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Comparison of two closed carriers for vitrification of human blastocysts in a donor program

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ABSTRACT

The survival of human blastocysts to vitrification with two different carriers is compared. Both vitrification carriers used in this study are in the category of closed carriers, as they completely isolate the samples from direct contact with liquid nitrogen or its vapours during cooling and storage, until warming. This characteristic is appealing because it reduces or eliminates the theoretical risk of cross-contamination during that period of time.

The two closed vitrification systems used present very different design and features: in the High Security Vitrification device, the carrier straw containing the embryos is encapsulated inside an external straw before plunging in liquid nitrogen, resulting in thermal insulation during cooling. On the other hand, in the SafeSpeed carrier embryos are loaded in a thin-walled, narrow capillary designed to maximize the thermal transference. Both closed carriers achieved comparable outcomes in terms of survival of blastocysts to the vitrification process, with 97.5% vs. 96.1% survival with HSV and SafeSpeed, respectively. In conclusion, the cooling and warming rates at which these carriers operate, in combination with the cytosolic solute concentration in the cells of the cryopreserved blastocysts attained after a cryoprotectant-loading protocol, result in successful vitrification of human blastocysts for human assisted reproduction.

1. Introduction

The cryopreservation of human embryos is a key element in the human assisted reproduction field: the ability to preserve the supernumerary embryos that are generated during in vitro fertilization treatments greatly improves treatment options [12]. Cryopreservation is particularly important in donation programs and in cases where the whole cohort of embryos obtained in an in vitro fertilization (IVF) treatment is cryopreserved, in a policy termed 'freeze all' [2,30]. The current method of choice for cryopreservation of human embryos is vitrification, and the clinical embryologist has a wide array of both commercial [8,11,13,15,18,19,36,39] and non-commercial [14,17,23,31,32,34,37] vitrification carriers —the devices in which embryos are loaded prior to cooling, and where they remain stored at cryogenic temperatures until warming— to choose from.

Due to the hypothetical risk of liquid nitrogen mediated cross-contamination during cooling and storage, vitrification carriers have been classified by the level of exposure of the embryos and the media surrounding them to the cooling agent liquid nitrogen [3,16,20,25]. From a lower to higher degree of isolation, there are: fully open carriers, which directly expose the embryo to liquid nitrogen during cooling and storage; open cooling and closed storage carriers; semi-closed, vapor-mediated cooling carriers; closed carriers composed of thin and narrow capillaries; and hermetically-sealed-into-container carriers [38]. There are also alternatives in which liquid nitrogen is sterilized [24].

Whether it is preferable to use open or closed carriers is a hotly debated topic in the human assisted reproduction field [9,10,38,42]. European directives do not impose the use of closed vitrification systems, but recommend laboratories to minimise the risk of contamination of tissues and cells [5]. However, most evidence on the efficiency of vitrification for cryopreservation of oocytes and embryos comes from studies using open-systems [23,26,37]. A recent meta-analysis showed similar survival but a tendency towards lower birth rates in transfers of blastocysts cryopreserved with closed system versus those cryopreserved with open systems, so the equality of both approaches cannot be assumed [41].

However, the performance of a vitrification carrier does not depend on just whether they are open or closed. Aside from the repeatability

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and ease of handling of the carrier to avoid manipulation mistakes, performance will depend on the cooling and, most warming rates it achieves and the volume of the solution containing the embryos that is loaded into it [6,21,27,33,40].

In this study, we compare the outcomes of blastocyst vitrification when using two different closed carriers, with different design: the high security vitrification carrier (HSV, Irvine Scientific, USA), and the capillary-based closed vitrification carrier SafeSpeed (SS, Safepreservation, Spain), specifically designed to maximize heat-transfer efficiency [13].

2. Material and methods

2.1. Study design

This retrospective study reports data from 79 cycles of elective transfers of supernumerary vitrified and warmed blastocyst from a fresh egg donation program. All treatments were performed in Instituto Bernabeu, between January and December 2015, when both devices were used concurrently. The data included in this study was framed in the routine clinical activity and retrospectively collected with Instituto Bernabeu's IRB approval.

