

Assessment of the developmental potential of different strains of cryopreserved murine embryos in a cryoprotectant medium.

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ABSTRACT

Cryopreservation can preserve organelles, cells, tissues, and other biological samples. In this method, samples are cooled keeping it in extremely low temperatures. The method adopted is cost-effective. It is an important method in cell and tissue research, essential in storing as well as preserving tissue for the long term. However, freezing kills the majority of live tissues because it leads to the formation of extracellular and intracellular ice crystals, which change the chemical makeup of the cell, causing harm. Excessive cooling of tissue may damage its original structure and function. The transition from the liquid to the solid phase is the main challenge that cells overcome at low temperatures. In this study, we examined the in vitro development of mouse embryos after cryopreservation through the vitrification method. Vitrification is a phenomenon in which a liquid solidifies without the formation of ice crystals. Thus, the process is referred to as a glass transition or ice-free cryopreservation. In our study, vitrification involved a glycerol-based cryoprotectant. The embryos were cultured in M16 culture medium and pretreated in Phosphate Buffered Saline (PBS) for 2 minutes at 25 °C and treated with vitrification solution for 60 seconds. In this method, the straws were thawed in air for 10 seconds and subsequently in water at 25°C for 20 seconds, and the embryos were further cultured in vitro for 48 hours to estimate the hatching efficiency of blastocysts. The in vitro blastocyst developmental rate with 0.6% BSA with glycerol was 82%, and morphologically normal embryos were 25%. The various mouse models used in this study were CD1, FVB/N and GU 2H. These mouse models were maintained in controlled room temperatures and environmental conditions.

Keywords: IVF, cryoinjury, slow freezing, vitrification.

INTRODUCTION

Cryopreservation is a process that maintains biological samples in a state of suspended animation at cryogenic temperatures for any considerable period and is used to preserve the fine structure of cells. (Mazur 1990) Moreover, the recent advances in the field of cryopreservation in animal tissues have revolutionized the field of reproductive medicine (Kasai 2002). In this study, we examined the in vitro development of mouse embryos after cryopreservation through a vitrification method. Vitrification is a phenomenon in which a liquid solidifies without the formation of ice crystals (Hans et al 2003). Thus, the process is referred to as a glass transition or ice-free cryopreservation. (Nagy et al., 2020). In our study, vitrification involved a glycerol-based cryoprotectant method. However, freezing kills the majority of the live tissues because it leads to the formation of extracellular and intracellular ice crystals, which change the chemical makeup of the cell, causing harm (Ho et al 1995). Excessive cooling of tissue may damage its original structure and function (Jin et al 2010). The transition from the liquid to the solid phase is the main challenge that cells overcome at low temperatures. Although the surface area of four- and eight-cell embryos is larger than that of two-cell embryos, the permeability of the plasma membrane of the embryos to water and cryoprotectants is as low as that of two-cell embryos. (Edashige,2016)

MATERIALS AND METHODS

Handling and use of mice.

Mice were maintained in the central animal facility of the Institute on a 12-hour light:12-hour dark schedule at 23°C–25°C. All the animal experiments were carried out according to the guidelines on the use of laboratory animals for research set by the Indian National Science Academy (INSA, New Delhi, India) and approved by the Institutional Animal Ethics Committee (IAEC).

Superovulation and oocyte collection.

Superovulation was performed as mentioned below Each female mouse was intraperitoneally injected with 7.5 IU of eCG (Cat. no. hor-272-a; ProSpec Bio USA, East Brunswick, NJ) or 100 µL of AIS plus 3.75 IU eCG (CARD HyperOva; Cosmo Bio USA, Carlsbad, CA) followed by intraperitoneal injection of 7.5 IU human chorionic gonadotropin (hCG; cat. no. hor-007; ProSpec Bio USA, East Brunswick, NJ) 48 post-AIS or eCG. The AIS had a titer of 1:1 000 000 as defined by the final dilution of the antiserum required to bind 50% of 125I-labeled bovine 32-kDa inhibin. Animals were euthanized via cervical dislocation 14-15 hours following the hCG injection. The oviducts were dissected out from each previously superovulated donor mouse

