A Comparison between Conventional Slow Freezing and Vitrification of Mouse Blastocysts using Cryo-E

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ABSTRACT

Objective: To compare the efficiency of two cryopreservations between conventional slow freezing and vitrification of mouse blastocysts using cryo-E.

Methods: ICR female mice (8 weeks) were superovulated with 5 IU/ml of pregnant mare serum gonadotrophin (PMSG), the successfulness of mating with males was verified by the presence of a vaginal plug. Blastocysts were obtained between 3.5 and 4.5 days per p.c. or 96-108 hours after hCG administration by flushing the uterus. Randomly selected blastocysts were simultaneously frozen by slow-rate freezing and a vitrification method. One month later, the embryos were thawed and cultured in the blastocyst medium (COOK; Sydney IVF, Australia).

Results: Based on 250 slow freezing and 310 vitrified mouse blastocysts, vitrification resulted in a slightly higher survival and hatching rates than the slow-freezing method (83.9% VS 82.0%, and 68.8% VS 66.8%, respectively).

Conclusion: Both slow freezing and vitrification of mouse blastocysts are useful methods for cryopreservation. These results showed that vitrification is better than slow freezing in terms of simplicity, duration, and cost-effectiveness.

Keywords: Blastocyst, cryo E, slow freezing, vitrification

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Cryopreservation involves the long term preservation of the preimplantation embryos of mammals with complete suspension of metabolism and development by freezing them at extremely low temperatures (liquid nitrogen: -196°C).[1,2] In the field of human assisted reproductive technology, the experimental system that includes maintenance and preservation of various stage embryos has developed such as two cells-four cells by slow freezing.[3] The first successful cryopreservation of a mouse embryo was reported by Whittingham et al., in 1972.[4,5] The slow freezing and thawing method which they used showed very high survival rates and excellent reproducibility and it is still widely used at present.[6-8] However, since this cryopreservation method required a computerized cryofreezer with a long time for freezing, the development and improvement of methods that were faster and simpler to perform continued.

Vitrification, known as the rapid freezing was developed by Rall and Fahy et al. in 1985.[9] In this approach, damaging ice crystal formation, are prevented by being vitrified to achieve a glass like solid state by short term exposure to a high concentration of cryoprotectant and then plunged directly into liquid nitrogen.[10-12]

Many studies have been undertaken to reduce the time of the freezing procedure and to try to eliminate the cost of expensive, programmable freezing equipment. One way to avoid ice crystallization damage is through the use of vitrification protocols.[10]

To date, however, the cryopreservation methods for freezing and thawing which suitably optimize the promised post-thawing embryo outcome have not been firmly established and there is no standard method yet for cryopreservation of embryos especially of blastocysts.[13,14] In the field of assisted reproductive technology, the embryo at the blastocyst stage may have greater

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implantation potential and result in a high pregnancy rate.[14-17] We will compare the efficiency of two cryopreservations between conventional slow freezing and vitrification of mouse blastocysts using cryo-E (sterilized stainless ear picks).

MATERIALS AND METHODS

ICR female mice (8 weeks) were superovulated with 5 IU/ml of pregnant mare serum gonadotrophin (PMSG) followed 48 hours later by 5 IU of hCG. The successfulness of mating with males was verified by the presence of a vaginal plug. Blastocysts were obtained between 3.5 and 4.5 days per p.c. or 96-108 hours after hCG administration. By cutting below the junction with the oviduct and placing the uterus into flushing media and flush both horns with about 0.2 ml of flushing medium using a 25 gauge needle and 1 ml tuberculin syringe. Randomly selected blastocysts were simultaneously frozen by slow-rate freezing and the vitrification method. One month later, the embryos were thawed and cultured in the blastocysts medium (COOK; Sydney IVF, Australia).