2.2. Laboratory procedures

Donor oocytes were fertilized by ICSI, and cultured to the blastocyst stage using microdrops of pre-equilibrated in vitro culture media (CCM, Vitrolife, Sweden), in 5% O_2, 6% CO_2, at 37 °C. Blastocysts were graded according to Istanbul consensus scoring on embryo assessment [1].

Briefly, based on their stage of development blastocyst were graded on a 1–4 scale, with grade 1 equivalent to an early blastocyst with a blastocoel that is half of the volume of the embryo, grade 2 equivalent to a blastocyst with a blastocoel that is greater than half of the volume of the embryo, grade 3 equivalent to an expanded blastocyst, and grade 4 equivalent to a blastocyst hatching or hatched. For each of the developmental stages, the inner cell mass (ICM) and trophectoderm (TE) were graded on a 1–3 scale. ICM grade 1 was defined as prominent, easily discernible, with many compacted and tightly adhered cells; grade 2 as easily discernible, with many loosely grouped cells, and grade 3, in which the ICM was difficult to discern, with few cells. The TE was assessed as follows: grade 1, many cells forming a cohesive epithelium; grade 2, few cells giving it an irregular aspect; or grade 3, very few large cells. Good quality blastocysts, defined as having an ICM and TE grade 1 or 2, were cryopreserved.

2.3. Vitrification and warming

All the blastocysts from each recipient were assigned exclusively to one of the carriers. Both vitrification and warming procedures were carried out according manufacturer's instructions for use. All solutions were at 23–27 °C room temperature except for TS warming solutions, at 37 °C.

2.3.1. Closed carrier 1: SafeSpeed (SS)

Prior to vitrification of the blastocysts with the SS carrier (Fig. 1), the blastocysts were placed in a 200 µl drop of equilibration solution (SS-ES) containing 7.5% v/v ethylene glycol (EG) and 7.5% v/v dimethyl sulfoxide (Me_2SO). The exposure to this solution lasted for a minimum of 8 and a maximum of 14 min, depending on the time the blastocysts took to re-expand to their isosmotic volume in a subjective assessment by the operator. Blastocysts were then transferred to 200 µl of vitrification solution (SS-VS, 15% v/v EG, 15% v/v Me_2SO, 0.5 M sucrose), rinsed repeatedly and loaded by aspiration in the SS capillary. The capillary and the back end of the straw were then thermo-sealed with a specific device [13]. Once hermetically closed, the straw was plunged in liquid nitrogen, keeping the capillary uncovered by the protective cap, and stirred for a few seconds. Then, while submerged in LN_2, the protective cover is slid down before releasing the straw. The total exposure time to the vitrification solution until quenching is of 60–90 s.

For warming, the capillary containing the embryos is exposed, remaining submerged in LN_2, transferred in a fast motion to a nearby sterile water bath at 37 °C, and stirred for two seconds. Afterwards the capillary is cut at the tip above the sealing, and the embryos are expelled in 200 µL of warming solution (SS-TS, 1 M sucrose) at 37 °C. After 1 min, they are rinsed for 3 min in 200 µl dilution solution (SS-DS, 0.5 M sucrose), followed by a 5 min rinse in 200 µl washing solution (SS-WS, no osmotic agents). Vitrification and warming media, and Safespeed carrier device sold by Safepreservation, Spain. All solutions contained 0.06–0.125 mg/mL of hydroxypropyl cellulose (HPC) for viscosity and as surfactant agent.

2.3.2. Closed carrier 2: high security vitrification (HSV)

Blastocysts were placed in a 50 µL drop of equilibration solution (HSV-ES) containing 7.5% (v/v) EG, 7.5% (v/v) Me_sSO in an M-199 HEPES Buffered Medium supplemented with 20% dextran for 7–10 min, until showing signs of re-expansion. They were then transferred to a 50 µL drop of vitrification solution (HSV-VS) containing 15% (v/v) EG, 15% (v/v) Me_sSO, and 0.5 M sucrose, washed thoroughly to eliminate leftover ES, and loaded in the tip of the HSV carrier (Irvine Scientific, USA). This carrier device containing the embryos is inserted in an outer resin straw, which is thermos-sealed before plunging in liquid nitrogen. The procedure, from exposure of the blastocysts to VS until the plunge in liquid nitrogen, is completed in 60–90 s.

