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TECHNOLOGICAL INNOVATIONS

A closed system supports the developmental competence of human embryos after vitrification

Closed vitrification of human embryos

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Abstract
Purpose Closed-system vitrification may enable the risk of contamination to be minimised. We performed three studies to compare the developmental competence of human embryos vitrified using either a closed vitrification system (CVS; Rapid-i®) or an open vitrification system (OVS; Cryo-top®).
Methods The first study was performed in vitro using 66 zygotes previously vitrified at pronuclear stage. These were warmed and randomised 1:1 to revitrification using either the OVS or the CVS. After re-warming, embryo development and blastocyst cell number were assessed. For the second study, also performed in vitro, 60 vitrified–warmed blastocysts were randomised 1:1:1 into three groups (OVS or CVS revitrification, or no revitrification). The proportion of dead cells was assessed by staining. The third study was performed in vivo, using 263 high-grade blastocysts randomly assigned to vitrification using either the CVS (n=100) or the OVS (n=163). After warming, single blastocyst transfer was performed.
Results There were no differences between the CVS and the OVS in survival rate (100 % vs. 97 %), blastulation rate (96 h: 50 % vs. 50 %; 120 h: 68 % vs. 56 %), proportion of good blastocysts (96 h: 32 % vs. 22 %, 120 h: 47 % vs. 41 %), or mean number of cells (137 vs. 138). The proportion of dead cells in blastocysts re-vitrified by CVS (31 %) was similar to that for OVS (38 %) and non-revitrification (32 %). In vivo, the implantation rate for blastocysts vitrified using the CVS (54 %) was similar to that with the OVS (53 %).
Conclusion Our studies consistently indicate that human embryos may be vitrified using a CVS without impairment of developmental competence.

Keywords Closed vitrification system · Human · Embryo · Blastocyst

Capsule The closed vitrification system overcame the majority of problems associated with direct liquid nitrogen contact that occurs in the open vitrification system without impairing developmental competence of human embryos.

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Introduction

Since the first successful pregnancy was achieved by the transfer of a frozen human embryo [29], attempts at controlling the rate of cooling have had varying success [7]. Embryo cryopreservation has recently been progressed by ultra-rapid vitrification, which was originally applied to murine embryos by Rall and Fahy [25]. Cryopreservation by vitrification occurs by glass formation both inside and outside the sample after dehydration. Two main challenges to refining the vitrification technique have been identified: i) high concentrations of cryoprotectant are required, and these may be toxic to oocytes, embryos, and ovaries; and ii) intracellular ice and chilling may occur if the applied cooling or warming rates are insufficient [17, 20, 30]. To overcome these problems, several methods have been developed. The most logical way to increase the cooling rate is to use the smallest possible volume of cryoprotectant medium to surround the sample and then expose it directly to liquid nitrogen without any thermo-insulation: this is the open vitrification system (OVS). This idea was initially proposed for freezing Drosophila embryos (Mazur et al. 1992). Animal data [11, 15, 22, 31] and recent analyses of clinical reports [18, 19] have revealed the benefits of vitrification such as low rates of cellular damage. However, the transition from freezing to vitrification is proceeding very slowly due to concerns regarding the sterility of liquid nitrogen and the risk of cross-

contamination during long-term storage [2, 3]. These concerns arise from direct contact of the solution containing the oocytes/embryos with liquid nitrogen.

To avoid the risk of contamination, several closed vitrification systems (CVSs) have been developed [5, 12–14, 23, 24, 32] However, these methods introduce new concerns such as a potential rise in temperature by using a heat sealer, and the risk of contamination during warming. In addition, with one type of CVS, the embryo recovery rate after warming has been shown to be lower than that achieved with an OVS [1].

A new CVS (Rapid-i®; Vitrolife, Tokyo, Japan) was developed using mouse embryos [16]. In this system, an embryo is inserted into a straw with super-cooled air for instantaneous vitrification, and then the open end of the straw is sealed using ultrasound adhesion to avoid the risks of temperature increase and contamination. For warming, only embryos and cooling device, which were not exposed to liquid nitrogen, were warmed in thawing solution, thus avoiding the risk of contamination. Recently, it has been reported that the implantation potential of human blastocyts vitrified with Rpid-i was comparable to that of counterparts with an open device [6]. However, the data was mixed with single and multiple blastocyst transfer, and the number of patients (22) was small.

