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FULL PAPER

In vitro viability of mouse zygotes vitrified in ethylene glycol

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Abstract

A study was made to determine if mouse zygotes can be effectively vitrified in 7 M ethylene glycol in modified Dulbecco's phosphate buffered saline (PB1) and to find out if the development of vitrified-warmed zygotes *in vitro* can be improved by renewing the culture medium. The results showed that without medium change, vitrification reduced the development of zygotes to the expanded blastocyst stage ($p<0.01$). With medium change, the development rate of vitrified-warmed zygotes exposed in 7 M ethylene glycol for 1 or 2 min was similar to that of unvitrified zygotes. However, prolonged exposure (5 min) markedly reduced the development rates of vitrified-warmed zygotes to the expanded blastocyst stage ($p<0.05$). When the zygotes were vitrified in 7 M ethylene glycol and diluted at 18°C to 22°C, a slower efflux of ethylene glycol from the cell might have occurred, leading to a toxic effect of ethylene glycol in culture. The development rates of vitrified embryos cultured with medium change at 24 hr did not significantly differ from the untreated control (89.0% vs 96.5%).

In conclusion, this study showed that mouse zygotes can be vitrified in 7 M ethylene glycol in PB1 and that changing the culture medium can improve the *in vitro* development rates of vitrified-warmed zygotes to the expanded blastocyst stage.

Key words: culture, embryos, ethylene glycol, mouse, vitrification

Introduction

Many vitrification protocols have been developed since the first successful vitrification procedure was reported by Rall and Fahy[13]. Kasai *et al.*[9] developed a vitrification protocol that uses a combination of a permeable cryoprotectant (ethylene glycol), a macromolecule (ficoll) and a sugar (sucrose). This combination results in the formation of stable glass at low temperatures, retaining the normal and ionic distributions of the liquid state and can be considered to be an extremely viscous liquid[12]. Thus, vitrification avoids the potentially detrimental effects of extracellular and intracellular crystallization[2].

Cryoprotective agents (CPAs) are organic solutes that protect cellular organelles during cryopreservation. However, at high concentrations required in vitrification, they are toxic and cause disruptive osmotic damage to the cell[6]. These effects depend on the choice and concentration of the CPA and the temperature of exposure[1]. Toxicity studies are performed as a

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prerequisite to the formulation of new vitrification solutions and provide vital information on the action of CPAs before and after cooling. The determination of the extent of injury to the cells has been studied exhaustively[1,2,4,5,7,8,11].

Several studies[1,3,5,8,9,11] have proven the effectivity of ethylene glycol in the vitrification of embryos and oocytes. Kasai *et al.*[9] obtained excellent results after one-step exposure of embryos to a vitrification medium containing only ethylene glycol as a permeating CPA plus ficoll and sucrose (EFS). Ali and Shelton[1] tested over 6,000 combinations of CPAs and subsequent toxicity tests led to the formulation of three promising vitrification solutions all containing ethylene glycol. In our previous study[3], we used 7 M ethylene glycol in the vitrification of mouse 8-cell embryos and obtained high *in vitro* development rates to the blastocyst stage.

Basically, there are two methods to improve the viability of cryopreserved embryos. The first and probably the most common method is to vary the cryopreservation technique, while the second method deals with the improvement of the viability of the cryopreserved embryos by changes in the culture methods. In the latter, Shamsuddin *et al.*[16] observed a significant effect of *in vitro* culture conditions on the post-thaw viability of bovine blastocysts. Semple *et al.*[15] showed that serum is not essential in the production of embryos that survive cryopreservation. Both groups sought to improve the survival of *in vitro*-produced bovine embryos by changing the media in which the embryos are cultured[10].

Zygotes are used in various biotechnologies, such as nuclear transfer, cryobanking of animal species, *etc.* The cryopreservation of zygotes, therefore, is of significance to the development of these technologies. This study was made to determine if mouse zygotes can be effectively vitrified in 7 M ethylene glycol and to find out if the *in vitro* viability of vitrified-warmed embryos can be improved by renewing the culture medium.

