Jpn. J. Vet. Res. **46**(1): 13–18 (1998)

FULL PAPER

In vitro viability of mouse oocytes vitrified in an ethylene glycol-based solution

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(Accepted for publication: Jan. 14, 1998)

Abstract

Ovulated mouse oocytes denuded of their cumulus cells, were vitrified in a solution containing 7 M ethylene glycol as the sole cryoprotectant using one or two steps of exposure before vitrification and were diluted in 1 M sucrose solution in 5 or 10 min after warming. The results proved that the viability of oocytes are detrimentally affected by exposure to the vitrification solution even without vitrification. At 5 min dilution time, the two-step exposure was superior to the one-step in terms of the post-warming recovery rate of vitrified oocytes with normal morphology and their subsequent development to the blastocyst stage ($p<0.01$) after fertilization *in vitro*. At 10 min dilution time, no significant difference between one- or two-step exposure was found. The effect of the addition of 0.5 M sucrose to the vitrification solution was also determined and did not result in a significant improvement in the viability of oocytes vitrified in one-step and diluted for 10 min.

In conclusion, the results in this study indicate that oocytes can be vitrified with 7 M ethylene glycol as the sole cryoprotectant in the vitrification solution, and that the recovery of normal oocytes after one-step exposure in the vitrification solution can be improved by 10 min dilution time. However, the improvement in the recovery rate of oocytes with normal morphology and their subsequent developmental *in vitro* was not improved by the addition of 0.5 M sucrose to the vitrification solution.

Key words: development, ethylene glycol, mouse, oocyte, sucrose

Introduction

The ability to store some types of mammalian gametes in liquid nitrogen at –196°C without damage or change has prompted the search for more efficient methods of cryopreservation adaptable to a variety of oocytes and embryos from various species[6]. Recent attempts to find a successful cryopreservation technique for oocytes have been directed to the use of vitrification[3,4]. Oocytes are especially difficult to freeze successfully since their meiotic spindles, microtubules and microfilaments may be damaged by cooling and exposure to cryoprotective agents (CPAs)[4,6,9,16].

The cryopreservation of mouse oocytes by vitrification using a mixture of permeable CPAs and sugars has been reported[4,7,8,5,14]. Recently, we used ethylene glycol as the sole CPA in the vitrification solution to determine the possibility

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of simplifying the vitrification technique for mouse 8-cell embryos[1] and zygotes[2]. In the present study, we used 7 M ethylene glycol as the sole CPA in the vitrification solution. In the first experiment, we determined if the one- or two-step method of exposure without vitrification of mouse oocytes was effective. In the second experiment, we tried to modify the dilution technique to determine if a prolonged dilution time improved the survival and subsequent development of oocytes. It has also been reported that the addition of sugars (i.e. sucrose) to the vitrification solution improved the post-warming viability of vitrified oocytes[4,13]. Thus, in the third experiment, we examined the effect of the addition of sucrose to the vitrification solution on the survival and subsequent development of vitrified oocytes.

Materials and methods

Collection of oocytes

Female F1 hybrid mice of C57BL/6J×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. Female mice were sacrificed by cervical dislocation 13.5 hr after the hCG treatment. Cumulus-oocyte complexes were excised from the oviducts and 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 (BSA, Sigma Chemical Co., St. Louis, MO, USA) and 100 IU/ml penicillin (PB1)[18]. Cumulus cells attached to the oocytes were removed by exposing them in PB1 with hyaluronidase (150 U/ml, bovine testis type I-S : Sigma Chemical Co.) for 3 min. Cumulus-free oocytes were washed three times in fresh PB1. Only morphologically normal oocytes were used in the experiments.

Vitrification procedures

The vitrification procedures used were modified from the methods reported by Bautista *et al.*[1]. Briefly, cumulus-free oocytes were exposed in 2 steps to the vitrification solution by exposing them initially to 2 M ethylene glycol in PB1 for 2 min and to 7 M ethylene glycol in PB1 for another 2 min at 18 to 22°C. Oocytes that were exposed in 1 step were directly exposed to 7 M ethylene glycol in PB1 for 2 min. Within 2 min, the exposed oocytes were drawn into 0.25 ml French straws (I.M.V. L'Aigle, France) that 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 oocytes, a bubble was added followed by a short column of 1 M sucrose in PB1, heat-sealed and cooled in liquid nitrogen (LN_2) vapor for at least 1 min before plunging into LN_2. The straws were then stored in LN_2 for 1 to 120 days. The composition of the vitrified straw has been described elsewhere[1].