For warming, the back end of the outer resin straw is cut open, keeping part of the carrier device containing the embryos submerged in liquid nitrogen at all times. Then, it is removed from the outer straw and the tip submerged in 250 µL of warming solution (HSV-TS; 1 M sucrose), as fast as possible. Blastocysts are recovered from TS in one minute, and transferred to dilution solution (HSV-DS, 0.5 M sucrose, room temperature) for three minutes, followed by 5 min in washing solution (WS, no osmotic agents, room temperature). Vitrification (VitKit - Freeze) and warming (VitKit – Thaw) solutions and HSV carrier sold by Irvine Scientific, USA.

After warming, embryos are then transferred to four well-dishes containing pre-equilibrated culture media (CCM, Vitrolife, Sweden), and cultured at 37 °C, 5% O_2, and 6% CO_2, until the moment of the embryo transfer, when a final survival and quality assessment was performed (> 2 h after warming). Blastocysts were considered as positive for survival and apt for transfer when > 50% of cells survived vitrification.

2.4. Clinical procedures

All donors started stimulation on day 2–4 of menstrual cycle with an initial dose of 150–300 UI/day of FSH (Fostipur®, Angelini-IBSA, Barcelona, Spain) according to antral follicular count (AFC) and body mass index. Donors were monitored from day 5 of stimulation by transvaginal ultrasounds scans every 2 or 3 days and doses were adjusted individually. When lead follicle reached 13–14 mm a GnRH antagonist (Cetrotide®, Merck-Serono, Madrid, Spain) was administered daily, and GnRH agonist (Triptoreline, 0.2 mg, Decapeptyl®, Ipsen Pharma, Barcelona, Spain) was used for final oocyte maturation when at least 3 follicles were > 18 mm in diameter. Oocyte aspiration was performed 36 h after GnRH agonist injection by transvaginal ultrasound-guided needle-aspiration.

Recipients were subjected to substitutive hormonal therapy with either a) transdermal oestradiol (Evopad 50, Janssen-Pharmaceutica, Belgium): applying 50 µg patches from day 1–8 of the cycle, 100 µg from day 9–12, and 150 µg from day 13 onwards. Patches were changed every 48 h; or b) oral oestradiol valerate (Progynova, Delpharm Lille, France): 2 mg daily from day 1–8, 4 mg daily from day 9–12 and 6 mg

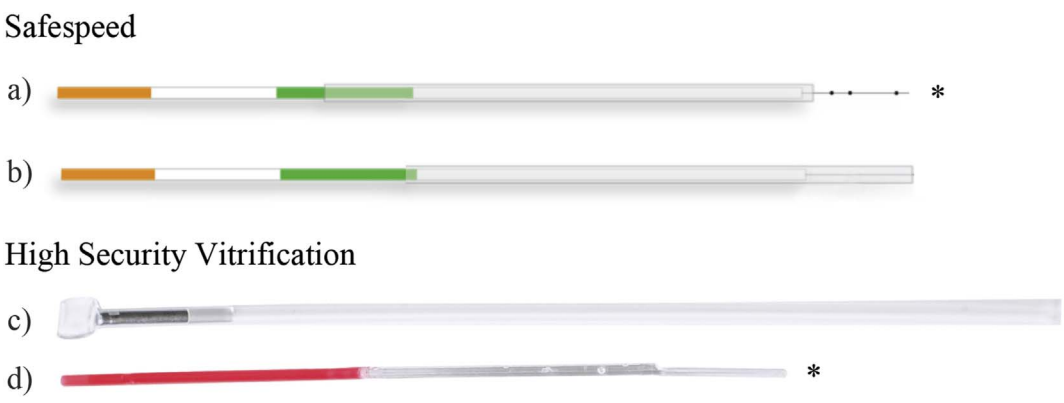

Fig. 1. The SafeSpeed carrier (a, b) presents a capillary (*) where the embryos, while floating in the vehicle solution (vitrification volution), are loaded by aspiration. This capillary is sealed before plunging in liquid nitrogen and cut open before expelling the embryos in warming solution.
The sealed capillary must be uncovered by the protective cap during cooling in liquid nitrogen and warming in a 37 °C water bath (a). During storage and handling, it is protected by a sliding cover, in grey (b).
The High Security Vitrification device is composed of a carrier straw (d), with a v-shaped tip in which the embryos are loaded with a minimum volume of vehicle solution (*). This carrier straw is then introduced in the outer straw (c), which is sealed before plunging in liquid nitrogen for cooling. For warming, the outer straw is cut open and the carrier straw extracted; the embryos are re-warmed by immersion of the tip of the carrier straw in 1 mL of warming solution.

