A Comparison of the Effects of Different Degrees of Zona Pellucida Damage Followed by Cryopreservation on the Postthaw Development of Mouse Embryos

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Submitted: September 4, 1996

Accepted: November 1, 1996

Purpose: The totally intact zona pellucida is not essential for the development of embryos. It is still unclear how much effect the degree of damages to the zona pellucida will have on the developmental potential of postthaw embryos after cryopreservation. We compared the developmental potential of cryopreserved mouse embryos after induction of two degrees of mechanical damage to the zonae pellucidae by micromanipulation.

Methods: In experiment I, the development of 124 cryopreserved ICR mouse embryos to the blastocyst stage after zona pellucida penetration of two-cell embryos as in the procedures of subzonal sperm insertion (SUZI) was compared with the development of zona-intact cryopreserved embryos. In experiment II, the zonae pellucidae of 93 two-cell mouse embryos were dissected as in the procedures of partial zonal dissection (PZD), following which the embryos were frozen. This postthaw development was also compared with that of zona-intact two-cell cryopreserved embryos. All the embryos were thawed and cultured to the blastocyst stage. Additional controls were provided by culturing zona-intact and zona-penetrated or zona-dissected embryos without cryopreservation.

Results: The development of unfrozen mouse embryos was not affected by either zona penetration ($P = 0.433$) or zona dissection ($P = 0.659$). The developmental potential of cryopreserved mouse embryos was significantly affected after zona dissection (blastocyst rate, 31% ZD vs 72% control; $P < 0.001$) but not after zona penetration (blastocyst rate, 59% ZP vs 64% control; $P = 0.441$).

Conclusions: The quality of cryopreserved embryos was affected by a large hole on the zona pellucida created by zona dissection but not by simple zona penetration.

KEY WORDS: cryopreservation; micromanipulation; zona pellucida.

INTRODUCTION

The presence of a zona pellucida appears to be necessary for the development of early embryos, as demonstrated in zona-free rabbit embryos, which showed reduced growth in vitro and failure to implant in synchronized recipients (1). However, a totally intact zona pellucida is not essential for the development of early mouse embryos (2–5). A few reports have demonstrated that development of early mouse embryos in which zonae pellucidae were damaged by mechanical embryo biopsy or zona drilling is not necessarily affected by cryopreservation, compared with zona-intact frozen mouse embryos (3,4,6–8). It is still unknown whether the size of the hole created in the zona pellucida will affect the ability of the embryo to survive cryopreservation. We designed this study to determine the developmental potential of mouse embryos undergoing cryopreservation after two degrees of micromanipulation-induced mechanical damage to the zona pellucida.

MATERIALS AND METHODS

Embryo Collection

Six-week-old ICR mice were given intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin (PMSG) to induce superovulation. These injections were followed by 5 IU human chorionic gonadotropin (hCG) 48 hr later and the mice were mated on the same day. Two-cell embryos were flushed from excised oviducts 42–48 hr after hCG injections. The embryos

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were randomly divided into eight groups as described below under Experimental Design.

Micromanipulation of the Zona Pellucida

Experiment I. Zona Penetration (ZP). Micromanipulation was performed under an Zeiss Axiovert-100 inverted microscope using a pair of Narishige hydraulic remote control micromanipulators, B2152 for holding pipettes and MM-188 and MO-188 for penetrating pipettes; Narishige, Tokyo. The holding pipettes were made from borosilicate glass capillaries (Sutter B-100-75-10; O.D., 1.0 mm; I.D., 0.75 mm) which were flame-pulled and polished under a Narishige MF-83 microforge to create outer and inner diameters of 100 and 20–25 μm , respectively. The penetrating pipettes were made from Sutter borosilicate glass capillaries and single pulled to an outer diameter of 6–10 μm using a Narishige PD-5 micropipette puller.

Mouse embryos were placed in M-16 culture medium with 0.3% bovine serum albumin (BSA) under equilibrated mineral oil (SIGMA, M8410, lightweight). The inverted microscope was contained in a hood at 37°C under 5% CO_2 in room air. The two-cell mouse embryo was held by the holding pipette while the penetrating pipette was pushed through the zona pellucida into the subzonal space to create a penetrating defect as in the procedure of subzonal sperm insertion (SUZI).

