TECHNICAL SESSION IV

Oocyte cryopreservation - a cost effective method

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Abstract

Embryo cryopreservation is a widely used and relatively well-established procedure. By contrast unfertilized mature metaphase (M11) oocyte freezing technique has not been developed as yet. The oocyte's surface to volume ratio, single membrane, temperature sensitive spindle and zona, susceptibility to parthenogenetic activation and chill injury have been proposed as contributory factors for the failure in attempts to freeze them. Embryo cryopreservation for human IVF programmes has raised a lot of ethical issues making it necessary to develop oocyte freezing technology.

Female albino mice aged 8-10 weeks were super ovulated by 5IU of Pregnant Mare Serum Gonadotrophin (PMSG) followed by human Chorionic Gonadotrophin(hCG) at 46-48 hours. 18-20 hours after the hCG injection, cumulus masses were retrieved and collected into M_2 culture media. In the experimental group, mouse oocytes were cryopreseved with different concentrations of DMSO, EG, glycerol with different concentrations of sucrose. These oocytes with cumulus masses were placed in 0.5ml paillets which were cooled at 0^0C for 20 minutes and transferred into liquid nitrogen vapour (LN_2) for 5 minutes and were immersed in LN_2 slowly before storing in LN_2 tanks for one week. These experimental frozen oocytes were thawed and inseminated with sperms. Fresh oocytes with cumulus masses were used as controls and were inseminated with sperms. Both groups were incubated at 37^0 C with 5% CO_2 for six hours. Presence of two polar bodies or 2 pronuclei were taken as confirmation of fertilization.

Chi-Square test was used to compare the fertilization rates (FR) of experimental with the control group. The FR for the control group was 72.74%. The highest FR of 71.4% was obtained for the experimental oocytes cryopreserved in 3.5M DMSO with 0.25M sucrose. The best protocol giving a 71.4% success rate at fertilization was by 3.5M DMSO with 0.25M sucrose, which is comparable to the control.

Introduction

Embryo cryopreservation is a widely used and relatively well-established procedure. By contrast ovarian tissue and unfertilized M11 oocytes are rarely cryopreserved, due to non availability of perfect technology. Developing a reliable technique for oocyte cryopreservation provides numerous advantages. If oocytes can be cryopreserved, ethical problems linked to embryo cryopreservation can be avoided. There is the possibility of providing a chance for a woman who is at risk of losing ovarian tissue through surgical or medical procedures to preserve her own oocytes for future use. Oocyte cryopreservation can be of great importance in modern day commercial animal husbandary and in gene cryopreservation programmes.

Ovarian tissues with metaphase one (M1) stage oocytes are difficult to cryopreserve because it involves invasive and harmful procedures to the mother. Nevertheless immature M1 stage oocytes frozen with ovarian tissue can be used after maturity to M11, with difficulty. It has been shown that unfertilized M11 stage oocytes are much more difficult to cryopreserve than cleavage stage embryos of the same species. Some factors which contribute to this observation include the oocyte's surface to volume ratio, single membrane, temperature sensitive metaphase spindle and zona, its susceptibility to parthanogenetic activitation and chill injury.

The cryoprotectants commonly used in freezing protocols for biological materials are Dimethyl sulphoxide (DMSO), 1-2 propanediol (1-2 PROH), Ethylene glycol(EG), glycerol (membrane permeating cryoprotectant) and sucrose (membrane non permeating cryoprotectant). All the commonly used cryoprotectants for cryostorage of oocytes, embryos and sperm such as PROH, DMSO, glycerol, EG and Polyethyleneglycol are to some extent toxic to cells. The current embryo-freezing protocols use 1-2 PROH, DMSO, glycerol or EG [1,2,3]. The toxicity of these cryoprotectants varies with their concentration and their exposure time. The low concentration and the short exposure time may reduce toxicity to embryo development [4].

Objective

The objective was to develop a reliable and cost effective manual for oocyte freeze-thaw protocol.

Materials and Methods

Animals

Albino mice aged 8-10 weeks were obtained originally from the Medical Research Institute and thereafter they were naturally bred in the Faculty of Medicine animal house. For this study mice weighing 20 to 30 g were used. Four animals were housed per cage with food and water *ad libitum*.

