In vitro and in vivo viability of human blastocysts collapsed by laser pulse or osmotic shock prior to vitrification

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Abstract

Purpose This study was designed to investigate whether artificial shrinkage, induced by a laser pulse or hyperosmotic sucrose solutions, improves in vitro survival and/or implantation of vitrified-warmed human expanded blastocysts.

Methods Before Cryotop vitrification, the blastocoelic cavity was collapsed either by a laser pulse or sucrose solutions. Non-treated blastocysts were used as control. Post-warm blastocyst survival and implantation after transfer were examined. Implantation rate outcome was retrospectively analyzed by morphological grading and developmental kinetics of post-warm blastocysts.

Results Survival rates in the three groups were high. Implantation rates in the laser-pulse group (59.7%) were comparable with those in the sucrose group (49.3%), and were significantly higher than those in the control group (34.2%). The proportion of blastocysts showing fast development tended to be higher when the blastocysts underwent artificial shrinkage treatment before vitrification. There was no clear correlation between morphology of post-warm blastocysts and implantation rate.

Conclusion Artificial shrinkage treatment before vitrification is associated with an increased probability of fast-developing embryos, resulting in higher implantation rates.

Keywords Artificial shrinkage · Clinical pregnancy · Laser pulse · Sucrose · Vitrification

Introduction

The first pregnancy from cryopreserved human embryos [1] and the birth of the baby [2] have been achieved using conventional slow freezing regimen and early cleaving embryos. Since the last decade, improvement in human embryo culture systems [3] has increased the opportunity for transfer and cryopreservation of blastocysts. Transfer of a viable blastocyst offers high efficiency for maintaining pregnancy rate and minimizing multiple gestations in the human in vitro fertilization (IVF) program [4]. The conventional slow freezing regimen applied to human blastocysts had a moderate success rate [5] and the conventional vitrification procedure improved the viability of post-warm blastocysts [6]. Both of these techniques used 0.25-ml plastic straws as the cryodevice. Thereafter, the minimum-volume cooling (MVC) vitrification procedure characterized by extremely high cooling rates was found to be more effective in improving the viability of post-warm blastocysts. Several cryodevices, such as Cryotop [7], microgrid [8], hemi-straw [9], Cryoloop [10], and Cryotip [11], have been used to accelerate the cooling rate for cryopreservation of human blastocysts. It is unlikely that the ovarian-uterine conditions during stimulated cycle equally support the implantation of transferred blastocysts when compared with those in non-stimulated cycles. Thus, the potential risk of low pregnancy rate in the fresh embryo transfer (ET) program of the treated cycle can be avoided by transferring the cryopreserved blastocysts at subsequent cycles.

Capsule Artificial shrinkage of human blastocysts prior to Cryotop vitrification is associated with an increased probability of fast-developing embryos, resulting in the higher clinical pregnancy rates.

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Recent development of the *in vitro* culture system for human embryos [12, 13] has resulted in effective production of expanded blastocysts. The expanded human blastocysts are probably more sensitive to cryopreservation than early blastocysts because detrimental ice crystal formation may occur in the blastocoelic cavity due to insufficient dehydration. In fact, cryotolerance of expanded blastocysts improved if the blastocoelic cavity was artificially collapsed prior to cryopreservation [14, 15]. Mechanical stimuli by micro-needle [14, 15], 29-G needle [16], laser pulse [15], and pipetting [17] have been used to induce artificial shrinkage of the blastocoelic cavity in expanded human blastocysts. In addition, treatment with hyperosmotic sucrose solutions made it possible to induce artificial shrinkage of blastocoelic cavity without special equipments, instruments, and/or micromanipulation skills (unpublished information; Hayashi et al. presented their data at the 2006 Annual Meeting of Japan Society of Fertility and Implantation).

In the present study, it was investigated whether the artificial shrinkage of blastocoelic cavity, induced by applying a laser pulse or exposing to hyperosmotic sucrose solutions, improves *in vitro* survival and/or implantation of vitrified-warmed human blastocysts.

