2-3 month-old) were superovulated by intraperitoneal injection of pregnant mare's serum gonadotropin (PMSG) at the dose of 5 IU per mouse and human chorionic gonadotropin (HCG) at the dose of 5 IU per mouse at 48 hour interval (Hogan *et al.*, 1994). Each female mouse was mated by a male from the same strain (single mating). In the following morning, its vaginal plug was examined for copulation sign. Animal was sacrificed by cervical dislocation and its uterine tubes were dissected out and placed in petridishes containing modified phosphate buffered saline (mPBS), supplemented with 0.3% bovine serum albumin (BSA) (Djuwita *et al.*, 2009). The blastocysts were collected at 96-98 hours following hCG injection by flushing the uterus and washing it three times in mPBS. Only blastocyst with an intact inner cell mass and trophectoderm with blastocoel no greater than half of the volume of embryo was used in this experiment.

### Vitrification and Warming

Vitrification solution consisted of ethylene glycol (EG) in mPBS supplemented with 1% BSA and 0.5M sucrose (S) (Djuwita *et al.*, 2005) was used. Blastocysts were pretreated by exposing them in mPBS+0.25M S and mPBS+0.5M S for 3 minutes each in one step equilibration and in

mPBS+0.25M S and mPBS+0.5M S + 10% EG for 3 minutes each in two steps equilibration. Blastocysts were then equilibrated in vitrification solution i.e: (a) mPBS+0.5M S+30% EG for 60 sec (one step equilibration method) or (b) in mPBS+0.5M S+10% EG followed by mPBS+0.5M S+30% EG for 60 sec each (two steps equilibration method). Blastocysts were loaded into 0.25 ml plastic straw each of which was exposed immediately for 10 seconds to the vapour of liquid nitrogen and then plunged into liquid nitrogen. Blastocysts were warmed by holding in air for 10 sec and then directly immersed into a water bath at 30°C. Blastocysts were diluted with mPBS+ 0.5M S followed by mPBS+0.25M S for 3 min each and washed three times with mPBS three times and three times with tissue culture medium (TCM) 199. The blastocysts were resuspended in TCM 199 and incubated in 5% CO<sub>2</sub> incubator at 37°C.

### Blastocyst Morphology and Viability Assessment

**Morphology Assessment.** The blastocyst morphology was examined for abnormality under light microscopes after vitrification and warming processes. Normal blastocyst was characterized by the presence of intact plasma membrane and zona pelucida, and homogenous cytoplasmic blastomeres. Abnormality of blastocysts were characterized by fracture of zona pelucida and degeneration or lysis of cytoplasm (Kaidi *et al.*, 2001).

**Viability Assessment.** The viability of blastocysts was determined based on the proportion of live and dead blastocysts; and their re-expansion and implantation rates.

**Live and dead of blastocysts.** After vitrification and warming steps, blastocysts were evaluated by differential fluorochrome staining (Bis-benzimidize-propidium iodine). In these steps, blastocysts were washed with mPBS (supplemented with 0.3% BSA) and they were stained for live and death cells by incubating them in 100ml of mPBS containing bizbenzimidize (Hoechst incubateing them in propidium iodine (10 mg/ml) for 30 minutes. The cells were examined under the fluorescence microscope. The presences of live and dead cells were characterized respectively by blue and pink nuclei. (Kaidi *et al.*, 2001)

**Blastocyst re-expansion rate.** Vitrified blastocysts were reconstituted at 37°C and cultured in TCM 199 supplemented with 50 µg/ml gentamicin and 20% *New Born Calf Serum*

(NBCS) for 24 h in 5% CO<sub>2</sub> incubator. Re-expanded blastocysts were examined and counted under microscope.

**Embryo implantation rate.** The re-expanded blastocysts following vitrification were transferred into the oviduct of pseudopregnant females on day-4 after the females were mated with the vasectomized male. Each female received 4 blastocysts. A week after transfer, the recipients were examined for the implantation rate (Hogan *et al.*, 1994).