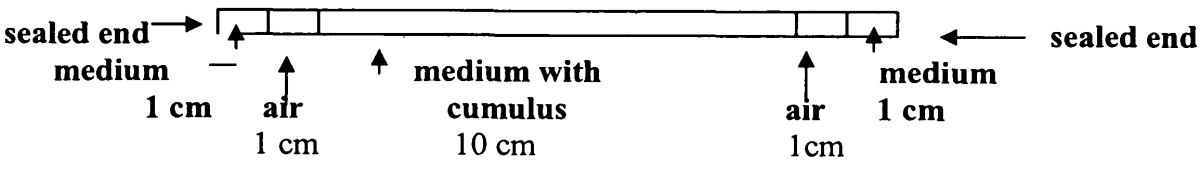
Super ovulation

The female mice were super ovulated by intra peritoneal injections of 5 IU pregnant mare serum gonadotrophin (PMSG)) followed by 5 IU of human chorionic gonadotrophin (hCG) 48 hours later. Under ether anaesthesia the female mice were sacrificed 18-20 hours after the hCG injection and cumulus masses with ovulated oocytes were retrieved by cutting their oviducts and collecting them into 10 ml of prepared M_2 culture media in Petri dishes. They were then dissected and the cumulus masses were collected into 0.7 ml of medium in a Nunc four well dish. These oocytes were checked for maturity (metaphase 11) and incubated for 1 hour before freezing. In all the experiments cumulus cells were not dispersed before freezing.

Freezing method

The freezing solutions were prepared with M_2 media. The permeative cryoprotectants used were DMSO, EG, 1-2 PROH or glycerol in different concentrations and combinations. The nonpermeative cryoprotectant used was sucrose in different concentrations. Cumulus masses were transferred into Nunc four well dishes filled with freezing solution. The cumulus masses were immersed in the freezing solution for 10 minutes. Thereafter these oocytes were loaded into 0.2 cm diameter, 14.5 cm long paillets in the order given below.

1 cm of freezing medium , 1 cm of air, 10 cm length of medium containing cumulus mass followed by 1 cm of air and 1 cm of medium. Both ends of the straw were sealed with a polythene sealer prior to cooling.



The paillets with cumulus masses were transferred to a deep freezer at 0°C temperature for 20 minutes. They were then transferred into goblets with ice cubes and kept at the liquid nitrogen (LN_2) vapour margin for 5 minutes. Finally they were slowly immersed into the LN_2 (-196° C) before storing in LN_2 tanks. Thereafter the paillets were transferred into visotubes in goblets in the storage tank. The duration of cryopreservation was 7 days.

Thawing method

The straws that were stored at -196° C were transferred into a goblet with ice cubes exposed to LN_2 vapour for 5 minutes. The goblet was taken out and thereafter ice was melted by running tap water at room temperature (RT). The paillets were then transferred into a water bath at 37°C for 5 minutes.The paillets were cut at the site of an air column and the contents were released into a Nunc four well dish. The paillets were flushed with pre heated (37°C) M_2 medium. Teasing out oocytes from the cumulus masses were done at this stage. The cumulus masses were thereafter transferred into M_2 medium in Nunc four well dishes.

Sperm preparation

Twelve week old male mice were isolated for five days. They were anaesthetized with ether inhalation and the epididymis dissected out and squeezed into M_2 medium. The sperms were covered with mineral oil and incubated at 37° C under 5 % CO_2 for one and half hours for capacitation. Sperm concentration of the suspension was determined and the concentration was adjusted to be approximately 20×10^6/ ml, of which 0.1 ml was used for insemination.

Insemination

Two million sperms (0.1 ml of the sperm suspension) were deposited in each of the wells with cumulus mass. Fertilization was assessed 5-6 hours after insemination, by observing the presence of the second polar body or two pronuclei under 10x40 power under an inverted microscope. For each experiment eight female mice were selected to obtain cumulus masses and from each one two cumulus masses were collected. However the number of oocytes used for insemination varied from one experiment to another because some of the oocytes were lost during transfer through the media and some cumulus masses adhered to walls of the paillets during freezing. In addition, after thawing oocytes with plasma membrane not intact under perivitelline space, leaked cytoplasm, shrunken oocyte and presence of space between zona pellucida and cytoplasm were discarded.

Experiments

Experiments 1 – 4

The effects of varying concentrations of sucrose with 3.5 M DMSO in M_2 medium.

In this experiment unfertilized mouse oocytes were cryopreserved in 3.5M DMSO with varying concentrations (0.2M, 0.25M, 0.5M or 1.0M) of sucrose. The freezing solution was prepared in M_2 medium. Four wells in the Nunc four well dishes were used for different concentrations of sucrose. The permeative cryoprotectant was added by two steps and in the third step, the non permeative cryoprotectant was added. All the steps of each experiment were carried out at room temperature and a total volume of 0.7 ml solution was added at each well at each step.