Materials and methods

Experimental design

Prior to MVC vitrification, human expanded blastocysts were either treated with a laser pulse (laser pulse group) or exposed to hyperosmotic sucrose solutions (sucrose group) for artificial shrinkage of the blastocoelic cavity. The control group contained untreated human expanded blastocysts. From November 2007 to June 2010, single-embryo transfer was performed with a total of 242 vitrified-warmed blastocysts in 193 patients who failed to get pregnant in previous IVF/intracytoplasmic sperm injection (ICSI)-ET programs. The implantation rates were retrospectively analyzed by morphological grading and developmental kinetics of the post-warm blastocysts. Informed consent was obtained from all patients prior to the study. Approval of the Ethical Board Committee of our institution was obtained before initiation of the study.

Preparation of blastocysts

Ovaries were stimulated by a gonadotropin-releasing hormone antagonist (0.25 mg/day; Cetrotide®; Shionogi, Osaka, Japan) in association with a human menopausal gonadotropin (HMG150; Fuji Pharma, Tokyo, Japan) and a follicle stimulating hormone (Follistim®; Schering-Plough, Osaka, Japan). When the maximum diameter of two or more follicles reached 18 mm, human chorionic gonado-

tropin (hCG; HCG 5000; Fuji Pharma) was administered. Oocytes were transvaginally retrieved approximately 36 h after hCG injection, and inseminated either by IVF or ICSI [18]. Eighteen to 20 h after insemination, oocytes with two pronuclei and second polar body, defined as normally fertilized, were selected and cultured for 6 days in a potassium simplex optimized medium containing amino acids [19] (Global®; IVFonline, Guelph, ON, Canada) supplemented with 0.5% (w/v) human serum albumin (HSA; Irvine Scientific, Santa Ana, CA, USA) at 37°C in a humidified air of 5% CO₂, 5% O₂ and 90% N₂. All expanded blastocysts with thinning zonae pellucidae harvested on Day-5 or -6 were used for subsequent artificial shrinkage treatment and vitrification (Day-0 was defined as the day of IVF/ICSI), except for the blastocyst harvested on Day-5 and immediately transferred to the uterus of patients (one to two blastocysts per trial). The blastocysts were morphologically graded for inner cell mass (ICM) and trophoctoderm (TE) according to the Gardner's criteria [20]. Blastocysts with B/B or better score for the ICM/TE were defined as having "good" morphology.

Artificial shrinkage treatments

One of the two different treatments (application of a laser pulse or exposure to sucrose solutions) was employed to induce artificial shrinkage of blastocoelic cavity in expanded blastocysts at room temperature (26 to 28°C). In laser pulse treatment [15], using the laser system ZILOS-tk™ (Hamilton Thorn Bioscience Inc., Beverly, MA, USA), one single laser shot (500 μs) was delivered to the cell-to-cell junction of TE while the ICM was positioned far from the shooting spot (Fig. 1a). In sucrose treatment, a blastocyst was first exposed to a 50-μl droplet of HEPES-buffered human tubal fluid (modified HTF-HEPES; Irvine Scientific) supplemented with 20% (v/v) serum substitute solution (SSS; Irvine Scientific), and then exposed to 50-μl droplets of modified HTF-HEPES/20% SSS containing 0.125 and 0.25 M sucrose (Wako Pure Chemical Industries, Osaka, Japan) in a stepwise manner (Fig. 1b). Exposure period was defined as the time from loading of the blastocyst at the top of the droplet until it settled down at the bottom.