**Data Analysis**

The data obtained from this study were analyzed using one way Analysis of Variance (ANOVA) proceeded by Duncan’s multiple range test. Statistical significance was established at the P<0.05 level. The statistical analysis was performed on the SPSS 12.0 program.

**RESULTS AND DISCUSSION**

Injuries caused by vitrification of cells can be identified by morphological (physical) abnormality of cells. For example cell fracture can be easily identified by the physical dissection of the zona pellucida of the embryos.

The percentage of morphologically normal blastocysts in one step equilibration method of vitrification (89.6%) was higher (P<0.05) than in two steps method ( 82.6%)(Table 1) (Fig 1).

Zona pellucidal fracture and cell degeneration was detected after warming and dilution of blastocysts, especially in the two steps method.

By bis benzimidine propidium iodine staining, it was shown that the viability of vitrified blastocysts in the one step equilibration following reconstitution was higher (64.0%) than that in the two steps method (57.8%) (P<0.05) (Table 2). By re-expansion methods following 24 hours *in vitro* culture, the blastocyst viability declined in both methods. However, the blastocysts viability based on the percentage of re-expanding blastocysts in the one step equilibration method (40.0%) was still higher than that in the two steps method (35.6%) (P<0.05) (Table 3). when transferred to the recipient into, the implantation rate of vitrified blastocysts in the one step method was not significantly different (P>0.05) to that of the fresh unvitrified blastocysts, indicating that in the one step vitrification the blastocyst viability was maintained until implanted into the recipient oviduct.

The success of cryopreservation by vitrification process depends on the concentration of cryoprotectan in the solution to prevent intracellular and extracellular ice formation. Thus, toxicity of the vitrification solution and the osmotic swelling are the major obstacles in the vitrification process. The use of least toxic cryoprotectant such as ethylene glycol and stepwise dehydration and equilibration in the

Table 1. The morphology of mouse blastocysts after vitrification

| Method of vitrification | No. of blastocysts | Morphology of blastocysts (%) |               |                             |                        |
|-------------------------|--------------------|-------------------------------|---------------|-----------------------------|------------------------|
|                         |                    | Normal                        | Zona fracture | Abnormal Degenerate / lysis | Total                  |
| One step                | 106                | 95 (89.6) <sup>a</sup>        | 4 (3.8)       | 7 (6.6)                     | 11 (10.4) <sup>b</sup> |
| Two steps               | 115                | 95 (82.6) <sup>b</sup>        | 5 (4.3)       | 15 (13.0)                   | 20 (17.4) <sup>a</sup> |

Data from 5 replication. <sup>a-b</sup> Values with different letter are significantly different (P<0.05)

Table 2. The viability of vitrified mouse blastocysts after examined by bis-benzimidize-propidium iodine

| Method of vitrification | No. of blastocysts | Viability of blastocysts (%) |           |
|-------------------------|--------------------|------------------------------|-----------|
|                         |                    | Live                         | Dead      |
| One step                | 50                 | 32 (64.0) <sup>a</sup>       | 18 (36.0) |
| Two steps               | 50                 | 20 (40.0) <sup>b</sup>       | 30 (60.0) |

Data from 3 replication. <sup>a-b</sup> Values with different letter are significantly different (P<0.05)

Table 3. The viability of vitrified mouse blastocysts after cultured *in vitro* for 24 h (re-expansion) and transferred into oviduct of the pseudopregnant recipients

| Method of vitrification | No. of blastocysts | No. of viable blastocysts (%) |                   |
|-------------------------|--------------------|-------------------------------|-------------------|
|                         |                    | Expansion rate                | Implantation rate |
| One step                | 45                 | 26 (57.8) <sup>b</sup>        | 6/26 (23.1)       |
| Two steps               | 45                 | 16 (35.6) <sup>c</sup>        | NI                |
| Fresh (Control)         | 75                 | 72 (96.0) <sup>a</sup>        | 9/27 (33.4)       |

Data from 3 replication. <sup>a-b</sup> Values with different letter are significantly different ( $P < 0.05$ ). BI = Not identified.

