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MOUSE EMBRYO VITRIFICATION

Kent A. Lehman, Max A. Lehman, and Thomas A. Cole
Department of Biology, Wabash College, Crawfordsville, IN 47933
and Berne Animal Clinic, Berne, IN 46711

ABSTRACT: Cryopreservation of embryos is an important technique in genetic and developmental research as well as in the applied area of livestock production. We have studied the effects of freezing upon mouse embryos by comparison of slow freezing and vitrification regimens. This mouse model system is used with the possibility of direct application to livestock, particularly lines of dairy cattle. The slow-freezing technique involves use of a programmable cryopreservation system, which is time-consuming, expensive, and somewhat unwieldy in site use compared to vitrification, the direct exposure of embryos in straws to liquid nitrogen from 21° C. Thirteen embryos from superovulated mice were subjected to slow freezing; 69.2% (9) survived the storage period as judged by further development in tissue culture during 12 to 18 hour observation times. Vitrification was done in the presence of a cryoprotectant solution, glycerol-sucrose or glycerol-propanediol. These trials gave 30.8% (26 embryos) survival for glycerol-sucrose and 34.3% (35 embryos) for glycerol-propanediol. The simplest interpretation of these data is that slow freezing is superior to the vitrification procedures used here, but the approximately 30% survival suggests that further experiments should be undertaken.

INTRODUCTION

Cryopreservation of mammalian embryos is a technique useful in basic and applied research. Transplantation of frozen embryos for implantation in surrogate females is particularly important in the dairy industry. We have used a mouse system to test the effect of vitrification versus conventional freezing on the survival rate of embryos. Conventional freezing involves the use of a programmable, computerized freezer for repeatable cool-down rates. This freezer system is expensive, unwieldy, and time-consuming compared to the simpler system of vitrification by direct plunging into liquid nitrogen.

Vitrification involves the formation of a super-cooled liquid without the formation of ice crystals; that is, the conversion of the liquid to a glass. This formation of a glass is a property of all liquids that are cooled at a sufficiently high rate. The cooling rate is fundamental in bypassing the formation of crystals as the normal freezing point is reached (MacFarlane, 1987). In this work, the embryos were cooled rapidly to ensure vitrification, and the question of viability of embryos subjected to different freezing rates was addressed.

There are two factors in freezing embryos that prove to be detrimental to embryo survival. The first is over-dehydration of the cells and the second is intracellular ice (Pomp and Critser, 1988). As the embryo is cooled slowly, extracellular ice crystals form. As the water freezes, the solution remaining between the embryo and ice becomes hypertonic. Through osmosis, this salt solution dehydrates the embryo. Once the embryo has reached the critical dehydration level, it must be plunged into liquid nitrogen to prevent over-dehydration. With respect to intracellular ice, crystals form when the embryo is cooled too quickly, not allowing extracellular ice crystals to form. Large ice crystals form within the membrane, causing considerable damage.

MATERIALS AND METHODS

Mouse population. CD-1 mice were obtained from Harlan Sprague-Dawley in Indianapolis, Indiana, and maintained at 25° C with 16 hours of light per day with water and food available *ad libitum.* Conditioning in this environment before experimentation was for three weeks. Males were kept separately, and five or fewer females were kept together (standard laboratory cages).

Superovulation cycle. Five units of PMS (pregnant mare serum) were injected into each mouse on day 0. Five units of HCG (human chorionic gonadotropin) were injected after forty-eight hours. The female was placed in a cage with a male for twelve to twenty-four hours. At seventy-two hours, the females were sacrificed by cervical dislocation.

Embryo recovery. Morula or blastocyst embryos were flushed from extirpated uteri from which the ovaries and fat bodies had been removed. The irrigation medium was Dulbecco's phosphate buffered saline containing calcium and glucose. Each uterine horn was flushed 3 to 4 times with 2.5 to 3.0 ml medium with a 25 gauge needle (3/8" on a 3 ml syringe). After settling in the flushing vessel (100 x 15 mm Petri dish), the embryos were moved to a 35 x 10 mm holding dish until all recoveries had been made.

Freezing solutions. The embryos were then placed in their respective freezing solutions and equilibrated for thirty minutes before freezing. The solution for programmed cooling was constituted from 1.725 ml Dulbecco's medium containing 4% BSA (bovine serum albumin), 0.275 ml DMSO (dimethyl sulfoxide), and 0.5 ml calf serum (final concentration = 1.5 M DMSO). Two cryoprotectant solutions were used for vitrification. The first contained 8 parts Dulbecco's medium containing 4% BSA, 1 part glycerol, and 1 part 1.5 M sucrose. The second solution was the same as the first except that 1 part propanediol was added in place of sucrose.

Freezing and storage. After equilibration in freezing medium the embryos were loaded into a 1/4 ml semen straw with a tuberculin syringe. The controls (conventional freezing) were frozen with a computer freezer which followed the following regimen: from 21° C to -6° C, the temperature decreased at a rate of 4° C/min; at -6° C, the temperature was held for ten minutes, allowing the embryo to be seeded; then, the temperature was decreased at a rate of 0.5° C/min until it reached -30° C; at -30° C, the embryo was plunged into liquid nitrogen (-196° C). The experimental embryos (vitrification) were lowered into liquid nitrogen directly from 21° C. It was done over a period of twenty seconds to prevent explosion of the semen straw. The embryos were stored in liquid nitrogen until all embryos from the project had been harvested and frozen.

Thawing and observation. The embryos were thawed by placing the straws in a 21° C water bath until the ice was gone (about 10 to 20 seconds). Each embryo was then washed thru four separate Dulbecco's-4% BSA solutions to remove the cryoprotectant, which is toxic to the embryo. The embryos were then incubated at 37° C in Dulbecco's-4% BSA with 10% calf serum for twelve to eighteen hours. Embryo survival was determined by embryo progression as viewed with 400x magnification. If the embryo developed to the expanded blastocyst or hatched blastocyst stage, then it was considered alive.

Table 1. Cryosurvival rate (formulations for control and experimental cryoprotectant solutions are given in the text).

Condition	Number Frozen	Number Survived	Survival Percentage (%)
Controls	13	9	69.2
Glycerol and Sucrose	26	8	30.8
Glycerol and Propanediol	35	12	34.3

RESULTS AND DISCUSSION

The percentage of surviving embryos from the vitrification (Table 1) is an indication that the procedures used in this project had a relatively high success rate when compared to other laboratories. Ideally, manipulations leading to the vitrification process should be carried out at 5° C (cold room operation vs room temperature), because it lessens the toxicity of the cryoprotectants (Rall, 1987). Also, the toxicity of the cryoprotectant and the amount of osmotic damage are directly influenced by the concentration of cryoprotectant along with the time and temperature of exposure (Rall, 1987).

While these percentages are encouraging, it must also be remembered that the survival rate was based on *in vitro* incubation and not on actual pregnancies after implantation of the thawed embryos. The percentages usually show a significant decline when based on actual pregnancies. The simplest interpretation of these mouse studies is that conventional freezing is superior to vitrification and that the use of vitrification procedures in cattle is not warranted at the present time.

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