We performed three studies (two in vitro and one in vivo) to compare the developmental competence of human embryos vitrified using the new CVS with those vitrified using an OVS.

Materials and methods

These studies were approved by the ethics committee of the IVF Namba Clinic. Embryos donated by patients who had completed fertility treatment were used for the in vitro studies. All donors provided signed informed consent. For the in vivo study, patients received full explanation of the methodology and gave signed informed consent to their participation.

Vitrification

The Rapid-i Kit is a CVS containing a plastic stick made from polymethyl methacrylate (termed 'Rapid-i') and a thermoplastic elastomer storage straw (termed 'RapidStraw'). The Rapid-i has a 50-nL loading hole, designed for receiving an embryo from a pipette under microscopy [16]. The Rapid-i Kit also contains a stainless steel rod which is inserted into the RapidStraw for cooling prior to insertion of the Rapid-i (the rod is removed 20–30 s before insertion of the Rapid-i). Cryotop® (Kitazato Corporation, Tokyo, Japan) [13] was used as the OVS.

Embryos were equilibrated in 7.5 % (v/v) ethylene glycol (EG, 054-0983; Wako Chemical, Osaka, Japan), 7.5 % (v/v) DMSO (D2650; Sigma-Aldrich, St. Louis, MO, USA), 20 % (v/v) Serum Substitute Supplement (SSS, 99193; Irvine Scientific, St. Ana, CA, USA) and TCM 199 medium (12350-039; Invitrogen, Tokyo, Japan) for a maximum of 10 min, confirming shrinkage and re-expansion, and transferred in vitrification solution which consisted of 15 % (v/v) EG, 15 % (v/v) DMSO, 0.5 M sucrose (192-00012; Wako Chemical), 20 % (v/v) SSS and TCM 199 medium. Each embryo was picked up with a small amount of vitrification solution (approximately 50 nL) and pipetted into the hole of a Rapid-i. Immediately afterwards, it was introduced into super-cooled air inside the RapidStraw held in liquid nitrogen (the steel rod had been removed from the straw 20–30 s previously). The straw was then sealed using an ultrasonic sealer as described previously [16]. The sealed straw was stored in liquid nitrogen for several weeks. For the OVS, after equilibration in vitrification solution, each embryo was picked up using the same method as for CVS and placed on the fine polypropylene strip that is part of the Cryotop system. The strip was then immediately submerged into liquid nitrogen.

Warming

For warming of embryos vitrified using the CVS, after clipping the end of the straw, the Rapid-i stick was removed and immediately warmed in 1 mL TCM199 containing 20 % SSS and 1 M sucrose, which was warmed at 37 °C for 1 min. This was followed by dilution in TCM199 containing 20 % SSS and 0.5 M sucrose, and then dilution twice in TCM199 containing 20 % SSS for 5 min each at RT. For embryos vitrified using the OVS, the device was warmed and diluted similarly.

Study 1: In vitro development

To assess developmental competence to the blastocyst stage, 70 zygotes vitrified by OVS at pronuclear stage were warmed and cultured for 14 h. Sixty-six of these zygotes, upon development to the 2–4 cell stage, were divided randomly into two groups for revitrification using either CVS or OVS. The embryos were then warmed for a second time and cultured individually for 120 h in a 0.01413-μL microwell [10] filled sequentially with culture media (Cleavage medium then Blastocyst medium; COOK Medical, Queensland, Australia) at 37 °C under 5 % CO_2, 5 % O_2 and 90 % N_2.

After 120 h of in vitro culture, the number of cells in each blastocyst was counted by using confocal microscopy (CellVoyager™ CV1000; Yokogawa Electronic, Tokyo, Japan) after staining with 10 μg/mL of bisbenzimide H 33342 trihydrochloride (Hoechst 33342, 591-01721; Wako Pure Chemical Industries, Osaka, Japan).

