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IN VITRO CULTURE OF FROZEN AND THAWED MOUSE OVA*

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Seventy mouse ova at the 4-8-cell stages were collected at room temperature from mice treated with pregnant mare's serum gonadotrophin and human chorionic gonadotrophin. Seventy ova were divided into 5 groups, and each group was placed in a 0.5 ml plastic straw with 0.2 ml of Brinster's medium. Then, the straws were immersed into an ice bath (0°C) for 5 minutes. Next, an equal volume of 2 M dimethyl sulfoxide was added to the sample straw. The medium with ova was then seeded at -6°C and cooled to -100°C at a rate of 0.4 to 0.5°C per minute using an inexpensive freeze-thaw apparatus. After reaching -100°C, the sample straws were directly immersed in liquid nitrogen at -196°C and stored for 1-9 days.

Following the storage period, the sample straw were thawed at 12-15°C per minute from -196°C to 0°C. The samples were then diluted with fresh medium and placed inside a petri dish at room temperature (20-25°C). The ova were moved immediately to a fresh medium 3 times, then incubated at 37°C for 72 hours. Sixty out of the 70 ova were recovered from the straws after the freezing experiment, and 41 out of the 60 ova (68.3%) developed in vitro to the blastocyst stage within 72 hours.

INTRODUCTION

The demonstration of the survival of mouse ova after freezing and thawing at -196°C by WHITTINGHAM et al. ('72) and WILMUT ('72) was an exciting report for basic researchers as well as for people who were involved in the embryo transfer program. It presents a unique procedure for the preservation of valuable genetic materials, and in addition, information on the adjustment of recipient synchronization as well as the advantages in transportation of ova for embryo transfer by commercial establishments.

Recently, because of the amount of research done on the freezing and thawing of mammalian ova after WHITTINGHAM et al. ('72) and WILMUT ('72), a number of commercial devices which give a controlled, variable cooling rate have become available.

This study using an inexpensive and simple apparatus describes the in vitro culture of mouse ova after freezing and thawing.

* A part of this work was presented at the 68th meeting of the Japanese Society of Zootechnical Science in April 3, 1978.