MVC vitrification procedure

Collapsed or non-collapsed blastocysts underwent MVC vitrification using Cryotop (Kitazato BioPharma Co., Shizuoka, Japan) as the cryodevice [7]. Blastocysts were equilibrated with 7.5% ethylene glycol (EG; Wako Pure Chemical Industries) and 7.5% dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) in modified HTF-HEPES/20% SSS for 7 to 12 min at room temperature. This

Fig. 1 Artificial shrinkage of blastocoelic cavity in human expanded blastocysts. **a** Treatment by a laser pulse or **b** treatment with hyperosmotic sucrose solutions (1) before and (2) after the treatment. Scale bar=50 μ m

equilibration period was determined by the 90% recovery of the original embryonic volume. The blastocysts were then transferred to a vitrification solution containing of 15% EG, 15% DMSO and 0.5 M sucrose in modified HTF-HEPES/20% SSS for 60 to 90 s at room temperature. During this period, blastocysts were loaded on the top of a polypropylene strip of a Cryotop with a minimum amount ($<0.1 \mu$ l) of vitrification solution, and then quickly immersed into liquid nitrogen (LN_2).

After storage for several months in LN_2 , blastocysts were warmed by immersing the strip in 3 ml of the modified HTF-HEPES/20% SSS containing 1.0 M sucrose at 37°C for 1 min. The blastocysts were transferred to the modified HTF-HEPES/20% SSS at room temperature in a stepwise manner (0.5, 0.25, and 0 M sucrose for 3, 4, and 9 min, respectively). During the dilution process in 0.25 M sucrose solution, assisted hatching treatment (the quarter segment-zona opening) was applied to the blastocysts using the laser system ZILOS-tk™ laser system.

Culture and transfer of post-warm blastocysts

Vitrified-warmed blastocysts were cultured in a 50- μ l droplet of Global®/0.5% HSA for 2 to 4 h at 37°C in a humidified air of

5% CO_2 , 5% O_2 and 90% N_2 . Their cryosurvival was assessed by the presence of the blastocoelic cavity 2 h after the in vitro culture. Simultaneously, the survivors were morphologically graded according to Gardner's criteria [20] and were classified into two categories based on their developmental kinetics [21]. Surviving blastocysts with $\geq 50\%$ increase in their embryonic diameter were defined as fast-developing embryos.

All blastocysts that survived MVC vitrification were transferred to the uterus of patients 2 to 4 h after warming (one blastocyst per transfer in each attempt). A vaginal suppository of progesterone (400 mg/day), an intramuscular injection of progesterone (Progeston depot 125 mg/week; Fuji Pharma), and a transdermal estradiol (Estrana 1.44 mg/day; Hisamitsu Pharmaceutical, Tokyo, Japan) were administered to provide luteal support. Implantations were confirmed by ultrasonic monitoring of the uterine cavity (gestational sac) 4 weeks after transfer.

Statistical analysis

Proportional data and efficiency of artificial shrinkage were analyzed by Fisher's exact probability test and

Student's *t*-test, respectively. For cryosurvival and implantation, $P<0.017$ was considered statistically significant following Bonferroni correction. For other parameters, $P<0.05$ was considered statistically significant.

Results

Profiles of blastocysts in the laser pulse ($n=129$), sucrose ($n=75$), and control ($n=39$) groups were comparable, with respect to mean age of patients (36.4, 34.7 and 35.4 years), proportion of blastocysts with “good” morphology (83.6, 86.7 and 76.9%), the proportion of blastocysts harvested on Day-5 (85.3, 89.3 and 97.4%), and IVF/ICSI ratio for blastocyst origin (1.11, 0.79 and 0.63). Mean diameters of collapsed blastocysts relative to their original ones were comparable as 0.77 ($n=8$) and 0.86 ($n=6$) in the laser-pulse and the sucrose groups, respectively, and the calculated ratios of surface area to volume (S/V) were 1.30 and 1.16, in the laser-pulse and the sucrose groups, respectively.