Table 1: Protocol for experiments 1-4 using varying concentrations of sucrose with 3.5M DMSO

Step	*Experiment*
A	**Freezing by addition of cryoprotectant**
A 1)	5 minutes.in 0.7 ml of 1.5 M DMSO in each of the four wells at RT
A 2)	5 minutes. in 0.7 ml of 3.5 M DMSO in each of the four wells at RT
A 3)	10 minutes.in 0.7ml of 3.5 M DMSO & 0.2 M sucrose or 0.25 M sucrose or 0.5 M sucrose or1.0 M sucrose* in each of the four wells respectively at RT
B	**Storage in paillets for 7 days in LN_2**
C	**Thawing and removal of cryoprotectant**
C 1)	5 minutes in 0.7 ml of 1.5 M DMSO & 0.2 M sucrose or 0.25 M sucrose or 0.5M sucrose or 1.0 M sucrose in each of the four wells respectively at RT
C 2)	5 minutes in 0.7 ml of 1.0 M DMSO & 0.1 M sucrose in each of the four wells respectively at RT
D	**Washing**
D 1)	5 minutes in M_2 medium
D 2)	5 minutes in M_2 medium

DMSO = Dimethyl sulphoxide
RT = Room Temperature

Experiments 5 - 8

The effects of varying concentrations of sucrose with 1.5 M DMSO in M_2 medium.

In this experiment the effect of varying concentrations of sucrose (0.1M, 0.2 M, 0.3 M or 0.4 M) with 1.5 M DMSO in M_2 medium was studied. The steps of experiments are given in Table 2.

Table 2: Protocol for experiments 5-8 using varying concentrations of sucrose with 1.5 DMSO

Step	*Experiment*
A	**Freezing by Addition of cryoprotectant**
A 1)	5 minutes in 0.7 ml of 1.0 M DMSO in each of the four wells at RT
A 2)	5 minutes in 0.7 ml of 1.5 M DMSO in each of the four wells at RT
A 3)	10 minutes.in 0.7 ml of 1.5 M DMSO & 0.1M sucrose, or.0.2M sucrose or 0.3 M sucrose or 0.4 M sucrose* in each of the four wells respectively at RT
B	**Storage in paillets for 7 days in LN_2**
C	**Thawing and Removal of cryoprotectant**
C 1)	5 minutes in 0.7 ml of 1.0 M DMSO & 0.1 M sucrose, or 0.2 M sucrose or 0.3 M sucrose or 0.4 M sucrose in each of the four wells respectively at RT
C 2)	5 minutes in 0.7 ml of 1.0 M DMSO & 0.1 M sucrose except in the 1st well which was not transferred at RT
D	**Washing**
D1)	5 minutes in M_2 medium
D2)	5 minutes in M_2 medium

DMSO = Dimethyl sulphoxide
RT = Room Temperature

Experiments 9 - 12

The effects of 43% of varying concentrations of sucrose with 57% of 1.5 M DMSO in M_2 medium.

In this experiment the effect of 43% of varying concentrations of sucrose (0.1M, 0.2M, 0.3M or 0.4M) with 57% of 1.5M DMSO in M_2 medium was studied. The steps of experiments are given in Table 3.

Table 3: Protocol for experiments 9-12 using 43% of varying concentrations of sucrose with 57% of 1.5 M DMSO

Step	*Experiment*
A	**Freezing by addition of cryoprotectant**
A 1)	5 minutes in 0.7 ml of 1.0 M DMSO in each of the four wells at RT
A 2)	5 minutes in 0.7 ml of 1.5M DMSO in each of the four wells at RT
A 3)	10 minutes in 0.7 ml of 1.5 M DMSO (0.4 ml) & 0.1 M sucrose or 0.2 M sucrose or 0.3 M sucrose or 0.4 M sucrose (0.3ml)* in each of the four wells respectively at RT
B	**Storage in paillets for 7 days in LN_2**
C	**Thawing and removal of cryoprotectant**
C 1)	5 minutes in 0.7 ml of 1.0 M DMSO (0.4ml) & 0.1 M sucrose or 0.2 M sucrose or 0.3 M sucrose or 0.4 M sucrose (0.3ml) in each of the four wells respectively at RT
D	**Washing**
D 1)	5 minutes in M_2 medium
D 2)	5 minutes in M_2 medium

DMSO = Dimethyl sulphoxide
RT = Room Temperature

Experiments 13 - 16

The effects of 14% of varying concentrations of sucrose with 28.5% of 1.5 M DMSO, 28.5% of 1.5M EG & 28.5% of 1.5M Glycerol in M_2 medium.

