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Development of 4-cell mouse embryos after re-vitrification

Rouhollah Fathi, Mojtaba R. Valojerdi, Poopak E. Yazdi, Bita Ebrahim, Hiva Alipour, Fatemeh Hassani

A Department of Embryology, Reproductive Biomedicine Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
B Department of Anatomy, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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.

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 (trophectoderm) 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 ≥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 G1TM version 3 until the 8-cell stage. The embryos were then transferred into G2TM 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 µg/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 µg/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 trophectoderm (TE) stained blue and red, respectively (Fig. 1).

Statistical analysis

Data from all groups were analyzed with the following statistical tests. Initially, the Kolmogrov Smirnoff test was performed for data normalization. Differences between blastocyst formation, to-
Stimulation Syndrome) in infertile patients undergoing IVF was referred. Furthermore, reducing the risk of OHSS (Ovarian Hyperstimulation Syndrome) to prevent either these possibilities, further studies still required 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.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of developed embryos</th>
<th>4–8 cell (%)</th>
<th>Morula (%)</th>
<th>Expanded blastocyst (%)</th>
<th>Degenerated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-vitrified</td>
<td>80</td>
<td>98.95 ± 1.04 m: 79</td>
<td>98.95 ± 1.04 m: 79</td>
<td>83.75 ± 6.42 m: 67</td>
<td>16.25 ± 6.42 m: 13</td>
</tr>
<tr>
<td>Vitrified</td>
<td>90</td>
<td>97.77 ± 2.56 m: 88</td>
<td>94.44 ± 3.55 m: 85</td>
<td>81.11 ± 5.02 m: 73</td>
<td>18.88 ± 5.02 m: 17</td>
</tr>
<tr>
<td>Re-vitrified</td>
<td>63</td>
<td>88.30 ± 2.5 m: 55</td>
<td>74.60 ± 3.88 m: 47</td>
<td>69.84 ± 3 m: 44</td>
<td>30.15 ± 3 m: 19</td>
</tr>
</tbody>
</table>

Vitrified: one cycle vitrified embryos; re-vitrified: two cycle vitrified embryos. Data are presented as means ± 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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cells (n)</th>
<th>ICM cells (%)</th>
<th>TE cells (%)</th>
<th>Blastocyst diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-vitrified</td>
<td>55.23 ± 4.57 m: 30</td>
<td>31.81 ± 2.51 m: 30</td>
<td>68.18 ± 2.51 m: 30</td>
<td>125.56 ± 1.64 m: 37</td>
</tr>
<tr>
<td>Vitrified</td>
<td>57.95 ± 4.09 m: 33</td>
<td>31.56 ± 2.39 m: 33</td>
<td>67.73 ± 2.31 m: 33</td>
<td>110.71 ± 1.09 m: 40</td>
</tr>
<tr>
<td>Re-vitrified</td>
<td>40.50 ± 2.49 m: 23</td>
<td>22.97 ± 0.79 m: 23</td>
<td>77.02 ± 0.79 m: 23</td>
<td>112.63 ± 2.56 m: 21</td>
</tr>
</tbody>
</table>

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 ± 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

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References