Thai Journal of Obstetrics and Gynaecology
September 2003, Vol. 15, pp. 185-190

GYNAECOLOGY

Ultrarapid Freezing of Mouse Two-Cell Embryos in Conventional Straws and on Aluminum Foils

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ABSTRACT

Objective To compare the success rates of ultrarapid freezing of mouse two-cell embryos in straws and on aluminum foils.

Design Laboratory experimental study.

Setting Assisted conception laboratory, Chiang Mai University.

Subjects Two-cell embryos from superovulated and mated ICR mice.

Intervention Embryos were divided into 3 groups: 177 were not frozen (control), 172 were frozen on aluminum foils and 171 in straws. Freezing was done by exposing embryos to 3M DMSO/0.25M sucrose for 2.5 minutes and plunged into liquid nitrogen. Thawing was done by dipping foils into 0.25M sucrose at 37°C or straws into 37°C water-bath and expelled embryos into 0.25M sucrose. Control and frozen/thawed embryos were cultured in G1.2 medium for 24 hours and in G2.2 medium for another 48 hours.

Main Outcome Measures Survival immediately after freezing/thawing, proportions of survived embryos that underwent further cleavage and those that developed into blastocysts and hatching blastocysts.

Results Embryos frozen on aluminum foils had a significantly higher survival rate than those frozen in straws (94.8% versus 88.9%; p=.047). They underwent further cleavage at a higher rate (135/163; 82.8% versus 91/152; 59.9%, p<.001). Proportions of embryos that became blastocysts (105/163; 64.4% versus 64/152; 42.1%) and hatching blastocysts (48/163; 29.4% versus 14/152; 9.2%) were also significantly higher (p<.001). Controls had higher (p<.05) cleavage rate (161/177; 91%), and higher (p<.001) rates of blastocyst (136/177; 91%) and hatching blastocyst formation (99/177; 55.9%) than embryos that underwent freezing/thawing.

Conclusion Ultrarapid freezing on aluminum foils gave better results than ultrarapid freezing in conventional straws.

Key words: ultrarapid freezing, mouse embryo, cryopreservation

Embryo cryopreservation has a potential to reduce the risk of multiple pregnancy while increasing the number of transfers and hence the pregnancy rate per retrieval.[1] Successful cryopreservation depends not only on the quality and the stage of the embryos, but also on the techniques of freezing and thawing.[1,2]

Slow controlled-rate cooling is now the conventional procedure used by most assisted conception laboratory to freeze surplus embryos.[1-4] However, the method requires an expensive biological freezer, a large amount of liquid nitrogen and is also time consuming.[2,3]

Alternative approaches that are simple to carry out and do not require a controlled-rate cooling apparatus include ultrarapid freezing and vitrification.[2-4] Both methods rely on a relatively high concentration of cryoprotectant to achieve embryo dehydration, followed by very rapid cooling rate to reduce the likelihood of intracellular ice formation.[2-4] Recently a high embryonic survival rate was described for vitrification method by employing direct contact between the oocytes/embryos and the liquid phase nitrogen.[5,6]

It should, therefore, be possible to improve the success of ultrarapid freezing by using a container-less technique instead of the conventional plastic straw. The aim of this study was to evaluate the effectiveness of ultrarapid freezing of mouse two-cell embryos in conventional straws with that on aluminum foils, in terms of immediate survival, further cleavage rates, and subsequent development into blastocysts and hatching blastocysts.

Materials and Methods

Source of 2-cell embryos

Outbred ICR mice were bought from the National Animal Institute, Mahidol University, Bangkok, Thailand. They were caged in a ventilated room at the Animal Husbandry Unit, Faculty of Medicine, Chiang Mai University at 25 + 2°C under 60-70% relative humidity, and 12 hour-light/12 hour-dark cycle. Animals were maintained undisturbed for 5 days after arrival to avoid the effect of stress from transportation.

Five- to six-week old ICR female mice were given intra-peritoneal injection of 5 IU pregnant mare serum gonadotropin (Sigma Chemical, MO), followed 48 hours later by IP injection of 5 IU human chorionic gonadotropins (hCG; N.V. Organon, Holland). Super-ovulated females were paired with ICR males immediately after hCG injection. They were checked for mating by the presence of vaginal plugs 16 hours later. Mated female mice were sacrificed by cervical dislocation. Two-cell embryos were flushed from the oviducts 40 hours after hCG injection, using Earle's balanced salt solution (EBSS; GIBCO BRL, N.Y.) supplemented with 10 ul/ml 1M HEPES (Sigma Chemical, MO) and 0.3% bovine serum albumin (BSA) fraction V (Sigma Chemical, MO). Embryos were pooled in small drops of medium under oil (Medicult, Denmark) and were randomly divided into 3 groups: non-frozen control (group 1), ultrarapid freezing on aluminum foils (group 2), and ultrarapid freezing in conventional plastic straws (group 3). Only 2-cell embryos with intact zona pellucida and nearly equal blastomeres without any fragmentation were used in this experiment.

