

Vitrification solution without sucrose for cryopreservation in mouse blastocysts

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Objective: This study was designed to investigate the survival rate of vitrified mouse blastocysts depending on the presence or absence of sucrose in vitrification solution.

Methods: Mouse two-cell embryos were collected and cultured to blastocysts. Two vitrification solutions were prepared. The control solution was composed of 25% glycerol, 25% ethylene glycol, and 0.5 M sucrose (G25E250.5S) containing 2.5 mL glycerol, 2.5 mL ethylene glycol, 2 mL SSS, and 0.855 g sucrose in 5 mL PB1. The experimental solution was composed of 25% glycerol and 25% ethylene glycol (G25E25) and contained 2.5 mL glycerol and 2.5 mL ethylene glycol in 5 mL PB1. Artificial shrinkage was conducted by aspirating the blastocoelic fluid using an ICSI pipette. To examine the effect of sucrose in the vitrification solution on the survival rate of mouse blastocysts, the shrunken-equilibrated blastocysts were rehydrated or vitrified after being exposed to one of the two vitrification solutions. After exposure and the vitrification-thawing process, the re-expansion rate and hatching rate were evaluated after 6 hours of *in vitro* culture.

Results: The re-expansion rate of mouse blastocysts exposed to vitrification solution with and without sucrose were not different in the experimental solution (without sucrose) (98%) and the control solution (with sucrose) (92%) ($p > 0.05$). The hatching rate was higher in the experimental solution (95%) than in the control solution (88%), but did not differ across two treatments ($p > 0.05$). The re-expansion rate of mouse blastocysts vitrified in the control solution was 92% and 94%, respectively ($p > 0.05$), and the hatching rate was higher in the experimental solution (90%) than in the control solution (74%) ($p < 0.05$).

Conclusion: Sucrose need not be added in vitrification solution for freezing of artificially shrunken mouse blastocysts.

Keywords: Artificial shrinkage; Blastocyst; Sucrose; Vitrification

Introduction

The increased speed of developing embryos to the blastocyst stage through the development of continuous culture media and improved culture conditions has resulted in the transfer of blastocysts on day 5 after ovum retrieval instead of an 8-cell embryo on day 3 [1,2]. Further-

more, because cryopreservation of surplus blastocysts can reduce the burden on infertile patients and increase the chances of pregnancy, research has focused on improving the survival rate of the vitrified blastocysts after thawing [3-6].

Slow freezing was once used for blastocyst cryopreservation [7]. During slow freezing, crystallization occurs abundantly, with resulting relatively low blastocyst preservation. To overcome these problems, vitrification is now applied to freeze blastocysts [8-15].

Cohen et al. [7] first reported the use of slow freezing cryopreservation of human blastocysts. Vitrification for human blastocysts was introduced by Vanderzwalmen et al. [12] in 1992. Initially, the survival rate of frozen-thawed human blastocysts was also very disappointing. One contributing factor was intracellular and extracellular ice crystal formation due to incomplete dehydration [11,16,17]. Another factor was osmotic injury caused by the high concentration of vitrifi-

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cation solution and the long equilibration time for dehydration, due to the relatively huge volume of the blastocoele cavity [11,16,17]. These two factors decreased the survival rate after thawing. Lastly, the *in vitro* culture and freezing process can produce zona hardening, which lowers the hatching rate after thawing [11,18,19].

To overcome these problems, artificial shrinkage, assisted hatching, and special containers have been developed. Artificial shrinkage is a method in which the fluid of the blastocoele is aspirated artificially using a micro-pipette, 29-gauge needle, hand-drawn Pasteur pipette, or micro-needle [20-23]. Assisted hatching has been conducted by laser or mechanically using a micro-pipette. After assisted hatching, the embryos are contained by specialized containers such as the Hemi-straw, EM-grid, cryotop, or cryoloop [24-27]. These refinements have improved the survival rate of the vitrified blastocysts and the pregnancy rate.

Sucrose can be included in the vitrification solution to dehydrate free water and protect the cell membrane. However, blastocoele fluid has already been aspirated from an artificially shrunken blastocyst, and the tightly condensed condition of an artificially shrunken blastocyst is very similar to a somatic cell. Therefore, sucrose for dehydration may not need to be added to the vitrification solution used for artificially shrunken blastocysts.