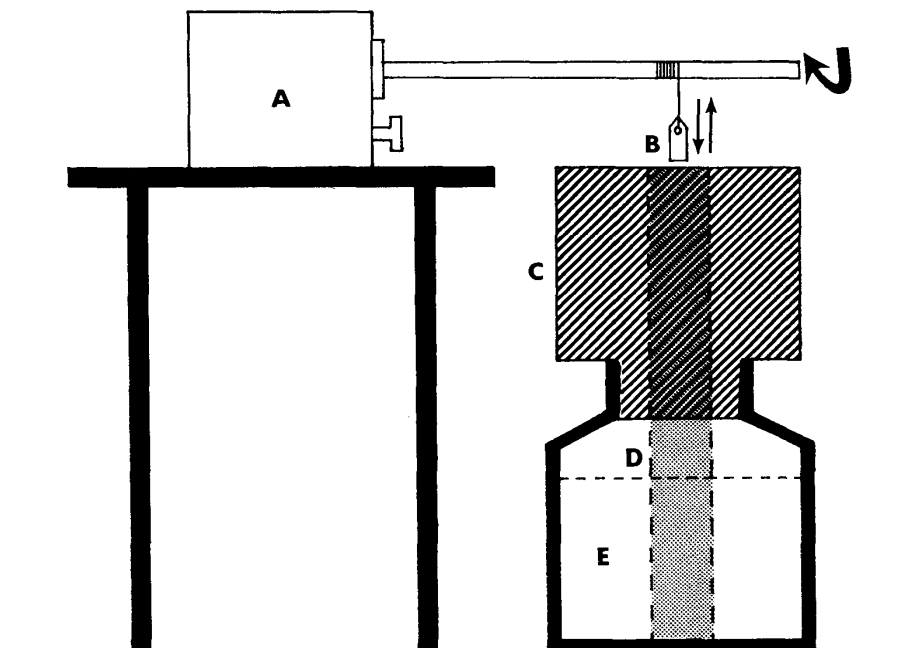
MATERIALS AND METHODS

Seventy fertilized ova were recovered from 10 gonadotrophin treated, C-57 Swiss mice. Fifteen 25-day-old female mice were subjected to superovulation by 5 IU of gonadotrophic hormones (PMSG, Ayerst Lab., Inc.) administered intraperitoneally. After 48 hours of treatment with PMSG, 5 IU of human chorionic gonadotrophin (APL, Ayerst Lab., Inc.) were injected intraperitoneally. Then, fertile males were introduced into the cages of hormone treated females overnight. The ratio of male to female in the cages was one male to three females. The following morning 10 females showing vaginal plug were selected for ova recovery.

One hundred ova at the 4- and 8-cell stages were recovered from the oviduct flush. Recovery was done under a dissection microscope with a 30 gauge needle and Brinster's medium (BMOC-3, Grand Island Biol. Co.) 48 hours after the injection of APL. Seventy good quality ova were selected from the 100 ova recovered morphologically under microscope.

The freezing unit consisted of a wide mouth (25 cm) liquid nitrogen container, an aluminum column with styrofoam, and a kymograph with a sample basket (fig. 1).

FIGURE 1 *Schematic drawing of the freezing and thawing unit*



- Notes: A Side position of kymograph
 B Sample basket
 C Styrofoam
 D Aluminum column
 E Liquid nitrogen container

When the container was filled with liquid nitrogen and the column was put in place with the mouth open, the liquid nitrogen vapor established a vertical temperature gradient ranging from 0°C at the top of the column to -196°C at the bottom of the liquid nitrogen container. When the sample basket was lowered into the column, it cooled down at a proportional rate. Conversely, when the basket was lifted up, it warmed up at a rate proportional to the speed of the basket. The speed was controlled by a kymograph and the temperature recorded by a thermocouple.

The ova were picked up from the flushed dish with 0.2 ml of BMOC-3 in a straw (0.25 ml plastic straw for frozen semen) at room temperature and immersed into an ice bath (0°C) for 5 minutes. At this temperature an equal volume of 2 M dimethyl sulfoxide (DMSO) was added as a cryoprotective agent. The samples were left for 15 minutes for equilibration. They were then moved to a -6°C cold bath for an additional 15 minutes. The medium was then seeded with an ice crystal and lowered into the container at a speed that gave a cooling rate of 0.4 to 0.5°C per minute between -6°C and -100°C. Once the samples reached -100°C, the straws were directly immersed into liquid nitrogen at -196°C and stored for 1-9 days. Following the storage period, the samples were raised with a speed giving a warming rate of 12-15°C per minute from -196°C to 0°C. Then the samples were placed in an ice bath. Next, the medium was diluted in a petri dish with fresh medium and the ova immediately located under a dissection microscope. The ova were moved into a fresh medium of BMOC-3 3 times to remove the DMSO.

The ova were then incubated *in vitro* as in the culture procedure¹⁾ and their viability assessed following 12 to 72 hours of incubation *in vitro*.

RESULTS

The summary results of the development of 60 ova to the blastocyst stage is shown in the table.

Ten ova were lost during the freezing and thawing procedure. After freezing and thawing, there was no damage morphologically either in the blastomere or the zona pellucida for 39 ova. However, there were degenerated blastomeres and 1 or 2 broken

TABLE *Summary results after freezing and thawing*

OVA FINDINGS	NO. OF OVA	
	FROZEN	DEVELOPED
No damage	39	27
Partially damaged blastomeres	12	8
Damaged or lost zona pellucida	9	6
Total	60	41 (68.3%)

membranes in 12 ova, and also damaged or completely lost zona pellucida for 9 ova after freezing and thawing.

Twenty-seven out of 39 normal ova (69.2%) developed into blastocysts after 24-72 hours culture in vitro. In 8 ova from the partially damaged blastomere's group, the rest of the blastomeres were recovered compensatorily and developed into blastocysts. From either the damaged or completely lost zona pellucida, 6 ova were still able to develop into blastocysts with a damaged zona pellucida or without the zona pellucida at all. In total, 41 out of the 60 ova (68.3%) developed into blastocysts after 24-72 hours culture in vitro (figs. 2-5).

