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Minimum volume Spatula MVD vitrification method improves embryo survival compared to traditional slow freezing, both for *in vivo* and *in vitro* produced mice embryos

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ABSTRACT

This study was conducted to compare the effect of minimum volume Spatula MVD vitrification (VIT) *versus* traditional slow freezing (SLF) of mouse embryos. A total of 2,617 8-cell *in vivo* derived and 2-cell *in vitro* produced B6D2 mouse embryos were subjected to freezing/thawing or vitrification/warming, while fresh embryos were used as control group. Embryo recovery, survival and development rate, pregnancy rate and offspring production were analyzed. In Experiment 1, 8-cell *in vivo* derived embryos were subjected to *in vitro* culture, resulting in greater survival and development rates at 3.5 days post coitum stage in VIT than in SLF group ($P < 0.05$). Although both methods reached an acceptable hatching rate (41.0% and 49.7% for VIT and SLF, respectively; P = NS), it was significantly lower respect to the control group (67.8%, $P < 0.01$). In Experiment 2, 2-cell *in vitro* produced mouse embryos showed a similar recovery rate from the device after freezing/thawing or vitrification/warming (~84%), however survival rate was significantly higher for vitrified/warmed (94.7%) than frozen/thawed embryos (85.1%; $P < 0.01$). Vitrified/warmed and control fresh embryos were transferred to surrogate mothers, revealing no differences both in pregnancy and offspring production rates. Our data demonstrate that minimum volume Spatula MVD method is a simple home-made useful technique for vitrification of 2-cell and 8-cell mouse embryos produced either *in vitro* or *in vivo*.

1. Introduction

Embryo cryopreservation is a widely long-term storage method used for domestic, experimental and wild animals, and humans. In general, vitrification has come to be the preferred technique for cryopreservation of pre-implantation embryos in several species [1,7,9], since it has the advantage of being simpler, faster and cheaper than conventional slow freezing. In addition, in some species, usually vitrification has greater embryo survival rate than slow freezing, mainly in *in vitro* produced embryos [7,18,26]. More recently, the vitrification technique has been enhanced by new protocols that imply a very rapid or ultra-fast cooling rate (i.e. 200–2500 °C/min) using a minimum volume of cryopreservation solution (i.e. ~0.1 µl) [8,16].

Different systems and devices has been used for vitrification, e.g. open-pulled straws, double straw system, electron microscope grids, cryotop, cryoloop, nylon and metal mesh [16,24]. Some of these methods allow direct contact between the embryos and the liquid nitrogen (LN_2) which may risk samples for pathogen contamination; others are complex home-made devices or trademarks that increases the final cost of cryopreserved samples. Recently, an ultra-fast and minimum volume method called vitrification spatula was reported, which enables embryo cryopreservation using an easy home-made spatula prepared with a gel-loading tip, and home-made media [23]. Since this method requires a cryotube for spatula storage into the LN_2 dewar, demanding more space than other systems, this tool was modified by dos Santos Neto et al. (2015; Spatula MVD), using a 0.5 mL straw to store inside the spatula device and eliminating the cryotube, reducing thus the LN_2 storage space [4].

In mice, *in vitro* fertilization (IVF) has become the method of choice in many laboratories [19,21], allowing the production of supernumerary embryos in a single cycle and reducing the number of used females and males. In this *in vitro* system, cryopreservation of early

Abbrevation: MVD, Montevideo; SLF, slow freezing; VIT, vitrification

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stage embryos is preferred instead of subjecting the embryo to *in vitro* culture to reach blastocyst stage. Additionally, cryopreservation at 2-cell stage embryos is a widely used strategy for storage and transport of mouse embryos among research centers [21,22]. Although cryopreservation of early stage embryos is important in the standard routine procedures of mice facilities, the available scientific information comparing the use of vitrification *versus* traditional freezing is scarce.

The aim of this study was to evaluate the efficiency of minimum volume Spatula MVD vitrification *versus* slow freezing methods of *in vivo* derived 8-cell and *in vitro* produced 2-cell mouse embryos. Embryo recovery from the device, survival and *in vitro* developmental rates, and pregnancy rate and offspring production were evaluated.

2. Materials & methods

2.1. Animals

Hybrid F1 female mice (B6D2, 8-12 week-old; used as donors and foster mothers) and F1 male mice (B6D2, 6-8 month-old) were housed under Specific Pathogen Free conditions in individually ventilated cages (IVC, Tecniplast, Milan, Italy) at the Experimental and Transgenic Animal Unit, *Institut Pasteur de Montevideo*, Uruguay. The animals received free access to autoclaved food (Labdiet 5K67, PMI Nutrition, IN, USA) and filtered fresh water, and were managed according to national and international guidelines for ethical conduct in the care and use of animals for research. Experimental protocols were approved by the Institutional Animal Ethics Committee, in accordance with National Law 18.611 and international animal care guidelines (Guide for the Care and Use of Laboratory Animal) [14] regarding laboratory animal's protocols.

2.2. Experimental design

Two experiments were carried out in order to compare the cryopreservation techniques for *in vivo* derived and *in vitro* produced B6D2 F2 embryos. In both cases, for each embryo production session the embryos were randomly allocated into three experimental groups: cryopreservation by slow freezing (SLF), vitrification (VIT), or were maintained fresh as control group. The experimental design is depicted in Fig. 1.