Study 2: Cell membrane damage after vitrification

To assess cell membrane damage after CVS vitrification, 60 vitrified–warmed blastocysts that developed on Day 5 after

insemination were divided randomly into three groups (OVS [n=20], CVS [n=20], and non-revitrified [n=20]). After revitrification (n=40) or culture (n=20), the blastocysts were stained with Hoechst 33342 and propidium iodide (PI, 160-16723; Wako Pure Chemical Industries). The proportion of dead cells was determined by the number of nuclei stained with PI divided by the total number of nuclei (Hoechst and PI stained) obtained using confocal microscopy. We included blastocysts whose degree of expansion was categorised as BL4/5, whose inner cell mass (ICM) was categorized before freezing as A or B, and whose trophectoderm (TE) grade was categorized before freezing as B or C, according to Gardner's criteria [8]. To obtain a numerical blastocyst morphology grading system based on Gardner's criteria, the blastocyst grade was converted to the multiplicative blastocyst quality score (BQS) [27, 33]. The BQS is a measure of blastocyst quality based on established morphologic criteria, and is defined as the product of the degree of embryo development and ICM and TE grades, where grade A is given the value 3; grade B is 2; and grade C is 1. For example, a 3AB blastocyst has a BQS of 3×3×2, giving a total of 18. After warming, the embryos were cultured for 3 h individually (Blastocyst medium; COOK Medical) at 37 °C under 5 % CO_2, 5 % O_2 and 90 % N_2.

Study 3: Implantation competence after embryo transfer

To assess the developmental competence of CVS-vitrified blastocysts, 153 patients scheduled for single vitrified–warmed blastocyst transfer under a hormone-replacement cycle between 7 November 2011 and 18 September 2012 were randomly divided into two groups (CVS, n=100; OVS, n=163). Blastocysts that were scored at least 3AA without C by Gardner's criteria were vitrified as good blastocyst. The endometrium was prepared using methodology described previously [9], modified by administration of GnRH agonist (600 μg/day, Suprecur® nasal solution 0.15 %; Mochida Pharmaceutical, Tokyo, Japan) for 3 weeks followed by incremental doses of oral oestradiol valerate (Progynova®; Bayer Schering Pharma, Zürich, Switzerland) from 1 to 4 mg for 2 weeks. After using ultrasonography to confirm the endometrial thickness to be more than 8 mm, 6 mg/day chlormadinone acetate (Lutoral®; Shionogi & Co., Osaka, Japan) was administered.. Progesterone (Progeston depot® 125 mg; Fuji Pharma Co., Toyama, Japan) was administered intramuscularly on the day of embryo transfer, with two additional doses after conception. Blastocyst transfer was carried out on the 5th day of chlormadinone acetate administration. Daily doses of 3 mg oestradiol valerate and 6 mg chlormadinone acetate were maintained until pregnancy testing. In case of confirmed pregnancy, transcutaneous oestradiol patches (2.88 mg every 2 days, Estradna®; Hisamitsu, Saga, Japan) and transvaginal progesterone (400 mg of progesterone/day, Utrogestan® 200 mg; Ferring Pharmaceuticals, West Drayton, UK) were administered until 9 weeks of gestation.

Implantation was determined by the detection of a single intrauterine gestational sac by transvaginal ultrasound around 3 weeks after embryo transfer. On-going pregnancy was defined as pregnancy developing beyond 8 weeks of gestation with foetal heart beat confirmed by ultrasound.

Statistical analysis

Differences between pairs of groups were determined using an unpaired Student's t-test. Differences among three groups were determined by analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. P-values <0.05 were considered significant. Data are presented as mean ± standard error. Statistical analysis was performed using StatView version 5 (SAS Institute Inc., Cary, NC, USA).

