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Published in:
Cryobiology

DOI (link to publication from Publisher):
[10.1016/j.cryobiol.2011.11.003](https://doi.org/10.1016/j.cryobiol.2011.11.003)

Publication date:
2012

Document Version
Early version, also known as pre-print

[Link to publication from Aalborg University](#)

Citation for published version (APA):

Fathi, R., Valojerdi, M. R., Yazdi, P. E., Ebrahimi, B., Alipour, H., & Hassani, F. (2012). Development of 4-cell mouse embryos after re-vitrification. *Cryobiology*, *64*(1), 23-6. <https://doi.org/10.1016/j.cryobiol.2011.11.003>

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Development of 4-cell mouse embryos after re-vitrification

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ARTICLE INFO

Article history:

Received 2 March 2011

Accepted 14 November 2011

Available online 23 November 2011

Keywords:

Mouse embryos

Re-vitrification

CPS method

ABSTRACT

This paper reports studies on the effects of re-vitrification by the CPS (Closed Pulled Straw) method on the development of 4-cell stage mouse embryos. The procedure involved culturing 2-cell mouse embryos in G-1 medium until the 4-cell stage followed by the division of the normal 4-cell stage embryos into a control group (non-vitrified) and two experimental subgroups (vitrified and re-vitrified). Embryos in the vitrified subgroup were cryopreserved by the CPS vitrification method. In the second experimental subgroup (re-vitrified), embryos that were already vitrified were warmed and cryopreserved again by the same method. There was no significant reduction in the rate of blastocyst formation after vitrification and re-vitrification. However, re-vitrification reduced the total cell number, ICM (inner cell mass) percent and blastocyst diameter ($P < 0.05$). These results showed that vitrification and re-vitrification by the CPS method did not negatively affect the development of vitrified-warmed 4-cell mouse embryos, whereas re-vitrification significantly reduced both the cell number and diameter of blastocysts.

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Introduction

Until now, embryo cryopreservation by methods such as slow freezing [1] and vitrification [2] has been applied to preserve genetic variants of laboratory animals, livestock breeding and assisted reproduction in humans. Such methods are advantageous because of the use of submicrolitre volumes of cryoprotectants [3].

The process of refreezing embryos can be used in conjunction with various techniques of assisted reproduction. Matsumoto et al. [4] have reported that previously frozen embryos resulting from in vitro fertilization (IVF) could be thawed, biopsied for sexing or genetic analysis and then refrozen for transfer at a later date. This technique may at some times be critical, particularly when the straws or ampoules contain additional embryos, and can enhance the results of in vitro development. The concept of repetitive embryo freezing is not new and has been applied to mice [5,6] and human [7,8] cryopreserved embryos.

There is insufficient data on the effect of repeated use of vitrification and slow freezing; there have been several studies on the efficiencies of two- or more cycle vitrification [5,6] and double slow freezing [8–11] on embryo viability.

In this study, to estimate the extent of damage, re-vitrified-warmed embryos were allowed to develop into expanded blastocyst and subsequently stained to count total, ICM (inner cell

mass) and TE (trophoectoderm) cell numbers. In each group, the embryos were compared for a range of blastocyst diameters.

Materials and methods

Preparation of embryos

Six-to-eight week-old female Naval Medical Research Institute (NMRI) mice were provided by Raezi Institute. Mice were induced to superovulate with injections of 7.5 IU pregnant mare's serum gonadotropin (PMSG; Intervet Inc., Boxmeer, Netherlands) followed by 7.5 IU human chorionic gonadotropin (HCG; Pregnil; Organon, Oss, Netherlands) given 48 h apart. Females were mated with males from the same strain and inspected for the presence of a vaginal plug the following day. Those with vaginal plugs were considered pregnant and sacrificed 44–48 h post-HCG by cervical dislocation. The 2-cell embryos were flushed from the oviduct with T6 medium (NaCl: 473, KCl: 11, KH₂PO₄: 50, CaCl₂·2H₂O: 300, MgSO₄·6H₂O: 10, NaHCO₃: 210, Na lactate: 200, Na pyruvate: 3, D-glucose: 100, penicillin G: 6, streptomycin: 5, phenol red: 1 and EDTA: 0.6 mg/100 cc deionized water), supplemented with 4 mg/ml bovine serum albumin (BSA; Fraction V, Sigma). The 2-cell embryos were cultured in G-1 (version 3; Vitrolife, Kungsbacka, Sweden) with 10% human serum albumin (HSA, Vitrolife) until the 4-cell stage. Morphologically normal 4-cell stage embryos cultured under normal conditions were used as controls and the two experimental subgroups. Embryos in the control group (non-vitrified) were left to develop under

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the same conditions while the embryos in the first experimental subgroup (vitrified) were immediately cryopreserved by the CPS vitrification method as described by Chen et al. [12] with some modification. In the second experimental subgroup (re-vitrified), already vitrified embryos were warmed and cryopreserved once more using the same method.