Warming and dilution procedures

Warming was done according to the method of Bautista *et al.*[2] Thereafter, the oocytes were transferred to about 4 ml of fresh PB1 kept at 18 to 22°C to rehydrate the oocytes for 5 min. All of the oocytes that were recovered were transferred to 30 μl drops of Toyoda, Yokoyama and Hoshi (TYH) medium[15] under paraffin oil and incubated for at least 30 min in an atmosphere of 5% CO_2 in air at 37°C before fertilization *in vitro*.

Fertilization in vitro

All treated and untreated oocytes with normal morphology underwent insemination *in vitro*. Fertilization *in vitro* was performed in insemination drops of 0.4 ml TYH medium under paraffin oil according to the method of Toyoda et al.[15]. Sperm were collected from the cauda epididymides of mature F1 hybrid males of C57 BL6/J× CBA. The sperm were allowed to disperse in 0.4 ml TYH medium under paraffin oil and were

incubated in an atmosphere of 5% CO_2 in air at 37°C for at least 30 min. Thereafter, the sperm concentration was determined using a haemocytometer, and the sperm were incubated for a further 1 to 1.5 hr. A suitable volume (4 to 10 μl) of the sperm suspension providing a final sperm concentration of 150 cells/μl was added to the insemination drops containing the oocytes and were incubated for 4 to 5 hr.

Culture in vitro

After co-incubation with the sperm, the oocytes were washed three times and cultured in 25 μl drops of Whitten's medium[17] under paraffin oil at an atmosphere of 5% CO_2 in air at 37°C for 120 hr.

Determination of the viability of treated and untreated oocytes

The viability of the oocytes was assessed by the number of morphologically normal oocytes recovered after treatment (survival), the number of oocytes that cleaved to the two-cell stage 24 hr after insemination *in vitro* and their development to the expanded/hatching blastocyst stage after 120 hr of culture. The mean cell number of all blastocysts was determined according to the air-dry procedure described elsewhere[2].

Experimental design

Experiment 1: Effect of exposure to ethylene glycol

To determine the effects of exposure to ethylene glycol on the viability of the oocytes, groups of 20 oocytes were exposed to 7 M ethylene glycol in PB1 at 18 to 22°C in one step or in two steps without vitrification. After the required exposure, dilution (5 min) and rehydration (5 min) periods were attained, the exposed oocytes as well as similar groups of untreated oocytes underwent in vitro insemination and culture.

Experiment 2: The effects of different exposure and dilution procedures on the viability of vitrified oocytes

To determine if the viability of oocytes is affected by the exposure and dilution procedures, groups of 20 oocytes were vitrified according to the one- or two-step methods. The dilution of ethylene glycol was carried out in either 5 or 10 min and the oocytes underwent in vitro fertilization and culture.

Experiment 3: The effect of the addition of sucrose to the vitrification solution

To determine if the addition of sucrose can improve the viability of oocytes vitrified in 7 M ethylene glycol in PB1, 0.5 M sucrose was added to the vitrification solution. Groups of 20 oocytes were exposed in one-step for 2 min and then vitrified in 7 M ethylene glycol with or without 0.5 M sucrose. Ethylene glycol was diluted out of the vitrified oocytes for 10 min. The viability of the vitrified oocytes were compared after *in vitro* insemination and culture.

Parthenogenetic activation

Oocytes that were vitrified using either of the one- or two-step method described above were incubated for 4 to 5 hr in 0.4 ml of TYH medium without sperm. Thereafter, the oocytes were cultured in Whitten's medium for 120 hr to determine if spontaneous activation occurs.

Statistical analysis

The data on Experiments 1 and 3 were analyzed by Student's t-test. Those on Experiment 2 were analyzed using two-way analysis of variance with Fisher's protected least significant difference test as a post-hoc test.