DISCUSSION

The general technique for freezing mouse ova has been described previously (WHITTINGHAM et al., '72), and so far, the preimplantation stages have been shown to survive freezing and thawing only in the mouse (WHITTINGHAM, '74).

It appears that to be effective in the ova, dehydration by osmotic loss of water requires very low cooling rates before the whole ova itself becomes frozen. MIYAMOTO & ISHIBASHI ('76) reported that the highest survival rate of mouse ova after freezing experiments was obtained in the medium of 1.2 M DMSO with cooling rates of 0.2-0.5°C per minute and with warming rates of 2-15°C per minute. MIYAMOTO et al. ('77) reported that a modified TCM 199 was a relatively satisfactory medium for freezing mouse ova. If dehydration occurs too rapidly, there is damage to the cell membrane of the blastomere, which is associated with changes in the chemical composition of the extra- and intra-cellular solution. Also, a high concentration of chemical components may damage irreversibly the fine structures in the cytoplasm. DMSO in a PBS solution was usually added directly to mouse ova at 0°C (WHITTINGHAM et al., '72; LEIBO et al., '74). However, in the present study, DMSO in a BMOC-3 solution was used during freezing and thawing, as well as for cultures in vitro after thawing.

It appears that these very low cooling rates in the ova are necessary for effective dehydration by osmotic loss of water before the whole ova itself becomes frozen. These prevent or minimize the amount of intra-cellular ice formation, which is one of the main factors causing damage during thawing. Any factors that cause a sudden increase in the rate of cooling, e. g., supercooling or transferring of the ova directly to liquid nitrogen before the ova have become adequately dehydrated, are deleterious to the ova. For optimal survival of mouse ova, slow cooling to approximately -60°C is necessary before transferring them to liquid nitrogen (LEIBO et al., '74), and as a safeguard, ova are usually slowly cooled to -70 or -80°C before being transferred directly to liquid nitrogen.

The apparatus described in this study is an economic approach to a controlled cooling and warming rate for small samples in the laboratory. It is inexpensive to

build, very economical to run, and flexible cooling rates can be generated. Some commercial devices giving a controlled, variable cooling rate are available, however, most of them are expensive and have a fixed range of cooling rates.

The present author lost 10 ova in the course of the freezing and thawing experiment; it is difficult to clarify the reason, but perhaps the small straws were not adequate containers for a freezing experiment, or the broken zona pellucida or loss to the zona pellucida during freezing and thawing contributed to the loss of the ova.

SCHNEIDER et al. ('74) reported that 36.1-75.5% of ova developed into blastocysts after freezing experiments with mice, and 9.7% exhibited one or more damaged blastomeres after thawing. However, they were still capable of developing into fetuses and later into viable young. In this study, 12 ova had one or more damaged blastomeres after freezing and thawing; the rest of the blastomeres were recovered compensatorily and developed into blastocysts. The recovery of the damaged blastomeres in vitro culture is very interesting because there is a possibility that a single blastomere will develop from a 4--8-cell mouse ova.

Survival of mouse ova after freezing and thawing has been assessed in two ways: 1) development to the blastocyst stage in vitro and; 2) development to full term fetuses and liveborn after transfer (WHITTINGHAM, '74). However, the overall survival of frozen-thawed mouse ova to fetuses and live births in a recent fairly extensive study was approximately 20-30% (WHITTINGHAM et al., '77).

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EXPLANATION OF PLATE

PLATE

- Fig. 2 4-cell stage ova just after collection from the oviduct
Approx. $\times 200$
- Fig. 3 4-cell stage ova after freezing and thawing
A : Two normal blastomeres and 2 degenerated blastomeres
without zona pellucida
B : Naked blastomeres attached to another ovum
C : Two blastomeres and 1 degenerated blastomere attached
to another ovum
D : Irregularity of zona pellucida
Approx. $\times 200$
- Fig. 4 Morula stage ova in vitro culture after freezing and thawing
One ovum developed without zona pellucida
Approx. $\times 200$
- Fig. 5 Developed blastocysts at 72 hours in vitro after freezing
and thawing
Approx. $\times 200$