All embryos in the two experimental subgroups were warmed and kept in fresh G-1 medium until development to the 8-cell stage. Embryos were then transferred to G-2 (version 3; Vitrolife, Kungsbacka, Sweden) droplets for further development.

Preparation of pretreatment, vitrification and dilution solutions

The solutions for pretreatment, vitrification and dilution were prepared using T6 medium. Pretreatment solutions contained 1.5 M ethylene glycol (EG; Sigma), whereas the vitrification solution contained 5.5 M EG and 1.0 M sucrose (Sigma). The dilution solutions contained 0.5, 0.25 and 0.125 mol/l sucrose.

Vitrification of embryos in CPS

The 4-cell stage embryos were first pretreated with 1.5 M EG solution for 5 min and then transferred to the vitrification solution at room temperature for 1.5 min. Then, dehydrated embryos were loaded within the straw which was prepared as follows: a 0.25 ml French straw (IMV, L'Aigle, France) was first heat-softened over a hot plate before being pulled manually until the inner diameter and the wall thickness of the central part were 0.8 and 0.07 mm, respectively [13]. The straw was allowed to cool to room temperature, cut at the narrowest point with a razor blade, and then filled using a tuberculin syringe with vitrification solution, air (2 mm), vitrification solution that contained six embryos (2 mm), air (2 mm) and vitrification solution (2 mm). The loaded straw was immediately plunged into liquid nitrogen.

Warming procedure

After 1 week, straws were transferred from liquid nitrogen to room temperature and touched by fingers for 5 s after which the contents were transferred into a drop of 0.5 M sucrose in T6 medium supplemented with 20% HSA. The 4-cell stage embryos were then diluted in stepwise sucrose solutions (0.5, 0.25 and 0.125 M sucrose; 2.5 min in each solution). Only the surviving embryos (retaining $\geq 50\%$ of intact blastomeres) were included in this experiment whereas damaged embryos (retaining $< 50\%$ of damaged blastomeres) were excluded. After embryo warming, the 4-cell embryos were randomly divided into two subgroups. Embryos of the first subgroup (vitrified group) were immediately cultured in G-1 medium until the 8-cell stage. Then, 8-cell embryos were transferred into

G-2 medium for subsequent development. One hour after warming, the healthy warmed 4-cell embryos of the second subgroup (re-vitrified group) were again vitrified by the above-mentioned procedure.

Embryo development

After warming, all groups of embryos were washed several times and kept in fresh G1_{TM} version 3 until the 8-cell stage. The embryos were then transferred into G2_{TM} version 3 droplets for further development. At the end of the experiment (day 5), the numbers of embryos in expanded blastocyst stages were evaluated in all groups. The quality of the blastocysts was determined using an inverted microscope (Olympus, Tokyo, Japan). Expanded blastocysts were then randomly selected for recording the number of blastomeres and expansion rate.

Blastocyst measurements

Quantitative measurements of the blastocyst's morphological features were obtained using the measurement program for the laser hatching system (Zilos-tk, Hamilton, USA). Blastocyst diameter (from outer zona to outer zona) was recorded, along with the longest length and widest perpendicular width (μm).

Number of blastomeres in blastocysts

The expanded blastocysts were first incubated in 500 μl of solution one (BSA-free human tubal fluid [HTF] medium with 1% Triton X-100 [Sigma] and 100 $\mu\text{g/ml}$ propidium iodide [Sigma]) for up to 30 s. The blastocysts were then immediately transferred into 500 μl of solution two (fixative solution of 100% ethanol with 25 $\mu\text{g/ml}$ Bisbenzamide [Hoechst 33258, Calbiochem, San Diego, USA]) and stored at 4 °C overnight. Fixed and stained blastocysts were then transferred from solution two directly into glycerol (Sigma), taking care to avoid carryover of excessive amounts of solution two. The blastocysts were then mounted on a glass microscope slide in a drop of glycerol, gently flattened with a cover slip and visualized for cell counting. Cell counting was performed on a fluorescence microscope (Olympus BX51) fitted with an ultraviolet lamp and excitation filters (380 and 420 nm for blue and red fluorescence, respectively) [14]. The inner cell mass (ICM) and trophoctoderm (TE) stained blue and red, respectively (Fig. 1).