Experiment II. Partial Zona Dissection (PZD). The dissecting pipettes were made from Sutter borosilicate glass capillaries with a single pull to an outer diameter of 8–12 μm using Narishige PD-5 micropipette puller. The zona pellucida of the two-cell mouse embryo was penetrated from 2 to 10 o'clock with the dissecting pipette. The incorporated portion of the zona pellucida was removed with a rubbing motion between the holding and the dissecting pipettes to create a linear hole on the zona pellucida, as in the procedure of PZD.

Embryo Cryopreservation

Before freezing, the mouse embryos were placed directly into 0.5 M PROH in a phosphate-buffered saline (PBS) solution supplemented with 20% fetal cord serum (FCS) at room temperature for 5 min. The embryos were then moved to 1.0 M PROH for 5 min, to 1.5 M PROH for 10 min, and finally, to 1.5 M PROH with 0.2 M sucrose for 5 min. The embryos were placed into plastic straws during the last 5-min period. Cooling and freezing were then carried out in a freezer (Cryo-Med; 990C Freezing Chamber and

1010 Controller). The straws were cooled from room temperature down to -7°C at a rate of $2^\circ\text{C}/\text{min}$. The temperature was held at -7°C for 5 min and ice crystallization was induced by touching each straw with cold forceps. The straws were then cooled at a rate of $0.3^\circ\text{C}/\text{min}$ down to -30°C and held for 15 min. The straws were finally plunged into liquid nitrogen.

Thawing was carried out by quickly removing the straws from the liquid nitrogen and plunging them into a 37°C water bath until thawing was completed. The embryos were then transferred to 1.0 M PROH and 0.2 M sucrose in PBS supplemented with 20% FCS at room temperature for 5 min. A stepwise procedure was followed for moving the embryos to a 0.5 M PROH solution with 0.2 M sucrose for 5 min, to 0.2 M sucrose for 5 min, and finally, to PBS with 20% FCS for 5 min. The mouse embryos were then placed in M-16 medium with 0.3% BSA.

Culture System

Embryos in each control and study group were cultured to the blastocyst stage in M-16 medium with 0.3% BSA at 37°C under mineral oil equilibrated by 5% CO_2 in room air. The development of the embryos was observed every 24 hr for 4 days.

Experimental Design

In experiment I, 109 zona-intact embryos were cultured without having been frozen, as a control for the culture environment. Another 96 embryos were cultured immediately following zona pellucida penetration without freezing. One hundred forty zona-intact embryos were frozen, thawed, and cultured to control for the effects of cryopreservation on the development potential of mouse embryos. An additional 124 mouse embryos in which the zona pellucida had been penetrated were frozen, thawed, and cultured.

In experiment II, 89 zona-intact mouse embryos were cultured without freezing as the control group. Ninety-seven embryos in which the zonae pellucidae had been dissected were also cultured without freezing. Eighty-nine zona-intact mouse embryos were frozen, thawed, and cultured as a control for cryopreservation effect. Ninety-three embryos in which the zonae pellucidae had been dissected were frozen, thawed, and cultured.

Statistical Analysis

Data were analyzed using the chi-square test. Differences reported were considered significant at $P \leq 0.05$.

Table I. The Effect of Zona Penetration (ZP) and Cryopreservation on the Developmental Potential of Mouse Embryos^a

	Control	ZP	Freezing	ZP + freezing
No. of 2-cell embryos	109	96	140	124
No. of blastocyst formation	97	82	90	74
Rate of blastocyst formation (%)	89.0*.*.*.*	85.4*	64.3*.*.*.*.*	59.5**

^a Analysis by chi-square test.* $P = 0.433$.** $P = 0.441$.*** $P < 0.001$.