Results

Study 1: In vitro development

There was no significant difference (P=0.54) in the mean age of the female donors of the 2-4 cell embryos revitrified using the CVS (34.4±0.5 years old) or the OVS (33.9±0.6 years old). All parameters indicating developmental competence after revitrification (survival rate; CVS: 100±0 % vs. OVS: 96.9±3.1 %, blastulation rate; CVS: 67.6±8.1 % vs. OVS: 56.3±8.9 %, percentage of good blastocysts; CVS: 47.1±8.7 % vs. OVS: 40.6±8.8 % and mean number of cells; CVS: 136.9±13.6 vs. OVS: 138.0±18.3) showed no significant difference between CVS and OVS (Table 1). The blastulation rates at 96 hand 120 h of

Table 1 Developmental competence of embryos revitrified using either a closed vitrification system (CVS) or an open vitrification system (OVS) (Study 1). Data are shown ± standard error

	CVS-revitrified embryos (n=34)	OVS-revitrified embryos (n=32)
Survival rate (%)	100±0 (34/34)	96.9±3.1 (32/32)
Blastulation rate, 96 h (%)	50.0±8.7 (17/34)	50.0±9.0 (16/32)
Good blastocysts, 96 h (%)	32.4±8.1 (11/34)	21.9±7.4 (7/32)
Blastulation rate, 120 h (%)	67.6±8.1 (23/34)	56.3±8.9 (18/32)
Good blastocysts, 120 h (%)	47.1±8.7 (16/34)	40.6±8.8 (13/32)
Mean number of cells[a]	136.9±13.6 (n=23)	138.0±18.3 (n=18)

[a] Cell numbers of all blastocysts were counted at 120 h

There were no significant differences

embryos cultured without vitrification which were performed at the same period between January 2011 and December 2012 were 51 % (112/220) and 55 % (121/220), respectively. Morover, the percentages of good blastocysts at 96 hand 120 h were 24 % (52/220) and 30 % (66/220), respectively. These values of vitrified blastocysts were almost same leve as those of fresh counterparts.

Study 2: Cell membrane damage after revitrification

There were no significant differences between embryos revitrified using the CVS, revitrified using the OVS, or non-revitrified, in relation to mean age of the female donors (CVS: 33.3±0.7 years old; OVS: 33.2±0.9 years old; non-revitrified: 32.4 ± 0.8 years old) and BQS before revitrification (Table 2). The mean number of cells following CVS revitrification was the same as that following either OVS revitrification or non-revitrification (Table 2). Moreover, the percentage of dead cells in the CVS group (30.8±5.1) was the same as the OVS (38.1±3.9) and non-vitrified groups (32.4±3.3).

Study 3: Implantation competence after embryo transfer

There were no significant differences between embryos vitrified using the CVS (n=100) or the OVS (n=163), with respect to mean age of the female donors (34.8±0.4 years old vs. 35.2±0.3 years old, respectively), or mean endometrial thickness (11.3±0.2 mm vs. 11.2±0.1 mm). As shown in Table 3, there was also no significant difference between the two groups of embryos in BQS before vitrification. All parameters relating to developmental competence of the embryos after vitrification indicated that CVS (implantation rate: 53.6±5.1 %, ongoing pregnancy rate: 45.4±5.1 %) was very similar to OVS (implantation rate: 53.2±4.0 %, ongoing pregnancy rate: 46.8±4.0 %; Table 3). Similarity between the groups in relation to the implantation rate and ongoing pregnancy rate is also shown in Table 3.

Table 2 Cell membrane damage among embryos revitrified using either a closed vitrification system (CVS) or an open vitrification system (OVS) (Study 2). Data are shown ± standard error

	CVS-revitrified embryos (n=20)	OVS-revitrified embryos (n=20)	Non-revitrified embryos (n=20)
Mean blastocyst quality score before revitrification	12.4±1.6	11.6±1.1	12.3±1.8
Mean blastocyst cell number	64.0±4.7	57.8±4.6	60.4±6.0
Dead cells (%)	30.8±5.1	38.1±3.9	32.4±3.3

There were no significant differences

Table 3 Developmental competence after single transfer of blastocysts vitrified using either a closed vitrification system (CVS) or an open vitrification system (OVS) (Study 3). Data are shown ± standard error