Statistical analysis

Data from all groups were analyzed with the following statistical tests. Initially, the Kolmogorov Smirnov test was performed for data normalization. Differences between blastocyst formation, to-

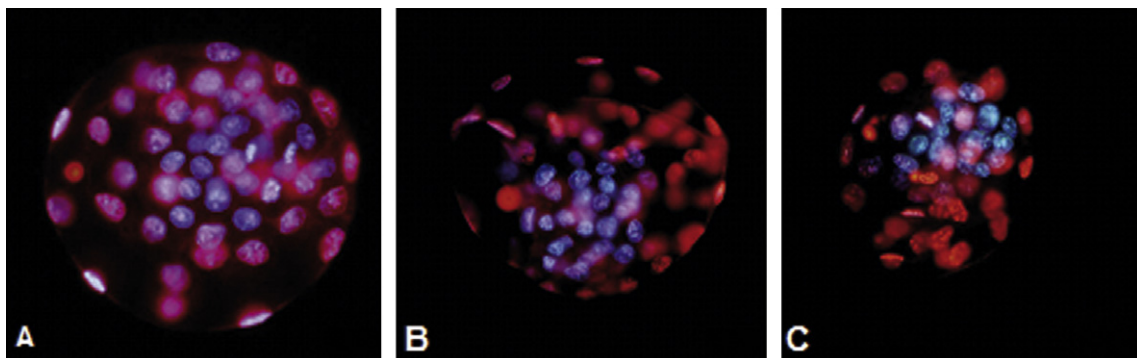


Fig. 1. Differential staining of (A) non-vitrified, (B) vitrified and (C) re-vitrified embryos. Magnification: 400 \times .

tal blastomeres, inner cell mass, trophoectoderm and blastocyst diameter were also compared using Tukey's HSD test after one-way analysis of variance. A *P* value of <0.05 was considered significant. All analyses were carried out using SPSS (Software program, Version 15; SPSS Inc., Chicago, IL, USA).

Results

To obtain sufficient number of vitrified-warmed embryos for this investigation, a total of 172 embryos were vitrified from which 160 with $\geq 50\%$ intact blastomeres were considered to be surviving embryos (93.02%). Of the surviving embryos, 90 were randomly selected for further development as the vitrified group and 70 (re-vitrified group) underwent re-vitrification. Survival rate following re-vitrification (90%) did not differ from those of vitrified embryos (93.02%).

Re-vitrification did not show significant reduction on blastocyst formation of 4-cell embryos compared to the vitrified and non-vitrified groups (Table 1).

The one cycle vitrification procedure did not affect total cell number, ICM and TE percent but it reduced blastocyst diameter compared to the non-vitrified group (Table 2). After re-vitrification, the total cell number and ICM percent were significantly reduced ($P < 0.05$). The diameter of the blastocysts derived from re-vitrified-warmed embryos was not different from those of the vitrified group, but significantly reduced compared to the non-vitrified group ($P < 0.05$; Table 2).

Discussion

The present research studied the effects of the CPS method of vitrification, performed either once or twice, on 4-cell mouse embryo development and quality. The results of this study showed high comparable survival rates of 93.02% and 90%, after one and two vitrification steps respectively with the use of a mixture of DMSO and EG. Our previous studies showed similar survival rates for mouse and human vitrification by DMSO and EG using straws [15] and cryotops [16].

Single embryo transfer may be more attractive to physicians and patients if an effective method for re-cryopreserving embryos is developed. Providing the option to re-cryopreserve a patient's previously stored embryos may encourage more patients to take a cautious approach with regard to the number of embryos transferred. Furthermore, reducing the risk of OHSS (Ovarian Hyper Stimulation Syndrome) in infertile patients undergoing IVF

(in vitro fertilization) treatment is an important clinical consideration. One of the methods to reduce this risk is the elective cryopreservation of all embryos and their subsequent transfer in nonstimulating cycles [1].

In the present study, after warming and embryo culture, the rate of blastocyst formation was reduced in the re-vitrified group (69.84%) with no significant difference compared to the other groups (control: 83.75% and vitrified: 81.11%). These results showed that vitrification performed either once or twice with the use of a simple method such as CPS did not have a notable negative effect on embryo's developmental potential. In similar studies, authors have reported successful culture of vitrified mouse and human embryos and oocytes using straws [15] and other vitrification methods such as cryotop [6,16], cryoloop [5] and EM grids [12]. Our results in survival and blastocyst formation were similar to these earlier studies.