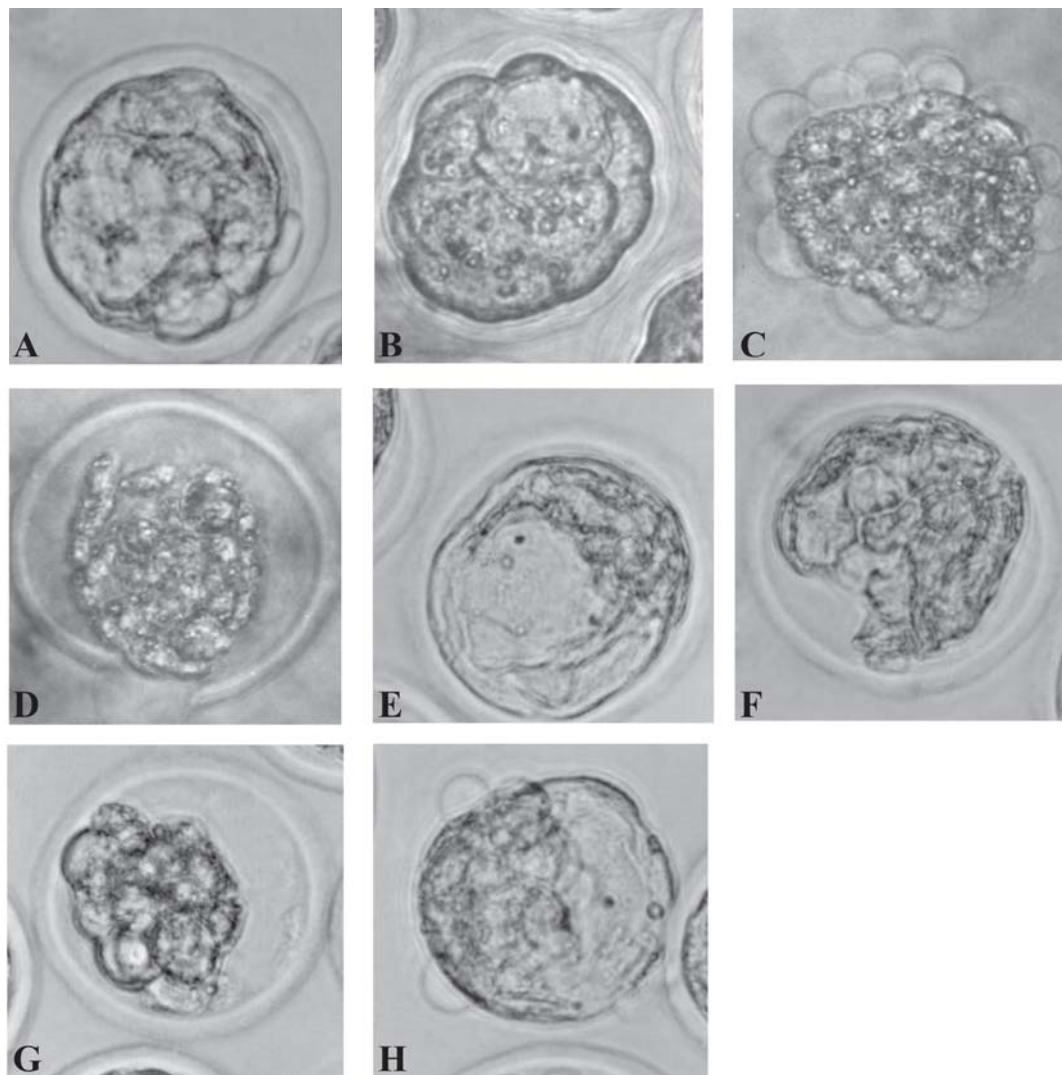


Figure 1. A. Mouse blastocyst before vitrification; B-H. Mouse blastocysts after vitrification; B. Normal blastocyst; C-G. Abnormal mouse blastocysts; C. Blastocyst cytoplasm protrusion; D. Zona pellucida fracture with cell degeneration; E-F. Blastocyst shrinkage; G-H. Blastocysts shrinkage with some degenerated cells. Bar scale: 27  $\mu$ m.

vitrification solution appeared to be able to maintain the viability of blastocysts following vetrification process. Ethylene glycol has been reported as a permeating compound that play important role in stabilizing the cellular membrane during freezing (Kasai *et al.*, 1990). However, after warming and dilution in decreasing sucrose solutions, some morphological defects of the vitrified blastocysts such as zona pellucida fracture, and cell degeneration was still detected in both methods.