In Experiment 1, 1209 *in vivo* derived 8-cell embryos were collected at 2.5 days post-coitum (dpc) to be cryopreserved by SLF (n = 410) or VIT (n = 341), or remained fresh (n = 458). Cryopreserved embryos were thawed/warmed on the same day for survival evaluation, and a proportion of them were maintained in the incubator to assess *in vitro* embryo development and hatching rate.

In Experiment 2, 1408 *in vitro* produced 2-cell embryos were allocated to two trials. In the first trial the embryos were subjected to the same three groups than Experiment 1 (SLF = 531, VIT = 431 and fresh = 446), and evaluated for recovery and survival rate. In the second trial, vitrified/warmed (n = 264) or fresh embryos (n = 257) were transferred to synchronized 0.5 dpc recipient females to evaluate pregnancy and birth rates.

Unless otherwise indicated, chemicals used in both experiments were purchased from Sigma Chemical Company (St Louis, Missouri, USA).

2.3. Slow freezing and thawing

Slow freezing protocol and successive thawing was performed as described by Renard and Babinet [15]. Briefly, embryos were equilibrated in 1.5 M propylene glycol (PROH) for 15 min and loaded in groups of 20 into 0.25 mL plastic straw (Minitüb, Germany). The straw was heat-sealed and inserted in a LN2 pre-filled cryo-chamber in vertical position (Freeze control CL-5500, Cryologic, Mulgrave, Australia). Cooling curve initiates at −6 °C for 8 min; during this time manual seeding was performed, and decreases at 0.5 °C per min until −35 °C. Straws were immediately plunged into LN_2 and stored into a dewar.

Thawing was performed by keeping the straw in air for 40 s, followed by 2 min at 37 °C in a water bath. They remained for 2 min at room temperature and the embryos were expelled in sucrose-PROH medium for additional 3 min, and then washed several times in M2 medium.

2.4. Vitrification and warming

Vitrification was performed using a modified homemade spatula as described in dos Santos Neto et al. (2015) [4,23]. For that, embryos were placed into pre-vitrification solution (10% dimethyl sulfoxide (Me2SO), 10% ethylene glycol, 80% M2) for 30 s and immediately transferred to vitrification solution (15% Me2SO, 15% ethylene glycol, 10% M2 and 60% Ficoll) for another 30 s. With a glass pipette, embryos were loaded onto the spatula with minimum volume, immediately plunged in LN2 and 5 s later inserted into a 0.5 mL straw cap under LN_2. Spatulas were then stored into a LN_2 dewar.

Warming was performed by removing the straws from the dewar and dipping them into a 0.5 M sucrose drop to release the embryos. They were immediately washed in 0.5 M and 0.25 M sucrose for 2 min each and rinsed in M2 several times.

2.5. In vivo derived embryos (Experiment 1)

B6D2 F1 females were superovulated by intra-peritoneal (i.p.) injection of 5 IU of equine chorionic gonadotrophin (eCG; Novormon, Syntex, Buenos Aires, Argentina) and 5 IU of human chorionic gonadotrophin (hCG; Chorulon, MSD, Boxmeer, The Netherlands) 46–48 h later. Inmediately after hCG, females were mated with B6D2 F1 males. On 2.5 dpc, 8-cell B6D2 F2 embryos were collected and divided in three groups, as described before. After survival rate evaluation (morphologically viable/recovered embryos), a subset of embryos were subjected to *in vitro* culture to continue with the experiment, while the others were used for a different study. For *in vitro* evaluation, cryopreserved and fresh embryos were cultured in M16 under mineral oil in 5% CO_2 in air at 37 °C until hatching. Developmental stage was assessed under a stereomicroscope (Olympus SZ2-LGB) at 45× recording the embryo development rate at 3.5 dpc (morulae and blastocysts/total embryos), 4.5 dpc (blastocysts/total embryos) and 5.5 dpc (hatched blastocysts/total embryos) as described in “Manipulating the mouse embryo” [12].

2.6. In vitro produced embryos (Experiment 2)

In vitro fertilization method was carried out according to CosmoBio Co., LTD (http://cosmobiousa.com/cellculivf.html) [20]. Briefly, females were superovulated by i. p. injection of 5 IU of eCG and 5 IU of hCG 48 h later. Fifteen to 17 h after hCG the females were euthanized by cervical dislocation, their oviducts excised and the Cumulus-Oocytes Complex (COCs) were collected in CARD medium. They remained in an incubator at 37 °C, 5% CO_2 for 30–60 min before sperm insemination.

Sperm was collected from the cauda epididymis and capacitated for 1 h in FERTIUP® (PM) medium at 37 °C, 5% CO_2. Oocytes were inseminated with 3uL of sperm, rinsed in homemade human tubal fluid (HTF) medium 3 h later and kept in the incubator.

Twenty-four hours after insemination, cleavage was assessed and embryos divided randomly for two trials. In the first trial, the embryos were allocated to the three experimental groups as described before, and recovery rate from the device (recovered/total cryopreserved embryos) and survival rate were compared. Because in trial 1 vitrification method resulted in similar outcomes than fresh group and greater than SLF group, in the second trial only fresh and vitrified/warmed embryos were transferred to recipient females. In this second trial, pregnancy rate (pregnant/total transferred females) and birth rate (pups born/transferred embryos) were recorded for both experimental groups.