In this experiment the effect of 14% of varying concentrations of sucrose (0.1M, 0.2M, 0.3M or 0.4M) with the combination of permeative cryoprotectants(28.5% of 1.5M DMSO, 28.5% of 1.5M EG and 28.5% of 1.5M Glycerol) in M_2 medium was studied. The steps of experiments are given in Table 4.

Table 4: Protocol for experiments 13-16 using 14% of varying concentrations ofsucrose with 28.5% of 1.5 M DMSO, 28.5% of 1.5M EG & 28.5% of 1.5M Glycerol.

Step	*Experiment*
A	**Freezing by addition of cryoprotectant**
A 1)	5 mts. in 0.2 ml of 1.0M EG (28.5%) 0.2 ml of 1.0M DMSO (28.5%) 0.2 ml of 1.0M gly (28.5%) & 0.1 ml of M_2 medium (14 %) in each of the four wells at RT
A 2)	10 mts. in 0.2 ml of 1.5M EG (28.5%) 0.2 ml of 1.5M DMSO (28.5%) 0.2 ml of 1.5M gly (28.5%) 0.1 ml of 0.1M sucrose (14%), 0.2M sucrose (14%), 0.3M sucrose (14%) or 0.4M sucrose (14%) in each of the four wells respectively at RT*
B	**Storage in paillets for 7 days in LN_2**
C	**Thawing and removal of cryoprotectant**
C 1)	5 mts.in 0.2ml of 1.0M EG (28.5%) 0.2ml of 1.0M DMSO (28.5%) 0.2ml of 1.0M gly (28.5%) 0.1ml of 0.1M sucrose (14%) or 0.2M sucrose(14%)or 0.3M sucrose (14%) or 0.4 M sucrose (14%) in each of the four wells respectively at RT
C 2)	5 mts.in 0.2ml of 1.0M EG (28.5%) 0.2ml of 1.0M DMSO (28.5%) 0.2ml of 1.0M gly (28.5%) 0.1ml of 0.1M sucrose (14%) in 2nd well, 0.2M sucrose(14%) in 3rd and 4th wells respectively at RT except in the 1st well which was not transferred
D	**Washing**
D 1)	5 minutes in M_2 medium
D 2)	5 minutes in M_2 medium

DMSO = Dimethyl sulphoxide, EG: Ethylene Glycol, gly: Glycerol
RT = Room Temperature

Control experiment

Freezing was not done and only the invitro fertilization was done. Spermatozoa were obtained from the cauda epididymis of 8-12 weeks old males and they were suspended in 0.5 ml M_2 media covered with mineral oil layer. The sperm suspension was incubated at 37° C under 5% CO_2 in air in a modular incubator for 1½ hours for capacitation. Eight female animals were super ovulated at each time and they were sacrificed 18-20 hours after hCG injection. The cumulus masses were collected into 1ml M_2 media covered with mineral oil layer. They were incubated about 1 hour for maturation. After 1½ hour incubation of sperms, 0.1 ml - 0.2 ml sperms (2 x 10^6/ml) were added to the wells in the Nunc 4 well dishes containing cumulus masses. The cumulus masses and sperms were incubated for 6 hrs in the modular incubator and presence of 2nd polar body was taken as fertilization. Three experiments were done in three days during the period of study.

Statistical Analysis

Chi-square test was used to compare the fertilization rates of experimental with control oocytes. Differences between groups were considered as statistically significant when P value is less than 0.05 ($P<0.05$)

Results

Control experiment (Direct insemination without freezing)

Control experiment	No of eggs	Fertilized eggs	FR(%)
1	32	24	75.0
2	64	46	71.8
3	42	30	71.4
Total	**138**	**100**	**72.7 (Mean FR)**

Experiments 1 - 4

The effects of varying concentrations of sucrose with 3.5 M DMSO in M_2 medium

Cryoprotectant	No of cryopreserved oocytes	Fertilized eggs	FR for cryopreserved oocyte (%)
3.5 M DMSO			
0.2M sucrose	14	08	57.1 ***
0.25M sucrose	**28**	**20**	**71.4 ***
0.5M sucrose	60	28	46.7
1.0M sucrose	16	00	0

***Combination which gave the best invitro fertilization rates (p=0.904513) in comparison with the mean FR of controls. *** p=0.2314416**

Experiments 5 - 8

The effects of varying concentrations of sucrose with 1.5 M DMSO in M_2 medium

Cryoprotectant	No of cryopreserved oocytes	Fertilized eggs	FR for cryopreserved oocyte (%)
1.5M DMSO			
0.1M sucrose	10	04	40.0
0.2M sucrose	16	08	50.0
0.3M sucrose	***12***	***08***	***66.6 *****
0.4M sucrose	08	02	25.0