There was, however, a notable difference in the total and ICM cell numbers. The total cell number and ICM percent of the control group was similar to those of the vitrified group but these rates significantly reduced after re-vitrification. This reduction in total cell number after double vitrification was comparable with the results of Vitale's study [11]. In Vitale's study, mouse embryos subjected to repeated freezing without an intervening culture time between each cycle after thawing contained lower mean cell numbers at hatching compared to unfrozen embryos. The adjusted regression coefficient of mean cell numbers as a function of the number of freeze-thaw cycles was 0.94, which suggested that freezing damage was cumulative [11]. In Courtney's study, 4-cell stage mouse embryos were re-vitrified by cryoloop and no significant differences in total, ICM and TE cell numbers were seen between control and re-vitrified embryos. It has been suggested that reduced cell numbers per embryo does not always indicate a decline in the subsequent in vitro or in vivo developmental capacity of embryos [17]. However, several points such as alteration of plasma membrane and enzyme activity by exposure of high concentration of cryoprotectants [18–20], toxic effects of cryoprotectants on morphological properties of embryos and any damage to sodium-potassium pump during blastocyst formation [21], may explain the reduction of cellularity and developmental rate after vitrification and re-vitrification. Moreover, Mozdarani and Moradi claimed that the appearance of chromosomal abnormalities such as deletion and incidence of aneuploidy may also reduce the viability of 8-cell mouse embryos after vitrification [22]. To prevent either these possibilities, further studies still required especially after re-vitrification.

We further measured the diameter of blastocysts derived from all groups. This rate significantly reduced in the single (110.71 μm)

Table 1
Mean developmental rates of non-vitrified, vitrified and re-vitrified 4-cell mouse embryos.

Group	No. of developed embryos	4–8 cell (%)	Morula (%)	Expanded blastocyst (%)	Degenerated (%)
Non-vitrified	80	98.95 \pm 1.04 <i>n</i> : 79	98.95 \pm 1.04 ^a <i>n</i> : 79	83.75 \pm 6.42 <i>n</i> : 67	16.25 \pm 6.42 <i>n</i> : 13
Vitrified	90	97.77 \pm 2.56 <i>n</i> : 88	94.44 \pm 3.55 ^a <i>n</i> : 85	81.11 \pm 5.02 <i>n</i> : 73	18.88 \pm 5.02 <i>n</i> : 17
Re-vitrified	63	88.30 \pm 2.5 <i>n</i> : 55	74.60 \pm 3.8 ^b <i>n</i> : 47	69.84 \pm 3 <i>n</i> : 44	30.15 \pm 3 <i>n</i> : 19

Vitrified: one cycle vitrified embryos; re-vitrified: two cycle vitrified embryos. Data are presented as means \pm SE, values within columns with different superscripts are significantly different ($P < 0.05$).

Table 2
Number of blastomeres and diameter of blastocysts derived from non-vitrified, vitrified and re-vitrified 4-cell mouse embryos.

Group	Total cells (<i>n</i>)	ICM cells (%)	TE cells (%)	Blastocyst diameter (μm)
Non-vitrified	55.23 \pm 4.57 ^a <i>n</i> : 30	31.81 \pm 2.51 ^a <i>n</i> : 30	68.18 \pm 2.51 ^a <i>n</i> : 30	125.56 \pm 1.64 ^a <i>n</i> : 37
Vitrified	57.95 \pm 4.09 ^a <i>n</i> : 33	31.56 \pm 2.39 ^a <i>n</i> : 33	67.73 \pm 2.31 ^a <i>n</i> : 33	110.71 \pm 1.09 ^b <i>n</i> : 40
Re-vitrified	40.50 \pm 2.49 ^b <i>n</i> : 23	22.97 \pm 0.79 ^b <i>n</i> : 23	77.02 \pm 0.79 ^b <i>n</i> : 23	112.63 \pm 2.56 ^b <i>n</i> : 21

Vitrified: one cycle vitrified embryos; re-vitrified: two cycle vitrified embryos; *n*: number of blastocysts; ICM: inner cell mass; TE: trophoectoderm. Data are presented as means \pm SE. Values within columns with different superscripts are significantly different ($P < 0.05$).

and double (112.63 μm) vitrification groups, compared to the control (125.56 μm) group.

A few cases of successful pregnancies have been reported after re-slow freezing embryos at the PN (Pronucleus) and blastocyst stage; therefore, progress has already been made in this aspect of clinical treatment [8,9]. In addition, the refreezing of blastocysts has also resulted in a successful birth [9]. However, these cases relied either solely or partially on slow freezing, which has been shown in some cases to result in less-favorable survival and embryonic development compared to vitrification [23,24].

In conclusion, the present study showed that re-vitrifying 4-cell mouse embryos using CPS was not discernibly detrimental to embryos. The rates of development and blastocyst formation were not affected by multiple vitrification cycles but cell number and blastocyst diameter reduced significantly after re-vitrification. Another investigation indicated that vitrification may be an efficient method for both oocyte cryopreservation and subsequent embryo re-vitrification [7].

Acknowledgment

This project has been supported by Royan Institute.

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