Slow freezing

Slow freezing was undertaken by conventional methods.[6,18] A 10-min equilibration in a 5% glycerol solution in HEPES-buffered EBSS media (Medicult, Denmark) was supplemented with 10% fetal bovine serum (Gibco, Invitrogen, USA) and was followed by a 10-minute equilibration in the same media with final concentrations of 9% glycerol and 0.2 mol/l sucrose. A Planer controlled rate freezer (Planer Kryo; T.S. Scientific, Perkasie, PA, USA) and 0.25 ml French straws (Cryo Bio System, l'Aigle, France) were used. The blastocysts were cooled from room temperature to $-7^{\circ}C$ at $-2^{\circ}C$/min. Manual seeding was performed, and this seeding temperature was maintained for 3 min. The freezer was subsequently cooled from $-7^{\circ}C$ to $-30^{\circ}C$ at $-0.3^{\circ}C$/min. At this point, the temperature was dropped at $-50^{\circ}C$/min to $-150^{\circ}C$, followed by immediately plunging the straw containing the blastocyst into liquid nitrogen for storage. This entire process required approximately 2.5 hours to finish. Thawing was accomplished by a two-step removal of the cryoprotectants as previously described.[6] Then, blastocysts were thawed quickly at room temperature with a 10-minute equilibration in HEPES-buffered EBSS media. This step was followed by an additional 10-minute equilibration in HEPES-buffered EBSS medium supplemented with 20% FBS and 0.2 mol/l of sucrose prior to transferring the blastocysts to culture medium (COOK, Australia). Blastocysts were considered surviving when greater than 50% of the inner cell mass and trophectoderm cells were undamaged by inverted microscopic observation.

Vitrification for mouse blastocysts

Vitrification was performed using a three step protocol.[19,20] Initially, the blastocysts were placed in the base medium containing 1.36 mol/l (10%) glycerol in 10% DPBS. After 3 minutes, they were moved to combined medium containing 1.36 mol/l (10%) glycerol and 2.7 mol/l (20%) ethylene glycol in 10% DPBS for 3 minutes. Then they were suspended in the last medium containing 3.4 mol/l (20%) glycerol and 4.5 mol/l (25%) ethylene glycol in 10 % DPBS. At the last step, the blastocysts were rinsed quickly in the cryoprotectant solution, placed into the tiny spoon of the cryo-E (sterilized stainless ear picks) in a small volume of 2-5 microlitres. After loading of the blastocyst, the spoon of the cryo-E was plunged immediately into liquid nitrogen within 30 seconds and kept stored in a liquid nitrogen tank.

Thawing of blastocyst

One month after freezing, thawing was performed with a three-step rapid thawing protocol in sucrose solution.[19,21] In a 4-well multi-dish, 0.8 ml of base medium containing 0.5 mol/l, 0.25 mol/l and 0.125 mol/l sucrose thawing solutions were warmed briefly in an incubator at $37^{\circ}C$ and then placed on the stage warmer of a dissecting microscope. The tiny spoon of cryo-E was placed in a solution of 0.5 mol/l sucrose for 3 minutes allowing the blastocyst to fall to the bottom of dish, and then each blastocyst was transferred to a solution of 0.25 mol/l sucrose followed by 0.125 mol/l sucrose for 3 minutes each before each blastocyst was rinsed and then cultured in blastocyst media (COOK, Australia).

Assessment of blastocyst survival

After 4 hours in culture after warming, survival was based on the clarity of blastomeres and blastocoel re-expansion. Further development was examined on survival and hatching after 24 h under an inverted microscope at X400 magnification.

Data analysis

Statistical significance of survival and further hatching rate after warming among treatment groups was compared using a Mann-Whitney U-test analysis.

RESULTS

Based on 250 slow freezing and 310 vitrified mouse blastocysts, vitrification resulted in slightly higher survival and hatching rates than the slow-freezing method (83.9% Vs 82.0%, and 68.8% vs 66.8%. respectively), the difference being non significant ($p<0.05$) by the Mann-Whitney U-test (Table 1). Furthermore, duration during slow freezing was longer than vitrification, and slow freezing required more

TABLE 1. Comparison of survival and hatching rates between two freezing methods.

Protocol	Number of Mouse blastocysts	Survival rate		Hatching rate	
		Number	%	Number	%
Slow freezing	250	205*	82.0	173**	66.8
Vitrification	310	260*	83.9	179**	68.8
Mann-Whitney U-Test: * p value = 0.89, ** p value = 0.18					

TABLE 2. Comparison of materials used in slow freezing and vitrification.