This study was conducted to evaluate the survival rate of vitrified mouse blastocysts depending on the addition or omission of sucrose from the vitrification solution.

Methods

1. Animals

Five-week-old female mice (C57BL/CBA) were superovulated by an intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG) followed 48 hours later by 5 IU of human chorionic gonadotropin (hCG, Sigma-Aldrich, St. Louis, MO, USA) and immediately paired with males of the same strain. On the following morning, mating was confirmed by checking for a vaginal plug. Forty-eight hours after hCG injection, two-cell embryos were collected and cultured in groups of 10 in 30 µL drops of medium under mineral oil. All embryos were cultured to the 8-cell stage in G1.1 culture medium (Vitrolife, Goteborg, Sweden) and to the blastocyst stage in G2.2 culture medium (Vitrolife).

2. Equilibration, vitrification, and thawing solutions

Equilibration solutions consisted of EBS1 (10% glycerol) and EBS2 (10% glycerol+20% ethylene glycol). EBS1 contained 1 mL glycerol, 2 mL serum substitute supplement (SSS, Irvine, Irvine, CA, USA), and 7 mL phosphate buffer 1 (PB1, Gibco, San Diego, CA, USA). EBS2 contained 1 mL glycerol, 2 mL ethylene glycol, 2 mL SSS, and 5 mL PB1. For vitrification, two different solutions were prepared. The control solution was composed of 25% glycerol, 25% ethylene glycol 25%, and

0.5 M sucrose and contained 2.5 mL glycerol, 2.5 mL ethylene glycol, 2 mL SSS, and 0.855 g sucrose in 5 mL PB1. The experimental solution was composed of 25% glycerol and 25% ethylene glycol and contained 2.5 mL glycerol and 2.5 mL ethylene glycol in 5 mL PB1. The thawing solution was composed of sucrose solution (0.5 M, 0.25 M, and 0.125 M), PB1, and 20% SSS.

3. Artificial shrinkage of expanding blastocysts and assisted hatching

The blastocysts were fixed with a holding pipette after turning the inner cell mass (ICM) to 6 or 12 o'clock. Then, an intracytoplasmic sperm injection pipette was inserted into the blastocoele cavity and about 70% to 80% of the blastocoele fluid was aspirated (Figure 1). After artificial shrinkage, the shrunken blastocyst was equilibrated in 10% glycerol and 10% glycerol+20% ethylene glycol solution for 3 minutes at room temperature, in sequence, and transferred to the vitrification solution. After 10 seconds, the blastocysts of the control group were re-equilibrated and the blastocysts of the experimental group were loaded in a capped-pulled straw and frozen [28]. After seven days of cryopreservation, for the thawing process, blastocysts were rehydrated with 0.5 M, 0.25 M, and 0.125 M sucrose for 3 minutes at