Materials and methods

Collection of mouse zygotes

Female F1 hybrid mice of C57BL/6 X CBA, 4 to 6 weeks old, were induced to superovulate with 5 IU equine chorionic gonadotrophin (Serotrophin, Teikoku Zoki, Tokyo, Japan) and 5 IU human chorionic gonadotrophin (hCG, Gonatrophin, Teikoku Zoki) given intraperitoneally 48 hr apart. After the hCG treatment, the female mice were paired with F1 hybrid males and were inspected the following day for the presence of a vaginal plug. The zygotes were collected from females with vaginal plugs 24 hr after the hCG treatment. Intact cumulus masses containing the zygotes were excised from the oviducts and were placed into a sterile petri dish (Nunc, Nunclon, Kamstrup, Denmark) containing Dulbecco's phosphate-buffered saline supplemented with 0.33 mM sodium pyruvate, 5.56 mM glucose, 3 mg/ml bovine serum albumin and 100 IU/ml penicillin (PB1)[18]. Cumulus cells attached to the zygotes were removed by repeated pipetting, and cumulus-free zygotes were washed three times in fresh PB1. Only morphologically normal zygotes with a pair of pronuclei were pooled in fresh PB1 until used.

Vitrification procedure

The vitrification method used was modified from the method reported by Bautista *et al.*[3]. Briefly, groups of twenty embryos were exposed initially in 100 μl and then in 40 μl drops of 7 M ethylene glycol in PB1 for 1, 2 or 5 min at 18°C to 22°C. Within the assigned exposure period, the zygotes were drawn into 0.25 μl French straws (I.M.V. L'Aigle, France) attached to a tuberculin syringe, which were prefilled with approximately 100 μl of 1 M sucrose solution in PB1, followed by a short column of air (bubble) and approximately 10 μl of 7 M ethylene glycol in PB1. After aspirating the 40 μl drop containing the zygotes, a bubble was added followed by a

short column of 1 M sucrose in PB1. The loaded straws were then heat-sealed and cooled in liquid nitrogen (LN_2) vapor (–170°C) by placing the sealed straw horizontally on a styrofoam plate (140×60×5 mm) with a stainless steel mesh on its upper surface and floating in a LN_2 bath[14]. Two min later, the straw was plunged into LN_2 and stored for 1 to 120 days. The straw was warmed by brief exposure to the air for 10 sec and then in a water bath at 18°C for 20 sec. The contents of the straw were then expelled into a petri dish and kept for 5 min at 18°C to 22°C. When the contents of the straw were emptied into the petri dish, the column of sucrose solution was mixed with the column of the vitrification solution containing the zygotes, thus diluting ethylene glycol out of the cells. Thereafter, the zygotes were transferred to about 4 ml of fresh PB1 kept at 18°C to 22°C to rehydrate for another 5 min. The zygotes were then washed three times in microdrops of Whitten's medium[17] and were cultured for 120 hr.

Evaluation of the viability of zygotes

The viability of all treated and untreated zygotes was assessed by their ability to cleave to the two-cell stage after 24 hr of culture, their development to the blastocyst stage at 96 hr and their development to the expanded/hatching blastocyst stage at 120 hr.

Experimental design

Experiment 1 : The effects of exposure with or without vitrification on the viability of mouse zygotes cultured without medium change

Groups of twenty zygotes were exposed to 7 M ethylene glycol in PB1 for 1, 2 or 5 min with or without vitrification. After dilution in 1 M sucrose solution and rehydration in fresh PB1, the zygotes were cultured for 120 hr without medium change.

Experiment 2 : The effects of exposure with or without vitrification on the viability of mouse zygotes cultured with medium change

Groups of twenty zygotes were exposed in 7 M ethylene glycol in PB1 for 1, 2 or 5 min with or without vitrification. The vitrified and unvitrified zygotes were then cultured for 120 hr with medium change at 24 hr.