Embryo container for ultrarapid freezing

Plastic straws (IMV, France), measuring 13 centimeters in length and 1 millimeter in outer diameter, were used to store embryos. Aluminum foil (Diamond, Richmond, U.S.A) was bought from a local supermarket. It was intended for household use to wrap and cook food. The foil was cut and folded into a long narrow strip, 3 mm wide and 7 cm long. The strip was bent at one end to hold a drop of freezing medium with embryos. (Fig. 1.)

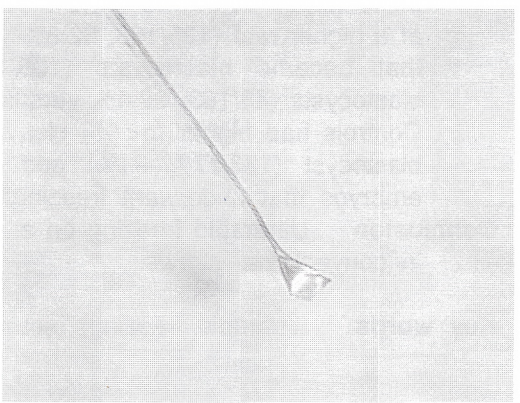

Fig. 1. Aluminum foil was cut, folded and bent into the shape of a spoon.

Ultrarapid freezing with dimethylsulfoxide (DMSO)

The method for ultrarapid freezing of mouse embryos was adapted from that described by Trounson et al.[7] In brief, two-cell embryos were transferred into the first well of a 4-well plate (Nunc, Denmark) containing 3M DMSO (Sigma Chemical, MO), 0.5% BSA (Sigma Chemical, MO), and 0.25M sucrose (Merck, Germany) in Dulbecco's phosphate buffered saline (DPBS; GIBCO BRL, NY) at room temperature (25 C). After a brief washing, embryos were transferred into the next well containing the same freezing medium.

For ultrarapid freezing in straws, a 2-3 cm column of thawing medium (0.25M sucrose + 0.5% BSA in DPBS) was first loaded into the straw, followed by an air gap of 1 cm in length. Freezing medium with a group of up to 5 embryos was then drawn into the straw, followed by another air gap and a column of thawing medium. The end of the straw was sealed with polyvinylpyrrolidone (PVP) powder (Sigma, MO). After 2.5 minutes of exposure to cryoprotective solution, the straw was plunged into liquid nitrogen. A group of straws was stored together inside a visitube, on aluminum cryostorage cane inside a liquid nitrogen tank.

For ultrarapid freezing on aluminum foils, up to 5 embryos were transferred in a small drop of medium (<25 ul) onto the bent part of the aluminum strip. After 2.5 minutes embryos were plunged into liquid nitrogen. The drop of freezing medium with embryos suddenly formed an ice-ball and adhered firmly to the aluminum foil (Fig. 2).

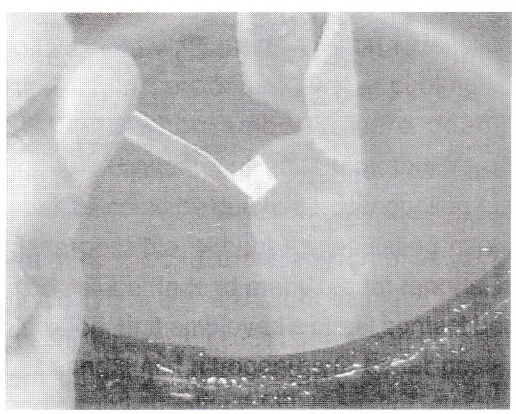

Fig 2. A drop of freezing medium with embryos formed an ice-ball that adhered firmly to the aluminum foil.

Thawing of embryos

Thawing was done by dipping the foil with a frozen drop of embryos into 0.25M sucrose + 0.5% BSA in DPBS at 37°C. The aluminum strip was removed when the frozen drop melted and sank to the bottom of the well. Embryos were then transferred into another well containing the same thawing medium.

Straws containing frozen embryos were thawed by plunging them into 37°C water-bath until the ice melted completely. Sterile scissors were used to cut the end off the straw and allowed its content to drain into a well containing thawing medium. Embryos were identified and transferred into the next well containing the same thawing medium.

Embryos were left in thawing medium for 10 minutes. They were then washed through G1.2 medium (Scandinavian IVF Science, Sweden) and observed for survival.

Mouse embryo culture

Control embryos (group 1) and those that survived immediate freezing and thawing (groups 2 and 3) were washed through G1.2 medium. They were then transferred into 25 ul drops of G1.2 medium under paraffin oil. Embryos were cultured in groups of up to 12 in a humidified atmosphere of 5% CO_2 in air at 37°C. After 24 hours, they were observed for further cleavage. Cleaved embryos were washed through G2.2 medium and then cultured in 25 ul drops of G2.2 medium under oil for another 48 hours. Blastocyst development was assessed after 72 hours in culture.

Statistical analysis

Stata Statistical Software (Stata Corporation, College Station, Texas) was used for data analysis. Fisher's exact and Chi²-tests were used as appropriate, to compare the proportions of embryos that survived immediate freezing and thawing, those that underwent further cleavage and those that developed into blastocysts and hatching blastocysts. Embryos that were lost during the thawing process were reported and treated as non-survived embryos in the final analysis. p-values of <0.05 were

considered significant.