RESULTS

The developmental potential of unfrozen mouse embryos was not affected by zona penetration ($P = 0.433$) or zona dissection ($P = 0.659$) compared with zona-intact unfrozen embryos (Tables I and II). Cryopreservation decreased the blastocyst formation rate of zona-intact mouse embryos from 89 to 64.3% in experiment I ($P < 0.001$) and to 72.3% in experiment II ($P = 0.009$). Zona penetration resulting in a small defect in the zona pellucida did not significantly affect the rate of blastocyst formation in cryopreserved embryos (59.5%) compared with zona-intact cryopreserved embryos (64.3%; $P = 0.441$). However, creating a larger linear hole by zona dissection did significantly reduce the rate of blastocyst formation in cryopreserved embryos (31.9%) compared with zona-intact cryopreserved embryos (72.3%; $P < 0.001$).

DISCUSSION

Cryopreservation is a standard procedure used to preserve supernumerary embryos obtained in IVF

Table II. The Effect of Partial Zona Dissection (PZD) and Cryopreservation on the Developmental Potential of Mouse Embryos^a

	Control	PZD	Freezing	PZD + freezing
No. of 2-cell embryos	89	97	89	93
No. of blastocyst formation	78	87	64	29
Rate of blastocyst formation (%)	89.1*.*.*.*	90.9*	72.3*.*.*.*.*	31.9**

^a Analysis by chi-square test.* $P = 0.6590$.** $P < 0.001$.*** $P = 0.009$.

cycles because stable and acceptable pregnancy rates have been achieved after improvement in the freezing and thawing techniques. In recent years, micromanipulation including partial zona dissection (PZD), subzonal sperm insertion (SUZI), and intracytoplasmic sperm injection (ICSI) have been developed to improve the fertilization of human eggs in which male factors account for a couple's infertility. A few reports have described pregnancy results of human cryopreserved embryos obtained after micromanipulation. Obasaju *et al.* reported that the human embryo survival rate and blastomere survival rate of SUZI embryos and zona-intact embryos followed by cryopreservation were similar. One clinical pregnancy was obtained of 16 patients who received thawed SUZI embryos (9). Similar clinical pregnancy rates have been achieved with human cryopreserved embryos obtained from ICSI procedures and from the traditional insemination of IVF cycles in the study by Steirteghem *et al.* (10). The clinical abortion rate was higher for cryopreserved ICSI embryos (40.9%) than for cryopreserved zona-intact embryos (27%) (10). The quality of the human embryos in the two above reports was not strictly controlled because the embryos which were cryopreserved had remained after the transfer of fresh embryos in IVF cycles.

In several reported mouse embryo studies, zona pellucida defects created by embryo biopsy did not compromise continued development of the embryos in vitro or in vivo (2,3,5), especially when fewer than three blastomeres of an eight-cell embryo were removed (4). Zona pellucida drilling also did not affect the development of cryopreserved embryos (6,8). The biopsied embryos in which the zonae pellucidae were damaged by mechanical puncture (3,4,7) were shown to survive and had a similar blastocyst formation rate after cryopreservation compared with zona-intact cryopreserved embryos. Thompson *et al.*, however, reported that embryo biopsy by zona slitting and zona piercing reduced blastocyst formation rates after embryo cryopreservation (2). Liu *et al.* found that removing more than three blastomeres from eight-cell embryos reduced both blastocyst formation rates and live offspring rates after embryo cryopreservation. These results suggest that the greater the trauma to embryos during embryo biopsy, the greater the susceptibility to freeze-thaw damage, making it more likely that there will be a decrease in the development potential of postthaw embryos (4).

It is still unknown how large a hole created by micromanipulation must be to affect the developmental potential of embryos after cryopreservation. In this investigation, a larger linear hole produced by zona

dissection rendered mouse embryos more susceptible to freeze-thaw damage, compared with a smaller hole created by zona penetration. Similar results were reported by Thompson *et al.*, who found that zona slitting resulted in a lower blastocyst formation rate than zona piercing after embryo cryopreservation (2). Zona dissection creating a persistent hole on the zona pellucida in our investigation has a more significant effect on the development of cryopreserved embryos, compared with zona slitting creating a zonal crack in Thompson and co-workers' investigation. According to our investigation, it appears that the small holes created by zona penetration with a small-diameter needle as is usually used for SUZI and ICSI does not significantly affect the developmental potential of cryopreserved embryos. However, embryos in which the zonae pellucidae have been dissected as for PZD are not suitable for cryopreservation.

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