The Effect of Prefreeze in Vitro Culturing on the Success of Embryo Freezing in Mice

ANTONIO PELLICER, 1-3 ABRAHAM LIGHTMAN, 1,4 and ALAN H. DeCHERNEY 1

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There is currently a controversy as to whether the prolonged in vitro culturing of embryos before freezing has a deleterious effect on their ability to survive freezing and thawing. We compared the survival rate of frozen/thawed mouse embryos after in vitro culturing, from the two-cell stage through the eight-cell, morula, and blastocyst stages, with the survival of embryos developed in vivo to the same stages. Following induced superovulation and mating, embryos in the desired cleavage stage were flushed from the oviducts and/or uterus and either cultured in vitro or frozen immediately in sterile glass ampoules to -40°C and plunged into liquid nitrogen for storage. Dimethyl sulfoxide (1.5 M) was used as cryoprotectant. After thawing, the survival rate (determined by the morphological appearance of the embryos) was significantly lower in the eight-cell stage embryos in the group grown in vivo \( (P < 0.05) \). The number of embryos developing into expanded and hatched blastocysts was not significantly different when the in vivo vs in vitro cultures were compared over each of the three cleavage stages: eight cells (82 vs 83%), morula (92 vs 87%), and blastocyst (33 vs 51%), respectively. There was a significant decrease in the development rate of blastocyst-stage embryos when compared with earlier stages under both culture conditions \( (P < 0.001) \). It is concluded that, compared to in vivo-grown embryos frozen at the same stages, prolonged in vitro culture does not reduce the embryos' ability to develop normally.

KEY WORDS: embryo freezing; mouse; in vitro culturing; in vivo development.

1 Department of Obstetrics and Gynecology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510.
2 Fulbright Fellow in Reproductive Endocrinology.
3 To whom correspondence should be addressed at Department of Obstetrics and Gynecology, Valencia University School of Medicine, Avenida Blasco Ibáñez, 17, 46010, Valencia, Spain.
4 Lalor and Bruner Foundations Fellow.

INTRODUCTION

All preimplantation embryonic stages have been successfully frozen in mice (1-3) and humans (4-6). The use of cryopreservation in human in vitro fertilization (IVF) entails a lengthy period of in vitro culturing, especially if advanced stages of embryo development are required.

Although it has been suggested that this period of in vitro culture may select against those embryos unable to display continued growth (5), it is also known that in vitro culture constitutes a less favorable environment than that found in the reproductive tract. It has been shown that mouse embryos cultured in vitro have a reduced rate of cleavage compared to those grown in vivo (7).

This experiment was designed to investigate the relative effects of in vivo development as opposed to in vitro culturing on the survival and growth rates of mouse embryos after freezing and thawing.

MATERIALS AND METHODS

Embryos

Female CB6F 1 mice, 6-12 weeks old, were superovulated by an intraperitoneal injection of 5 IU pregnant mare serum (PMS) (day -2, 3:00 PM), followed 46 hr later (day 0, 1:00 PM) by 5 IU human chorionic gonadotropin (hCG) and mating with males of the same strain. Mated females were sacrificed at different intervals in order to obtain eight-cell embryos (day 3, 8:00 AM), morulae (day 4, 8:00 AM), or unexpanded blastocysts (day 4, 3:00 PM) developed in vivo in the reproductive tract. Another group of females was sacrificed 50 hr after hCG injection (day 2, 3:00 PM) to obtain two-cell
embryos which were cultured in vitro until they reached either the eight-cell (day 3), the morula (day 4), or the blastocyst (day 5) stage.

Embryos were flushed from the excised tubes (2- and 3-day-old embryos) and horns (day 4) using a 30-gauge needle and Ham's-F10 medium (Gibco Co., Grand Island, NY) plus 1% bovine serum albumin (BSA) (Sigma Co., St. Louis, MO, fraction V). The pH and osmolarity of the culture medium were adjusted to 7.4 and 280 ± 10 mOsm. The same medium was used for in vitro culture until the embryos reached the stage desired for freezing. Embryos were cultured in groups of 10-20 per dish, in a humidified atmosphere of 5% CO2 in air at 37°C.

Only embryos which appeared morphologically normal under the dissecting microscope were selected for this study. Normal appearance is defined in this study as the presence of even blastomeres, in number according to the age of the embryos, and an intact zona pellucida.

Freezing-Thawing Procedure

The technique used for freezing and thawing was based on the method described by Whittingham et al. (8). Before freezing, embryos were transferred to dishes containing modified Dulbecco’s phosphate-buffered salt solution (PB1) (9) and 10% fetal calf serum (FCS) at room temperature. Between 5 and 10 embryos were placed in 0.1 ml of the medium in sterile tissue culture glass ampoules and cooled on crushed ice to 0°C. Subsequently, 0.1 ml of 3 M dimethyl sulfoxide (DMSO) (Sigma Co.), previously cooled to 0°C, was added to the ampoules (final concentration, 1.5 M DMSO). The mixture was allowed to equilibrate for 15 min.

After equilibration, the glass ampoules were flame-sealed and placed in a programmable biological freezer (Planer R-204). The temperature was lowered to -6°C at a rate of 1°C/min. Seeding was induced by touching each ampoule at the meniscus with a pair of forceps previously cooled in liquid nitrogen (LN2). After being held for a further 10 min at the seeding temperature, the ampoules were cooled to -40°C at a rate of 0.3°C/min and then plunged into LN2 at -196°C for storage.

Thawing was achieved by gently agitating the ampoules in a water bath at 37°C until all traces of ice had disappeared. Subsequently, 0.8 ml of PB1 plus 10% FCS was added to the ampoules at room temperature.

Survival Evaluation

The contents of each ampoule was emptied into culture dishes and the recovered embryos were washed in PB1 and Ham’s F-10 at room temperature. The number and appearance of embryos recovered after thawing were recorded. Embryos were classified into three categories: (i) good appearance (GA)—not damaged, or more than 50% of the blastomeres intact and zona pellucida apparently normal; (ii) zona broken (ZB)—zona pellucida damaged, regardless of the condition of the blastomeres; and (iii) bad appearance (BA)—totally degenerated embryos or less than 50% of the blastomeres apparently normal after thawing. All embryos except those wholly degenerated after thawing were further cultured in Ham’s F-10 plus 1% BSA under the conditions cited above.

After 24, 48, and 72 hr of culture, in vitro development of the embryos was assessed through the expanded and hatched blastocyst stages. Data were analyzed by chi-square tests. Differences between groups were considered statistically significant when P ≤ 0.05.

RESULTS

A total of 302 mouse embryos with a normal appearance under the dissecting microscope was used in this study. One hundred fifty-two embryos in different stages of development were obtained from the reproductive tract of 13 mice (mean, 11.7 ± 4.3 per mouse). The remaining 150 embryos were obtained at the two-cell stage and were cultured in vitro until they reached the stages identified above. These embryos were selected from 184 embryos obtained after flushing the oviducts of 11 mice on day 2 (mean, 16.6 ± 3.7 per mouse). The distribution of the embryos in each developmental stage and group is shown in Table I.

After freezing and thawing, the recovery rates were equivalent in all groups. There was no significant difference in appearance at the morula and blastocyst stages between the embryos grown in vivo and those grown in vitro. However, compared to the in vitro cultured embryos, there was a signif-