from day 13 onwards. In patients with regular ovarian function a GnRH analogue (Decapeptyl, Ipsen-Pharma, France) was administered in the midluteal phase for pituitary desensitization.

Progesterone supplementation started when endometrial thickness reached 6–8 mm and trilinear appearance at ultrasound was confirmed, with 200 mg intravaginal capsules (Utrogestan, SEID, Spain) every 8 h. The warming and transfer of embryos was performed on the same day, with at least two hours of difference.

Circulating ß-hCG levels were determined 12 days after the embryo replacement, and the presence of a gestational sac was confirmed by ultrasound after 5 weeks. In pregnant patients, the hormonal treatment was sustained for 12 weeks.

2.5. Outcomes of the study and statistical analysis

Donors and patients' baseline characteristics considered were: age of the donor at the time of ovarian puncture, age, endometrial thickness, duration of the follicular phase of the recipient in the cycle of the embryo replacement, and the quality of the replaced blastocysts. The primary outcome of the study is the survival rate of blastocysts to vitrification and warming. Secondary outcomes analysed are: number of patients with positive ß-hCG levels, number of patients with clinical pregnancy (CPR; presence of one gestational sac with heartbeat), implantation rate (number of gestational sacs per blastocyst transferred), miscarriage rates and live-birth rates. Quantitative variables are expressed as mean and standard deviation (SD). Differences between groups were analysed with Student's t-test or Mann-Whitney *U* test according to the distribution. Qualitative variables are expressed as percentage. Proportions were compared using the chi-square test.

3. Results

The baseline characteristics of donors and patients included in the study did not differ in between groups (Table 1). Both vitrification methods achieved excellent results, with 80/82 blastocysts (97.5%) vitrified and warmed with the HSV device presenting morphological survival at the time of the embryo transfer, and 50/52 (96.1%) with the SS carrier.

In Table 2, the clinical outcomes of the warmed blastocysts are presented: the number of biochemical and clinical pregnancies, implantation rates, miscarriage rates and live birth rates did not differ in between groups.

Table 1
Donor age at the time of the donation, recipient age at the time of the embryo transfer, endometrial thickness at the day of the last ultrasound check before the embryo transfer, duration of the follicular phase since estrogenic supplementation. Data expressed as mean (SD).

	HSV	SS	p
Number of cycles	50	29	
Donor age (y. o.)	24.7 (4.0)	26.0 (2.9)	0.138
Recipient age (y. o.)	40.9 (4.5)	41.7 (3.3)	0.388
Endometrial thickness (mm)	8.8 (1.5)	8.5 (1.2)	0.483
Duration of follicular phase (days)	15.8 (2.9)	16.4 (3.2)	0.387

Table 2
Data expressed as absolute values (percentage) or mean (SD). Positive ß-hCG (> 5 mIU/mL), clinical pregnancy rate (gestational sac with foetal heartbeat), ongoing pregnancy rate (after the first trimester) and live birth rate expressed per transfer performed. Implantation rate as number of gestational sacs per blastocyst transferred. Miscarriage rate and multiple pregnancy rates expressed per clinical pregnancy.

	HSV	SS	p
Blastocysts warmed per patient	1.6 (0.5)	1.8 (0.4)	0.182
Survival rate	80/82 (97.5)	50/52 (96.1)	0.641
Number of blastocysts transferred per patient	1.6 (0.5)	1.7 (0.5)	0.560
Positive ß-hCG	22/50 (44.0)	15/29 (51.7)	0.507
Clinical pregnancies	21/50 (42.0)	14/29 (48.3)	0.588
Ongoing pregnancies	16/50 (32)	11/29 (37.9)	0.592
Implantation rate	25/80 (31.2)	17/50 (34)	0.767
Multiple pregnancies (twin)	4/21 (19.0)	3/14 (21.4)	0.863
Miscarriage rate	5/21 (23.8)	3/14 (21.4)	0.869
Live birth rate	16/50 (32)	11/29 (37.9)	0.592