Experiment 3 : Comparison of the viability of untreated and vitrified zygotes

After determining the vitrification treatment that gave the best result (2 min exposure period), the viability of vitrified zygotes was compared with that of untreated zygotes. Both groups were cultured with medium change after 24 hr. To count the mean cell number, embryos that developed to the expanded/hatching blastocyst stage after 120 hr were placed in a 1% solution of sodium citrate for 3 min and to methanol : acetic acid : distilled water (5:1:4). The blastocysts were then fixed in methanol : acetic acid (3:1) and were stained with Giemsa stain. The mean cell number of the blastocysts was counted under a light microscope.

Statistical analysis

For experiments 1 and 2, data were analyzed using two-way analysis of variance (ANOVA). When interactions among the treatments were found, the data were analyzed further by one-way ANOVA with Fisher's protected least significant difference as a post hoc test. For experiment 3, a comparison between treatments was made using Student's t-test.

Results

Experiment 1

The period of exposure to 7 M ethylene glycol in PB1 did not affect the ability of the unvitrified zygotes to develop to the two-cell stage after 24 hr of culture (Table 1). However, the development of vitrified zygotes to the two-cell stage was affected by the exposure period. A prolonged (5 min) exposure period resulted in a significantly lower development rate to the two-cell stage than a 2 min exposure before vitrification ($p<0.05$). Zygotes exposed for 2 min before vitrification had a development

Table 1. Viability of mouse zygotes exposed with or without vitrification in 7 M ethylene glycol without medium change*

Treatment	Exposure time (min)	Number examined	% developed to 2-cell (24 hr)	% developed to blastocyst (96 hr)	% developed to expanded blastocyst (120 hr)
Unvitrified	1	80	93.7±7.5[a)]	57.5±18.5[a)]	47.5±27.5[a)]
	2	80	91.2±4.8[a)]	55.0±14.2[a)]	45.0±33.4[a)]
	5	80	90.0±5.8[a)]	40.0±18.7[ab)]	41.2±28.1[a)]
Vitrified	1	80	77.5±9.6[b)]	8.8± 8.5[c)]	12.5±15.0[ab)]
	2	80	90.0±8.2[a)]	20.0±14.7[bc)]	28.8±21.7[ab)]
	5	80	62.5±9.6[c)]	0.0± 0.0[c)]	1.2± 2.5[b)]

Values are mean±SD of 4 replicates.

a), b), c), d) Values with different superscripts within the same columns are significantly different ($p<0.05$).

*All treatment groups were cultured for 120 hr without medium change after 24 hr.

rate to the two-cell stage similar to those exposed without vitrification. Development to the blastocyst stage after 96 hr of culture was affected by vitrification regardless of the exposure period. Vitrified zygotes showed development rates that were significantly lower than those exposed without vitrification ($p<0.0001$). Development to the expanded blastocyst stage at 120 hr was similarly reduced by vitrification and vitrified zygotes showed lower development rates than those of the unvitrified group ($p<0.01$).

Experiment 2

Prolonged exposure (5 min) before vitrification reduced the development of the zygotes to the two-cell stage ($p<0.05$, Table 2). The development rate of the vitrified zygotes exposed to 7 M ethylene glycol for 1 or 2 min did not differ from the unvitrified group. The development rates to the blastocyst and expanded blastocyst stages (96 and 120 hr, respectively) were affected by the exposure period regardless of the treatment (with or without vitrification) used. Zygotes exposed for 5 min had significantly lower development rates than those exposed in 7 M ethylene glycol in 1 or 2 min with or without vitrification ($p<0.005$).

Experiment 3

No significant difference in the development rate to the expanded blastocyst stage after 120 hr

Table 2. Viability of mouse zygotes exposed with or without vitrification in 7 M ethylene glycol with medium change*

Treatment	Exposure time (min)	Number examined	% developed to 2-cell (24 hr)	% developed to blastocyst (96 hr)	% developed to expanded blastocyst (120 hr)
Unvitrified	1	100	96.0± 5.8[a)]	71.0±23.3[a)]	66.0±24.8[a)]
	2	100	98.0± 4.8[a)]	71.0±22.2[a)]	72.0±15.2[a)]
	5	100	93.0±10.9[a)]	58.0±24.6[a)]	53.0±29.9[a)]
Vitrified	1	100	90.0±11.7[a)]	73.0±13.0[a)]	72.0±11.5[a)]
	2	100	97.0± 4.5[a)]	78.0± 9.1[a)]	84.0± 8.2[a)]
	5	100	68.0±19.2[b)]	27.0±25.1[b)]	25.0±24.7[b)]

Values are mean±SD of 5 replicates.

a), b) Values with different superscripts within the same columns differ significantly ($p<0.05$).