Results

One hundred and seventy two 2-cell embryos were frozen on aluminum foils (group 2) and 171 were frozen in plastic straws (group 3). The numbers of embryos that did not survive or were lost during the freezing/thawing process were slightly higher in group 3 than those in group 2, but the difference did not reach statistical significance (Table 1). In this study, embryos with at least 1 intact blastomere post-thaw were defined as survived embryos. Under this criterion, the proportion of survived embryos was significantly higher in group 2 than that in group 3 (Table 1).

After 24 hours of culture, further cleavage rate in group 2 was significantly higher ($p<0.001$) than that in group 3 (Table 2). At 72 hours post thaw, embryos in group 2 developed into blastocysts and hatching blastocysts at a significantly higher proportion than those in group 3 ($p<0.001$). However, embryos that previously underwent ultrarapid freezing/thawing had significantly lower cleavage rates after 24 hours of culture than nonfrozen control ($p<0.05$). They also developed into blastocyst ($p<0.05$) and hatching blastocysts ($p<0.001$) at a significantly lower proportion.

Table 1. Immediate survival of 2-cell mouse embryos post freezing/thawing

	Freezing methods		
Results	**Foil (n=172)**	**Straw (n=171)**	**P**
Non-viable	4 (2.3%)	8 (4.7%)	0.236
Lost	5 (2.9%)	11 (6.4%)	0.122
Intact 1 blastomere	5 (2.9%)	5 (2.9%)	0.993
Intact 2 blastomeres	158 (91.9%)	147 (86.0%)	0.082
Survived embryos	163 (94.8%)	152 (88.9%)	0.047

Table 2. Further cleavage and development into blastocysts and hatching blastocysts

	Freezing methods		
	Foil (group 2)	**Straw (group 3)**	**Nonfrozen Control (group 1)**
Viable embryos	163	152	177
Further cleavage	135 (82.8%)	91 (59.9%)	161 (91%)
Total blastocysts	105 (64.4%)	64 (42.1%)	136 (76.8%)
Hatching blastocysts	48 (29.4%)	14 (9.2%)	99 (55.9%)

Discussion

The original technique of ultrarapid freezing (URF) was described by Trounsen et al.[7] in 1987. It allowed embryos to be plunged into liquid nitrogen after a brief exposure of 2-3 minutes to 2.5-4.5 M DMSO and 0.25 M sucrose.[2] Several reports had shown that URF was efficient for the cryopreservation of mammalian embryos.[8-11]

Mouse embryos were used in our freezing experiment because they could be easily obtained in quantity. Moreover, the results from freezing study of mouse embryos were broadly similar to those of human embryos.[12] Cleavage stage was chosen because our assisted conception program

cryopreserved human embryos at the 2- to 8-cell stage. The 2-cell stage was specifically employed in this study because it was most sensitive to cryodamage than any other cleavage stages.[13]

In a pilot study, 14 out of 15 embryos (93.3%) on aluminum foil and 12 out of 15 (80%) embryos in straw survived ultrarapid freezing/thawing. Given a type I error of 5% (two-tailed) and a type II error of 20%, we estimated that at least 97 two-cell embryos were required in each group.

In our study survival rate of 2-cell embryos frozen in straws (88.9%) was comparable to those reported by Wilson and Quinn (87%)[9] and by Macas et al. (84.8%)[14] This rate was similar to the result obtained from conventional slow programmable freezing of mouse 2-cell embryos (79%-86.2%).[14,15] Further cleavage rate of embryos frozen in straws in this report (59.9%) was also compatible with that reported by Macas et al. (55.9%) and similar to that after slow programmable freezing (69%).[14] In comparison, URF on aluminum foils (container-less technique) gave a survival rate of more than 94% with a further cleavage and blastocyst formation rates of 82.8% and 64.4% respectively, which were higher than previous reports using conventional straws.[2,8,9,14]

The mechanism that embryos survived ultrarapid freezing was still unknown.[2] The inward flow of DMSO in conjunction with the outward flow of water could theoretically raised intracellular concentration of DMSO to a level which intracellular vitrification could occur without ice formation when embryos were plunged into liquid nitrogen. In a thermodynamic model of cooling, Toner et al.[16] predicted that intracellular ice formation could be prevented at a cooling rate of 120°C/minute when embryos were adequately dehydrated. A better result after ultrarapid freezing on aluminum foil could be due to a faster cooling rate that was made possible without an insulating effect of a plastic container. Indeed many recent reports, mostly on vitrification, that employed a direct contact between embryos and liquid nitrogen gave better post-freeze embryonic survival rates.[5,6,17] However, electron grid and cryoloop described in those studies[5,6] were expensive and needed to be imported. Our preliminary homemade method of ultrarapid freezing on aluminum foils were, therefore, an attractive alternative. Further study was underway to compare the conventional slow programmable freezing and vitrification with URF on aluminum foils.

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