- Equilibrium media:
 - PBS medium containing 0.6% (W/V) BSA
 - 1.6 M glycerol in PBS medium containing 0.6%(W/V) BSA
 - 4.2 M glycerol in PBS medium containing 0.6% (W/V) BSA
 - 1.0 M glycerol in PBS medium containing 0.6% (W/V) BSA
- Vitrifaction solutions: VS3a solutions (Rall et al. 1985)
- Vitrifaction of Embryos (cryoprotectant media)
 - Pipette – 10 to 20 embryos – dish of PBS medium (0.6% (W/V) BSA)
 - Mix with the tip of the pipette – equilibrate at room temperature for 10 minutes
 - Pipette – dish of 1.6M glycerol in PBS medium containing 0.6% BSA
 - Mix the tip of the pipette – equilibrate at room temperature for 20 minutes
 - After 20 minutes exposure to 1.6M glycerol – pipette the embryos into the dish of 4.2M glycerol in PBS medium containing 0.6% BSA
 - Mix the tip of the pipette – equilibrate at room temperature (for dehydration)
 - Pipette the embryos into the vitrification column of each cryotube after 1-1.5 minutes' exposure to 4.2M glycerol
 - Exposure to the vitrification solution for 60 sec
 - Cryo-tube placed on LN₂ in a Dewar flask for more than 3 minutes before being immersed in liquid nitrogen

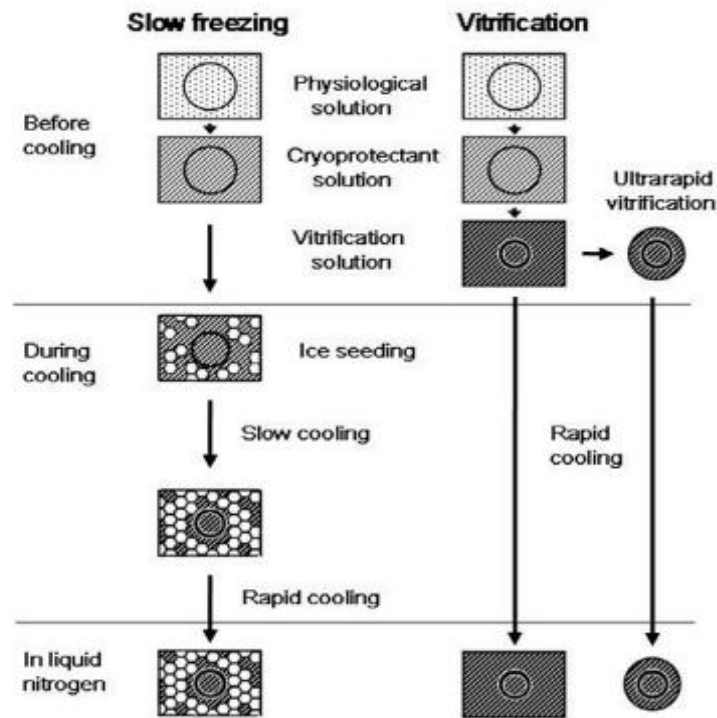


Figure 1. Schematic presentation of an embryo (circle) before cooling, during cooling and in liquid nitrogen in slow freezing, conventional straw vitrification, and ultrarapid vitrification. White hexagons represent ice crystals.

RESULTS

Morula-stage embryo in the vitrification solution with glycerol containing 0.6% BSA