****** The combination which gave the second best invitro fertilization rates.(p =0.7395740)**

Experiments 9 - 12

The effects of 43% of varying concentrations of sucrose with 57% of 1.5 M DMSO in M_2 medium

Cryoprotectant	No of cryopreserved oocytes	Fertilized eggs	FR for cryopreserved oocyte (%)
1.5M DMSO (57%)			
0.1M sucrose (43%)	14	08	57.1
0.2M sucrose (43%)	12	04	33.3
0.3M sucrose (43%)	06	02	33.3
0.4M sucrose (43%)	04	00	0

Experiments 13 - 16

The effects of 14% of varying concentrations of sucrose with 28.5% of 1.5 M DMSO, 28.5% of 1.5M EG & 28.5% of 1.5M Glycerol in M_2 medium

Cryoprotectant	No. of cryopreserved oocytes	Fertilized eggs	FR for cryopreserved oocyte (%)
1.5M EG (28.5%)			
1.5M DMSO (28.5%)			
1.5M gly (28.5%)			
0.1M sucrose (14%)	12	06	50.0
0.2M sucrose (14%)	missing eggs	00	0
0.3M sucrose (14%)	08	00	0
0.4M sucrose (14%)	10	02	20.0

Discussion

In our study we used different concentrations of DMSO, EG and glycerol with different concentrations of sucrose. In some experiments combinations of two to three permeative cryoprotectants with different concentrations of sucrose were used.

The oocytes frozen with EG as the cryoprotectant exhibited significantly lower survival and fertilization rates. The survival and fertilization rates for oocytes cryopreserved in different concentrations of DMSO are higher than in glycerol or EG alone. The highest fertilization rate was seen in DMSO with 0.25M sucrose (71.4%) which is comparable to the control.

In a slow freezing and rapid thawing technique using 1.5M DMSO and 0.2M sucrose the highest fertilization rate for mouse oocytes 71.9% has been recorded previously[5]. In this study the oocytes were slowly frozen in a programmable freezer at a cooling rate of 1^0C/min. The solution containing oocytes was seeded automatically by the machine and held for 10 minutes. The oocytes were again cooled to -70^0C (slow freezing) at a cooling rate of 0.5^0C/min and stored in LN_2. The cryoprotectant was prepared in a solution containing Dulbeco's phosphate buffered saline (PBS) with 20% heat inactivated fetal calf serum, whereas in our study a higher fertilization rate was obtained with prepared M_2 media and the temperature was lowered several steps without temperature measurements. Initially the paillets were at 0^0C at the deep freezer for 20 minutes and then exposed to ice in LN_2 vapour, at a temperature of at -70^0C for 5 minutes. Thereafter the paillets were slowly immersed into the LN_2 before storage in the tanks for 7 days. The technique we used in our study can be described as between slow and rapid freezing, manually done without programmable freezers, thus making the procedure cost effective.

In rapid thawing protocols, the frozen oocytes in straws are rapidly transferred to a water bath at 37^0C. In our study thawing was carried out at room temperature and the paillets frozen into -196^0C were exposed to warmth at 37^0C by the increasing temperature gradually (between slow and rapid thawing). Initially at -196^0C and therafter exposed into LN_2 vapour with ice -70^0C. The ice was melted by running tap water and the paillets were transferred to a warm water bath at 37^0C for 5 minutes.

In our study, eight animals were used for each experiment. From each mouse 10-20 eggs were harvested, but in all the experiments equal number of eggs could not be used as some cumulus masses were adhered to the walls of the paillets and some eggs were lost during transfer through the media in the Nunc-4 well dishes. In addition, after thawing oocytes with plasma membrane not intact under perivitelline space, leaked cytoplasm, shrunken oocyte and presence of space between zona pellucida and cytoplasm were discarded. This explains why equal number of eggs could not be used in each experiment.

In our preliminary studies cumulus digested eggs were frozen in DMSO and sugar.But fertilization rates were low. Therefore we used eggs with cumulus masses for freezing and obtained better invitro fertilization results. The presence of the cumulus in the mouse oocyte reduces the toxic effects of DMSO and found better rates of fertilization in cryopreserved mouse oocytes[6,7]. We too have confirmed that in cumulus intact oocytes the presence of the cumulus offers better protection against toxicity by cryoprotectants.

A cost effective manual oocyte cryoprotecting protocol for mice has been developed which may be attempted in other animals and humans.

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