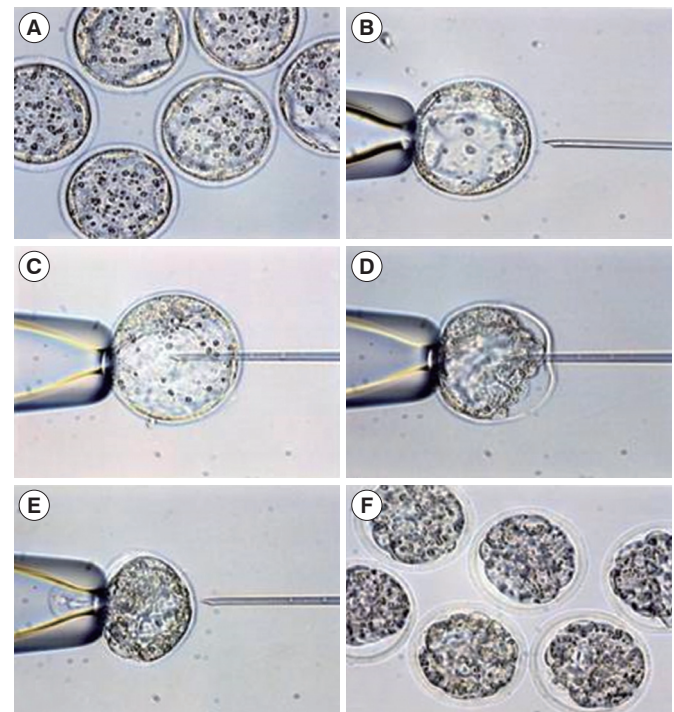


Figure 1. Stepwise photographs of artificial shrinkage ($\times 200$). (A) Expanding mouse blastocyst before vitrification. (B) Retention of an expanding mouse blastocyst with a holding pipette. (C) Insertion of an intracytoplasmic sperm injection pipette inside the blastocoele cavity. (D) Aspiration of blastocoele fluid. (E) Removal of pipette after aspiration of blastocoele fluid. (F) Artificially shrunken mouse blastocysts.

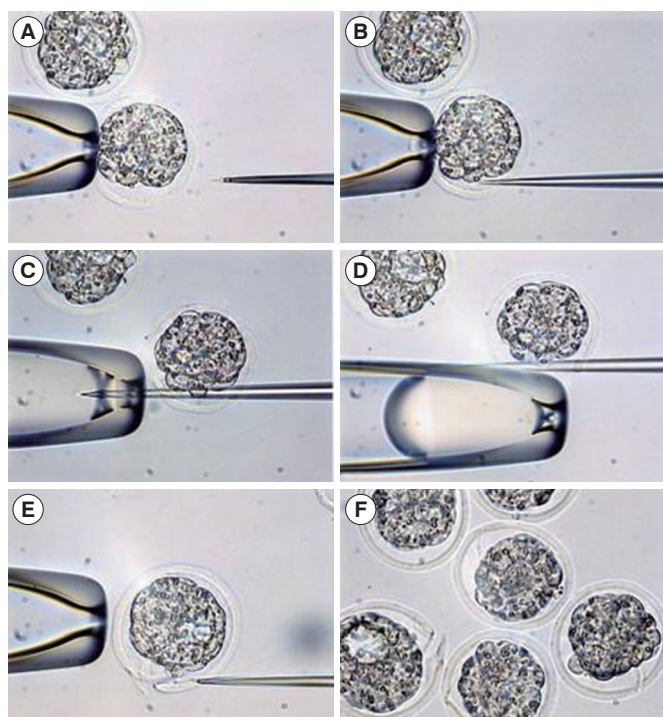


Figure 2. (A–F) Photographs of the assisted hatching procedure of an expanding mouse blastocyst with micropipette after thawing ($\times 200$).

room temperature, in sequence and rinsed with PB1 3 times. Assisted hatching was then performed (partial zona dissection). After fixing the embryo using a holding pipette, a hand-made hatching pipette was inserted in the perivitelline space and penetrated opposite the zona pellucida. The zona was split with holding and assisted hatching pipettes (partial zona dissection, Figure 2). The re-expansion rate and hatching rate were evaluated 6 hours after re-hydration or thawing. These procedures were repeated 5 times. In each experiment, 10 blastocysts of each group were observed. The results were analyzed with the Student's *t*-test.

Results

After the equilibration of the artificially shrunken blastocysts, they were exposed to different vitrification solutions. Ten seconds later, re-hydration was conducted without freezing. The aim of this experiment was to determine whether the absence of sucrose in the vitrification solution was harmful to the shrunken blastocysts. After 6 hours of culture, the re-expansion rate and hatching rate of the blastocysts exposed to vitrification solution with sucrose (control) were 92% and 88%, respectively. The re-expansion rate and hatching rate of the blastocysts exposed to vitrification solution without sucrose (experiment) were 98% and 95%, respectively. There was no statistically significant difference (Table 1).

Table 1. Re-expansion and hatching rates of mouse blastocysts exposed to vitrification solution without sucrose and with 0.5 mol sucrose after artificial shrinkage and assisted hatching

	Vitrification solution		<i>p</i> -value
	G25E25S0.5 (control)	G25E25 (experiment)	
No. of exposed blastocysts	50	50	-
No. of re-expanded blastocysts	46 (92%)	49 (98%)	NS
No. of hatching blastocysts	44 (88%)	47 (95%)	NS

NS, not significant (p -value > 0.05).