*All treatment groups were cultured for 120 hr with medium change after 24 hr.

Table 3. Comparison of viability rates and mean cell numbers of untreated and vitrified zygotes with medium change

Group	Number examined	Number & (%) recovered after vitrification	% developed to expanded-hatching blastocyst / number recovered	Mean cell number in expanded-hatching blastocyst (Number of blastocysts)
Untreated	140	—	96.5± 3.8	84.7±23.8(40)
Vitrified	140	132(94.3±6.1)	89.0±10.7	96.3±29.9(37)

Values are mean±SD of 7 replicates. Vitrified embryos were cooled after 2 min exposure in 7 M ethylene glycol in PB1. Both groups were cultured for 120 hr with medium after 24 hr.

of culture was observed between the treatment groups. Likewise, the mean cell numbers of the untreated and vitrified groups were not different, although the vitrified group had slightly higher mean cell numbers than the untreated control (Table 3).

Discussion

The results of this study show that without medium change, vitrification has a detrimental effect on the development of vitrified-warmed zygotes *in vitro*. With medium change after 24 hr, vitrification had no deleterious effect on the development to the blastocyst and expanded blastocyst stages of zygotes vitrified after 1 or 2 min exposure to 7 M ethylene glycol. Furthermore, even with medium change, zygotes were detrimentally affected by a prolonged (5 min) exposure period before vitrification.

When the zygotes were vitrified in 7 M ethylene glycol and were diluted at 18°C to 22°C after warming, a slower efflux of ethylene glycol from the cell might have occurred[3]. This could have resulted in some ethylene glycol remaining in the embryonic cells, leading to a toxic effect of ethylene glycol in culture. By renewing the culture medium, the embryos are rid of the residual ethylene glycol concentration in the medium enabling the embryos to resume normal development to the later stages. The exact mechanism responsible for the effect of the residual concentration of ethylene glycol on the development *in vitro* of the vitrified zygotes was not determined in this study. Further study is required to confirm this interpretation.

The poor development of vitrified-warmed zygotes exposed for 5 min indicates the toxic effect of ethylene glycol that is magnified by the deleterious effects of vitrification. Even if the depolymerization caused by the CPA can be reversed upon dilution, rehydration and subsequent culture[6] the damage could have been greater than what the cell can repair.

The development rate to the expanded/hatching blastocyst stages of the vitrified zygotes obtained in this study (89.0%) was higher than those previously reported for vitrification with EFS (62.0%) by Miyake *et al.*[11] and quick-freezing with 3 M ethylene glycol with 0.25 M sucrose (68.9%) by Rayos *et al.*[14]. The difference in the development rates between that obtained by Miyake *et al.*[11] and the result obtained in this study could be attributed to the change in the culture medium after 24 hr. In both studies, the zygotes were exposed for 2 min before vitrification, but the result obtained in this study was higher than that after vitrification with EFS. This indicates that the residual action of ethylene glycol has a detrimental effect on the subsequent development of vitrified-warmed zygotes *in vitro*. Furthermore, the lower development rate obtained by Rayos *et al.*[14] could be attributed to the fact that with quick freezing, the probability of intracellular ice crystal formation that is injurious to the cells is greater than with vitrification, hence the difference in the develop-

ment rates. This proves that the vitrification process is a more efficient cryopreservation technique than quick freezing due to the absence of intracellular ice crystal formation at subzero temperatures.

In conclusion, this study showed that mouse zygotes can be vitrified in 7 M ethylene glycol in PB1 and that without medium change, the development of the vitrified-warmed zygotes *in vitro* is affected by vitrification in LN_2. Furthermore, changing the medium after 24 hr improves the development of vitrified-warmed embryos cultured *in vitro*.

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