	CVS-vitrified embryos (n=100)	OVS-vitrified embryos (n=163)
Mean blastocyst quality score before vitrification	22.8±0.9	22.3±0.7
Survival rate after vitrification and warming (%)	97.0±1.7 (97/100)	96.9±1.4 (158/163)
Implantation rate (%)	53.6±5.1 (52/97)	53.2±4.0 (84/158)
Ongoing pregnancy rate (%)	45.4±5.1 (44/97)	46.8±4.0 (74/158)

There were no significant differences

The BQS before transfer, the implantation and on-going pregnancy rates in the case of fresh transfers which were performed at the same period between January 2011 and December 2012 were 20.9, 46 % (15/33) and 39 % (13/33), respectively. There were numerical differences in favour of the both vitrification in terms of BQS, implantation rate and ongoing pregnancy rate. Statistical analysis between fresh and vitrified blastocyst transfers was not carried out because of a large difference of population scale.

Discussion

There are two main risks of contamination during cryopreservation. The first occurs during the vitrification procedure, through direct contact with liquid nitrogen. The second occurs during storage under liquid nitrogen; the liquid nitrogen might become contaminated, introducing the risk of cross-contamination. In the CVS used in this study, each embryo is vitrified in super-cooled air and then packaged in a closed straw. As a result, the embryo is supposed to have low risk of contamination from liquid nitrogen [16] similar to conventional slow freezing [7] and packaged-straw vitrification [34]. However, this contrasts to direct contact with open vitrification [3].

There have been concerns with the Rapid-i system that a reduction in the cooling rate might cause ice crystal formation inside and outside the sample, potentially causing cell death. A key aim when developing the OVS was to maximise the rate of cooling, minimising the extent of ice crystal formation without the need for high concentrations of cryoprotectant [20]. This reflects a general focus on cooling rate in the development of vitrification for mammalian embryos [26]. Such focus has led to the use of miniature vitrification devices (e.g. CryoLoop™ [Hampton Research, Aliso Viejo, CA, USA], Cryotop, McGill Cryoleaf™ [Origio, Malov,

Denmark]) that support sub-microlitre volumes of cryoprotectant, permitting cooling rates >10,000 °C/min upon direct contact with liquid nitrogen [21].

To assess the effect of the CVS on human embryos, we compared the developmental competence of human embryos after CVS revitrification with that following OVS revitrification. In Japan, there are major restrictions on experiments using human embryos. This was the reason for performing the in vitro revitrification study to assess cell membrane damage. The outcome of that study reflected the in vitro development results, showing no significant difference between revitrification using the CVS versus the OVS. Moreover, there was no significant difference in cell membrane damage between blastocysts revitrified using the CVS and those that were not revitrified blastocysts.

Clinical comparison of the CVS with the OVS was only permitted after establishing in the in vitro studies that no increase in damage was observed wiht the CVS . To avoid differences between CVS and OVS in relation to maternal characteristics or blastocyst quality, patients scheduled for a single blastocyst transfer under a hormone replacement cycle were randomly divided into the CVS and OVS groups.

The CVS was shown to be comparable to the OVS in terms of implantation rate and developmental competence over 10 weeks of gestation. These results are consistent with previous data obtained in mice [16]. It has recently been reported that rapid warming may be more critical than the cooling rate [28]. When embryos vitrified using either the CVS and OVS were warmed, they were submerged directly into the warming solution. Thus, the warming rate was the same for both groups of embryos. This may explain the lack of differences in our studies between embryos vitrified using either the CVS or the OVS.

According to the latest available data, over 90,000 cryopreserved embryos were thawed or warmed in Europe in 2007 and more than 11,000 babies were born that year as a result of cryopreserved embryo transfer [4]. Thus, a CVS which enables aseptic vitrification without impairing the developmental competence of human embryos could potentially have a large impact in ART.

Our results indicate the feasibility of vitrifying human embryos in a closed system which is supposed to avoid contamination risks, without impairing developmental competence. Large-scale investigation including follow-up of children born from embryos vitrified using a CVS will be required.

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Conflict of interest None declared.

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