Results

Experiment 1 : Effect of exposure to ethylene glycol

High recovery rates of the oocytes with normal morphology were obtained after one- or two-step exposure to 7 M ethylene glycol without vitrification (88.7 and 91.2%) as shown in Table 1. After insemination *in vitro*, no significant difference in the cleavage rate to the two-cell stage was observed among the untreated oocytes and those exposed in one or two steps. Howev-

Table 1. *In vitro* viablity of mouse oocytes exposed to 7 M ethylene glycol in PB1 without vitrification

Group	No. exposed	% recovered with normal morphology	No. inseminated *in vitro*	% cleaved to 2-cell	% developed to blastocyst developed/ inseminated	developed/ exposed
Untreated	–	–	80	75.0±17.8	63.7±11.8[a)]	63.7±11.8[a)]
One-step	80	88.7±2.5	71	67.2±17.6	35.2± 6.9[b)]	31.1± 6.3[b)]
Two-step	80	91.2±4.8	73	65.9±15.9	49.5±12.5[ab)]	45.0± 5.4[b)]

% values are mean±SD of 4 replicates.

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

Oocytes were either exposed in one step to 7 M ethylene glycol in PB1 for 2 min or in two steps to 2 M and 7 M ethylene glycol in PB1 for 2 min each. Dilution was carried out for 5 min in both treatment groups.

er, the proportion of blastocysts per number of oocytes recovered with normal morphology after treatment in one step was lower than that of the untreated oocytes and of those exposed in two steps ($p<0.05$). The proportion of blastocysts per number of oocytes treated in one and two steps was significantly lower than the untreated group ($p<0.01$).

Experiment 2 : The effect of different exposure and dilution procedures on the viability of vitrified oocytes

The recovery rate of the oocytes with normal morphology after vitrification was affected by the dilution procedure regardless of the one- or two-step method. The vitrified-warmed oocytes with 10 min dilution time showed a significantly higher recovery rate than those diluted for 5 min ($p<0.001$) as shown in Table 2. Cleavage to the two-cell stage was not affected by the exposure and dilution procedures (one versus two steps, 5 versus 10 min dilution time). A significant interaction on the development to the blastocyst stage was found between the vitrification procedure and the dilution time. At 5 min dilution time, the two-step method was superior to the one-step method in the development to the blastocyst stage ($p<0.01$). At 10 min dilution time, no significant difference in the development rates to the blastocyst stage was observed between the one- and two-step methods.

Experiment 3 : The effect of the addition of sucrose to the vitrification solution

The addition of 0.5 M sucrose to the vitrification solution did not increase the survival rate of oocytes of normal morphology after vitrification and warming, nor did it contribute to a significant improvement in their development to the blastocyst stage (Table 3).

Parthenogenetic activation

After 120 hr of culture, no parthenogenetic activity was observed in vitrified-warmed

Table 2. Effect of different exposure and dilution procedures on the viability *in vitro* of oocytes vitrified in 7 M ethylene glycol

Exposure procedure	Dilution time (min)	No. vitrified	% recovered with normal morphology	No. inseminated *in vitro*	% cleaved to 2-cell	% developed to blastocyst developed/ inseminated	developed/ vitrified
One-step	5	80	40.0±15.8[a)]	32	52.9± 3.6	2.3± 4.6[a)]	1.2± 2.5[a)]
	10	80	90.0± 4.1[b)]	72	55.3±12.8	28.1±13.2[b)]	25.0±10.8[b)]
Two-step	5	80	36.2±14.4[a)]	29	74.5±18.3	38.1±15.0[b)]	13.7± 6.3[b)]
	10	80	78.7±13.1[b)]	67	58.3±14.9	26.8± 2.2[b)]	21.2± 4.8[b)]

% values are mean±SD of 4 replicates.

a), b) Values with different superscripts within the same column differ significantly ($p<0.01$).

Oocytes vitrified in one step were exposed to 7 M ethylene glycol in PB1 for 2 min while those vitrified in two steps were exposed in 2 M and 7 M ethylene glycol in PB1 for 2 min each.