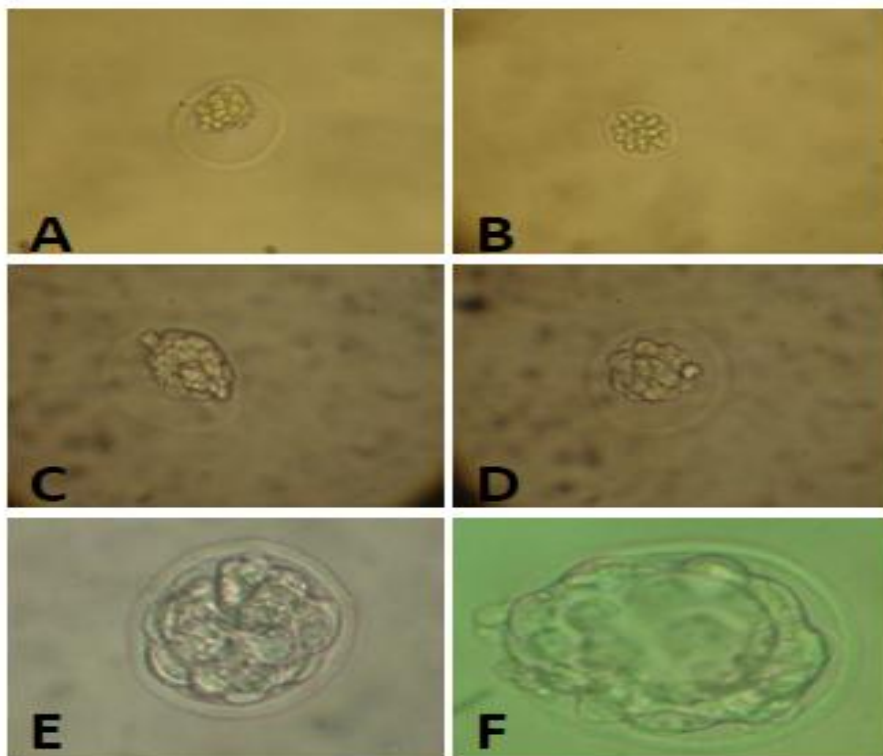


Figure 2.

- (A) Freshly recovered morula stage embryo in 1.6M glycerol solution.
- (B) Morula stage embryo in 4.2M glycerol.
- (C) Morula stage embryo in 6.5M glycerol.
- (D) Morula stage embryo in 0.5M sucrose in PBS.
- (E) The embryo develops into compacted morula after 24h culture with M16 culture medium.
- (F) The embryo develops into blastocyst stage after 48 h incubation.

Table 1. In vitro development of embryos after thawing Glycerol with 0.6% BSA

strains	Total no of tubes	Total no of embryos vitrified (%)	Recovered normal embryos(%)	Morphologically normal embryos(%)
CD1	10	100	80	25
FVB/N	12	200	160	51
GU 2H	3	100	90	24

Morphological Survival Rate:

The percentage of intact, viable embryos immediately after thawing/warming was observed. Intact plasma membranes and no lysed blastomeres indicate successful cryoprotection. In our study, we observed that around 25% of the thawed embryos were morphologically normal in CD1, GU2H and FVB/N strains of mice,

In Vitro Development (Blastulation Rate): The percentage of thawed embryos (usually frozen at the 2-cell or morula stage) that successfully develop into expanded or hatching blastocysts under culture conditions, as shown in Figure 2. The total recovered embryos were 380 out of 400 total vitrified embryos. We achieved a total of 82% blastocyst hatching efficiency

In Vivo Viability (Live Birth Rate): The ultimate measure of developmental potential, calculated by performing **embryo transfers** into pseudo-pregnant recipient dams and tracking implantation and full-term pup development. In my study, I noticed that the embryos were resorbed in the uterus of the pseudo-pregnant mother.

DISCUSSION

In this study, I chose to work with CD1 and FVB/N strains of mice, as they are the most commonly used background strains for transgenic mouse production (Wigglesworth et al. 1995). In conclusion, equilibrium vitrification using low concentrations of cryoprotectants can be performed for mouse embryos at multiple stages. The proposed method has several advantages: 1) The vitrification medium contains lower concentrations of permeating and non-permeating cryoprotectants, and is thus less toxic to embryos; 2) As this

method does not require ultra-rapid cooling/warming, skilled techniques or minute devices (for ultra-rapid vitrification) are not required; 3) The process is instant and quick; 4) The procedure can be performed at room temperature without a programmable freezer; 5) This method is useful for two-, four-, and eight-cell embryos and shrunken blastocysts; and 6) Vitrified embryos can be transported on dry ice.

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