As reported by Kasai *et al.* (2002, 2003), the cause of such damage appeared to be the formation of intracellular ice in the blastocoel cavity which reduces the post-warming survival of blastocysts. The ice formation is likely to be due to insufficient permeation of EG (30%) , shorter exposure, and low temperature. (Zhu *et al.*, 1993). Ethylene glycol has also been reported to be a very weak glass formation as compared to dimethyl sulfoxide (DMSO) (Valdez *et al.*, 1992). The use of 30% EG in this research appears to be too low as compared to the previous study which used 40% EG and Ficoll as the vitrification solution (Zhu *et al.*, 1993; Kasai *et al.*, 2002).

The percentages of life blastocysts following *in vitro* re-expansion were also lower than the percentages of the morphologically normal blastocysts, indicating the presence of damaged cells undetected by light microscope examination. This can be due to toxicity injury caused by the cryoprotectant which has also been reported in the previous study of Kasai *et al.* (2002).

During dilution, the damage of the cells caused by the osmotic changes is often more severe than that caused freezing and the high concentrations of the cryoprotectant which is toxic to the embryonic cells (Kuwayama *et al.*, 1994). Meanwhile after vitrification the damage of embryonic cells includes a decrease in numbers of microvilli in trophectoderm cells, loss of plasma membrane integrity, mitochondrial changes and swelling of the rough endoplasmic reticulum, formation of small vesicles, and distinct intra membrane particle aggregations in the plasma membranes (Vajta *et al.*, 1997, Ohboshi *et al.*, 1998, Kaidi *et al.*, 1999). Most of such embryonic damage cells are usually not detected by examination under the light microscope.

Some of morphologically normal blastocysts swell, after being transferred into mPBS medium and collapsed after 1 hour culture *in vitro*. The inability of the blastocysts to re-expand after *in vitro* culture is likely to be related to the effect of osmotic swelling during dilution. As reported by Endashige *et al.* (1999), embryos become more sensitive to the osmotic swelling after recovery from cryopreservation. After warming at 37°C, blastocysts were immediately exposed to mPBS containing a decreasing concentration of sucrose for recovery. In this step often causes zonal damage of embryonic cells (Shaw *et al.*, 1997). During dilution, water moved into the cell faster than cryoprotectant moved out from the cell, causing cells to undergo cytoplasmic protrusion and swelling (Fig 1C). However, some of these changes are reversible (Kaidi *et al.*, 1999) after *in vitro* culture, although cell degeneration and death are also occurs, depending on the severity of the damage.

In contrast to the present results, Valdez *et al.*, (1992) reported that both one and two step methods were effective for vitrification of mouse embryos. However, this previous research used mixed cryoprotectants (ethylene glycol, dimethyl sulfoxide and 1,3-butanediol) in both the equilibration and the vitrification solution. Whereas in our study, we used ethylene glycol as single cryoprotectant in both equilibration and vitrification solution.

Toxic injury caused by the cyoprotectant is also usually detectable after *in vitro* culture characterized by the shrinkage of the embryos (Fig. 1F) (Kasai *et al.*, 2002). The timing of exposure to vitrification solution is also important to obtain optimal concentration of intracellular cryoprotectant. However, if the equilibration time is too long, the cryoprotectant itself could be toxic to the cells. In two step method equilibration time of vitrification solution was longer than one step method, so this could cause degeneration of embryonic cells (Fig. 1D).

## CONCLUSION

The one and two steps equilibration method in this research are effective for vitrification and maintaining the viability of mouse blastocysts.

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