Table 3. Viability *in vitro* of oocytes vitrified in 7 M ethylene glycol in PB1 with or without 0.5 M sucrose

Sucrose addition	No. vitrified	% recovered with normal morphology	No. inseminated *in vitro*	% cleaved to 2-cell	% developed to blastocyst developed/ inseminated	developed/ vitrified	Mean cell no. in blastocyst
Without	80	81.2±7.5	65	47.7±11.5	30.9±18.5	25.0±14.7	45.6±14.1
With	80	77.7±9.6	62	59.7±20.3	47.1±11.1	36.2± 8.5	48.9± 6.6

% values are mean±SD of 4 replicates.

Oocytes were exposed to the vitrification solution with or without 0.5 M sucrose in one step for 2 min before plunging in liquid nitrogen. Dilution time was 10 min.

oocytes. All oocytes cultured without undergoing insemination *in vitro* degenerated by 72 hr of culture.

Discussion

The results in this study showed that oocytes are affected by exposure to the vitrification solution even without vitrification. Although it is difficult to specify which factor is responsible for this effect, Miyake *et al.*[7] summarized the probable causes as 1) chemical toxicity of the CPA, 2) osmotic shrinkage of the cells, 3) intracellular ice formation and 4) osmotic swelling of the cell during removal of the CPA. Furthermore, there is a possibility that a 5 min dilution time may not have been sufficient to remove all of the ethylene glycol in the cell since a dilution time of 10 min improved the survival rate of vitrified oocytes in Experiment 2. Hotamisligil *et al.*[4] reported that 90 to 100% of the recovered oocytes after vitrification in 6 to 8 M ethylene glycol in Hepes-buffered human tubal fluid (HTF) medium were lysed after warming, while Miyake *et al.*[7] did not recover any oocyte of normal morphology after vitrification in 40% (7.1 M) ethylene glycol with ficoll and sucrose. In both studies, the dilution of the CPA was performed for only 5 min in 1 M sucrose solution. A 5 min dilution time may have resulted in the insufficient removal of the intracellular ethylene glycol and this could have led to a detrimental effect on the recovery of oocytes with normal morphology after warming. Furthermore, the harmful effects of the rapid or excessive swelling of the cells could have occurred after a 5 min dilution time. A 10 min dilution time could have resulted in the significant removal of the intracellular ethylene glycol and was beneficial to the cells. However, in the present study, we only compared 5 and 10 min dilution time. Further experiments are needed to optimize the dilution period.

Although Hotamisligil *et al.*[4] indicated that 0.5 M sucrose can be effective in protecting oocytes from the deleterious effects of 6 and 8 M ethylene glycol concentrations, no beneficial effect on the post-warming viability of vitrified oocytes was observed with the addition of 0.5 M sucrose to 7 M ethylene glycol in Experiment 3. Without sucrose, a high recovery rate (81.2%) of oocytes with normal morphology was obtained with 7 M ethylene glycol in contrast to 0 to 10% for 6 and 8 M ethylene glycol by Hotamisligil *et al.*[4]. With the addition of 0.5 M sucrose, 77.7% recovery rate of oocytes with normal morphology was obtained with 7 M ethylene glycol in Experiment 3, which was definitely lower than the 90% recovery rate with 6 M ethylene glycol reported by Hotamisligil *et al.*[4]. However, the development rate to the blastocyst stage in this study (47.1%) was similar to the value (46.6%) that they obtained. Although differences in the concentration of ethylene glycol (7 versus 6 and 8 M), the basal medium of the vitrification solution (PB1 versus Hepes HTF) and the dilution procedures in 1 M sucrose solution (10 versus 5 min) exist between this study and that by Hotamisligil *et al.*[4], we have reason to believe that our

technique with 0.5 M sucrose added to the vitrification solution is comparable to theirs. Furthermore, we were able to achieve higher rates of recovery of oocytes with normal morphology (81.2% versus 0 to 10%) and subsequent development to the blastocyst stage (25.0% versus 0%) without adding 0.5 M sucrose to the vitrification solution.

In conclusion, the results in this study indicate that mouse oocytes can be vitrified with 7 M ethylene glycol as the sole CPA in the vitrification solution and that the technique of vitrification of oocytes can be simplified using the one-step procedure with 10 min dilution time.

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