Table 2. Re-expansion and hatching rates of mouse blastocysts vitrified in vitrification solution without sucrose and with 0.5 mol sucrose after artificial shrinkage and assisted hatching

	Vitrification solution		<i>p</i> -value
	G25E25S0.5 (control)	G25E25 (experiment)	
No. of exposed blastocysts	50	50	-
No. of re-expanded blastocysts	46 (92%)	47 (94%)	NS
No. of hatching blastocysts	37 (74%)	45 (90%)	0.037

NS, not significant (p -value > 0.05).

The re-expansion rate and hatching rate of the vitrified blastocysts were 92% and 74% in the presence of sucrose (control), and 94% and 90% in the absence of sucrose (experiment), respectively. There was a statistically significant difference in the hatching rate between the two groups ($p = 0.037$) (Table 2).

Discussion

Sucrose is a cryoprotectant and is used for several purposes during the freezing process. It is used for intracellular water removal to prevent ice crystal formation and also to protect the cell membrane and cytoplasm when the influx of highly concentrated permeating cryoprotectants into the cytoplasm occurs rapidly [29-33].

Sucrose is also used during the thawing process. While thawing in sucrose solution, the removal of the cryoprotectant and rehydration in cytoplasm can make the shrunken blastocysts re-expand [34-38].

The survival rate and hatching rate after thawing are affected by several factors such as ice crystal formation, shifting of blastocoelic fluid, and zona hardening. During the vitrification of blastocysts, if insufficient blastocoelic fluid is released, ice crystal formation inside the blastocoeles would occur extensively and the blastocysts would be severely damaged [11,16,17]. Also, during dehydration, the trophoblast cell might be damaged by the shifting of a large amount of water [11,16,17]. Zona hardening that occurs during *in vitro* culture and the freezing procedure affects the hatching rate [18,19].

Recently, to overcome low survival and hatching rates, several stud-

ies have been conducted. Martino et al. [24] attempted to use ultra-rapid cooling for cryopreservation. Researchers have also tried to use variations of the vitrification method such as the use of a hemi-straw carrier [26] or containerless vitrification [25]. Other studies have suggested that artificial shrinkage could increase the survival rate by preventing ice crystal formation [20-23].

Usually, the vitrification solution contains 30% to 40% of the permeating cryoprotectant and a non-permeating protectant such as Ficoll and sucrose. However, in this study, the vitrification solution contained 50% of the permeating cryoprotectant and Ficoll was not added because ice formation did not occur with higher concentrations of the permeating cryoprotectant. Sucrose, which is used for dehydration, also was not added to the vitrification solution in this study, because the blastocysts were already shrunken artificially.

Similar vitrification solutions, that is, those without sucrose, have been used for the vitrification of somatic cells such as stem cells. Because the cell size is relatively small and the amount of water in the cytoplasm is small in stem cells or somatic cells compared to an embryo, the cell membrane was not significantly damaged by dehydration.

The blastocyst is composed of the trophoectoderm and ICM. The shrunken blastocyst may be morphologically similar to somatic cells. Therefore, we hypothesized that sucrose may not need to be added to the vitrification solution in the case of shrunken blastocysts. The results of this study suggest that this hypothesis may be correct.

Additionally, sucrose may react more efficiently in the thawing process. During the thawing process, the blastocyst is first exposed to sucrose solution, and this exposure can make rehydration more effective.

In this study, the re-expansion rate was not statistically different in either group, but was higher in the group that was exposed to vitrification solution without sucrose. However, the hatching rate was significantly higher in the group exposed to vitrification solution without sucrose ($p=0.037$). This result suggests that blastocysts in vitrification solution without sucrose react more sensitively to a sucrose solution in the thawing process.

In conclusion, sucrose has been used for several decades in the cryopreservation of embryos, but when the artificial shrinkage method was used, the survival rate of mouse blastocysts was higher in the absence of sucrose than in the presence of sucrose [39]. In our clinic, we have used vitrification solution without sucrose in the cryopreservation of human blastocysts, and the outcomes are better than when vitrification solution with sucrose is used. Therefore, we think that further research should be carried out on effects of sucrose on the survival rate of the vitrified blastocysts.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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