Material	Slow freezing	Vitrification
Time finished	2 hr and 30 min	6 min and 30 sec, plunge
Equipment	Controlled cryofreezer	No machine used
Supporting	Straws	Stainless cryo-E
Cost	80 baht/straw	8-10 baht/piece
Volume of LN_2 used	70 litres/time	0.1 litre/time

Fig 1. Computerized programmable cryofreezer for slow freezing.

liquid nitrogen volume than vitrification. (2 hours 30 minutes VS 6 minutes 30 seconds and 70 liters/ time and 0.1 litres/time, respectively). Whereas slow freezing needed a computerized controlled rate cryofreezer, vitrification did not need any cryo-machine. The cost of slow freezing was also 10 times more expensive than the cost of vitrification. (Table 2) The mouse blastocyst after freezing and thawing is shown in Figure 1-4 respectively.

DISCUSSION

The objective of cryopreservation is to obtain a high number of post-thawing survival normal embryos which maintain structural integrity, viability and functional potential for development as before freezing. The traditional method of freezing known as computerized controlled slow rate freezing has been developed to eliminate the consequences of ice formation. Furthermore, vitrification is a process combining the use of concentrated solutions with rapid cooling to avoid the formation of ice and become a glassy state.[11,21,22] Currently, the standard methods for freezing-thawing blastocysts that will optimize survival rate as well as implantation have not yet been firmly established.[13] In the present study we, therefore, determined the cryodamage effects on mouse blastocyst, focused on survival rate, hatching rate and cost effectiveness. Those effects were compared between the slow freezing and the vitrification technique.

In this study, post-thaw mouse blastocyst survivals were clearly affected by the freezing-thawing process during both cryopreservation methods. This effect is explained in many ways including osmotic injury, thermal injury and toxic effects during the freezing process. In addition, our result demonstrated that the percentages of survival rate and hatching rate in populations of mouse blastocysts after vitrification were slightly higher than the percentages in the slow freezing group. The result in this study corresponded to previous studies.[23,24] However, the percentage of survival and hatching rate of the blastocysts after cryopreservation by both methods in our study were higher than the study from Tharnprisarn et. al.[23] An explanation may be related to the different techniques, cryostorage equipment and cryoprotective media, which were improved from the past.

Fig 2. Cryo-E (sterilized stainless ear pick) with microdrop of vitrified mouse blastocyst was plunged into liquid nitrogen.

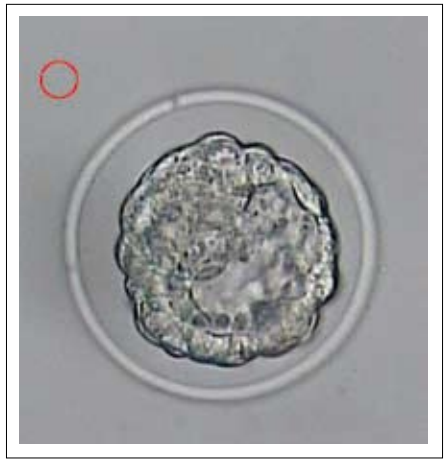

Fig 3. The mouse blastocyst was exposed to the three step vitrified freezing solution: Note the presence of a distinct shrinkage of cytoplasm.

Fig 4. A surviving blastocyst vitrified which expanded and hatching after thawing.

According to the cost-effective aspect, our study clearly showed the advantages using vitrification over slow freezing. Firstly, slow freezing required the use of a computerized programmable freezing equipment which can ensure controlled marginal temperature decline, but vitrification did not require sophisticated equipment. Secondly, the cost, time and volume of liquid nitrogen used for the slow freezing method were much higher than those for vitrification. Although this study proved that freezing blastocyst with vitrification seem superior to blastocyst freezing with slow freezing, we studied only in a mouse blastocyst population and the difference in the percentage was not significant. Further studies, therefore, are required to conclusively prove the effectiveness of vitrification on human blastocysts.

CONCLUSION

Both slow freezing and vitrification are useful for mouse blastocyst cryopreservation, but vitrification is better than slow freezing in terms of simplicity, duration, and cost-effectiveness.

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