4. Discussion

In the present study, the results of two closed devices for vitrification of human blastocysts were compared, with similar outcomes in terms of survival to the vitrification and warming process. For blastocysts cryopreserved with each carrier, the manufacturer's kit of solutions and recommended protocol of preparation for vitrification and rehydration after warming was used. However, both sets of solutions contain very similar concentration and types of cryoprotectants, and recommend similar exposure regimes to the solutions [7]. Hence, we can assume that the cytosolic solute concentration in the cells of the cryopreserved blastocysts was similar. The interest of this comparison lies then in the remaining variables affecting the probability of

vitrification that depend on the vitrification carrier: the rates of cooling and warming they attain. These rates are determined by how each carrier is exposed to the cooling and warming solutions, their thermal mass and overall heat exchange capabilities.

The HSV carrier presents several features that impair its cooling rate: the protective outer plastic straw and the sleeve of air thermally isolate the samples placed in the carrier. It also will produce more boiling when plunged into the liquid nitrogen, by evaporating more of it, due to its increased thermal mass [5,35]. The HSV does however allow retrieving the carrier from the protective straw for warming, which is performed in similar fashion to open carriers, by transferring the tip of the carrier containing the embryos into a volume of warming solution, allowing direct contact, which might compensate the deleterious effect of impaired cooling [21,27,33]. Warming rates achieved will be dependent on the volume of warming solution used [9]. On the other hand, in the case of SS carrier, the thin-walled capillary containing the embryos was developed to attain higher cooling (120000 °C/min, data provided by manufacturer) and warming rates (200000 °C/min) [13,29].

Despite the difference in terms of cooling rates and warming rates at which both carriers operate, they successfully cryopreserved blastocysts subjected to the recommended cryoprotectant agents loading and dehydration protocol, avoiding lethal ice formation during cooling to −196 °C and warming to 37 °C. The high survival rate obtained with HSV was not improved by using a carrier operating at higher cooling and warming rates. Similarly, in a recent meta-analysis, differences were also not detected when comparing survival of blastocysts from open (Cryotop, Cryoleaf, and Cryoloop) and diverse closed carriers (semi-closed: Rapid-I; capillary-based: Cryotip and hermetically sealed into a container: HSV-CBS), but a trend towards decreased live birth rates per transfer was however identified [41]. In our study, despite the higher gap in terms of heat-exchange rates in the carriers used, similar implantation and live birth rates were observed. However, the power of the comparison is very limited due to the small sample size, and potential confounding pre and peri-fertilization variables that can influence outcomes, as well as the use of top-quality donor eggs [4,26,28].

In terms of the risk of cross-contamination through LN_2 or its vapours during cooling, storage, and warming, both closed carriers protect the sample from direct contact with liquid nitrogen. That is not to say that they are totally risk-free: the cutting of the rear end of the protective straw could be a source of contamination in the HSV, as well as the warming of the SS capillary in a water bath and the cutting of said capillary to release the embryos [38]. It must be noted that the odds of nitrogen-mediated contamination have been questioned, considering the number of embryos cryopreserved worldwide and the lack of reported cases [22].

Despite both carriers used in this study avoiding contact of the embryos with liquid nitrogen and are hence 'closed', one belongs to the carrier-sealed-into-a-container category, while the other is capillary-based. As a result, they present vastly different thermodynamic aspects. The cooling and warming rates obtained, in combination with the cytosolic solute concentration obtained with a specific cryoprotectant-loading and dehydration regime, as well as the repeatability of the handling by the operators, is what ultimately determines their efficacy [9]. For that reason, grouping these two carriers in the same group of closed systems may not be completely accurate. Further evidence from prospective studies is necessary to clearly determine the influence of the combination of cooling and warming rates on the clinical outcomes of cryopreserved blastocysts; the thermodynamic characteristics of vitrification carriers should be the primary aspect taken into account for future research, which is necessary to achieve the optimal balance between the safety and efficacy of vitrification of human embryos.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cryobiol.2018.03.002.

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