As shown in Fig. 2, survival rates of all the vitrified-warmed blastocysts were high (97.4 to 100%). Implantation rates of vitrified-warmed blastocysts in the laser pulse group (59.7%) were comparable with those in the sucrose group (49.3%), and were significantly higher than those in the control group (34.2%; $P<0.017$). Proportions of vitrified-warmed blastocysts with “good” morphology were not different among the three groups (60.0 to 69.8%), and this parameter was not associated with implantation outcome (Table 1). In contrast, the proportion of fast developing blastocysts ($\geq 50\%$ increase in diameter after 2-h of culture) tended to be higher when the blastocysts underwent artificial shrinkage treatment prior to vitrification (49.6, 46.7 and 31.6% for laser pulse, sucrose, and control groups, respectively; Table 2). Transfer of fast- vs. slow-developing embryos resulted in significantly higher implantation rates in both artificial shrinkage groups (68.8 vs. 50.8% in laser pulse group, and 65.7 vs. 35.0% in sucrose group).

Fig. 2 Effect of artificial shrinkage treatments prior to vitrification of human blastocysts on in vitro survival and implantation rates after blastocysts transfer. **a-b** Different letters on the columns indicate significant difference ($P<0.017$: Bonferroni correction)

Discussion

Very high survival rates of vitrified-warmed blastocysts in vitro (97.4 to 100%, Fig. 2) were in agreement with the previous reports by Stehilik et al. [22] and Liebermann et al. [23], both of which used Cryotop as the cryodevice for cryopreservation of human blastocysts. In the present study, improved implantation rates of the vitrified-warmed blastocysts (49.3 to 59.7 vs. 34.2% in control, Fig. 2) were obtained if the blastocoelic cavity was collapsed prior to vitrification. A direct comparison of post-vitrification implantation rate and clinical pregnancy rate from collapsed human blastocysts between this study and previously published papers was summarized in Table 3. For examples, Vanderawallen et al. [14] reported that implantation rates of human blastocysts after vitrification with conventional 0.25-ml plastic straws increased from 7.1 to 18.4% following an artificial shrinkage treatment by micro-needle. Mukaida et al. [15] also reported that the clinical pregnancy rate of human blastocysts after Cryoloop vitrification increased from 34.1 to 61.5%

Table 1 Retrospective analysis for clinical pregnancy based on morphological grading of blastocysts 2 h after warming

Artificial shrinkage	No. of Survivors	Morphological grading ¹	No. (%) of blastocysts	
			Incidence ²	Implanted ³
Laser pulse	129	Good	90 (69.8)	52 (57.8)
		Fair	39 (30.2)	25 (64.1)
Sucrose	75	Good	45 (60.0)	26 (57.8)
		Fair	30 (40.0)	11 (36.7)
Non-treated control	38	Good	23 (60.5)	10 (43.5)
		Fair	15 (39.5)	3 (20.0)

¹ Morphological grading: Good; B/B or better by Gardner's criteria for ICM/TE cells, Fair; others.

² Percentages were calculated from survivors.

³ Percentages were calculated from incidence (=transferred embryos).

Table 2 Retrospective analysis for clinical pregnancy based on developmental kinetics during 2-h culture of post-warm blastocysts

Artificial shrinkage	No. of Survivors	Developmental kinetics ¹	No. (%) of blastocysts	
			Incidence ²	Implanted ³
Laser pulse	129	Fast	64 (49.6)	44 (68.8) ^a
		Slow	65 (50.4)	33 (50.8) ^b
Sucrose	75	Fast	35 (46.7)	23 (65.7) ^c
		Slow	40 (53.3)	14 (35.0) ^d
Non-treated control	38	Fast	12 (31.6)	5 (41.7)
		Slow	26 (68.4)	8 (30.8)

¹ Developmental kinetics: Fast; $\geq 50\%$ increase in embryonic diameter at 2 h of post-warm culture, Slow; others.

² Percentages were calculated from survivors.

³ Percentages were calculated from incidence (=transferred embryos).

a vs b, c vs d Significantly different at $P < 0.05$.

following an artificial shrinkage treatment either by laser pulse or by micro-needle. In expanded mouse blastocysts, artificial shrinkage induced by laser pulse prior to vitrification was effective in decreasing the proportion of embryonic cells with fragmented DNA at 3-h post-warm culture and increasing the total cell number of blastocysts at 24-h post-warm culture [24].

In the present study, no significant difference in implantation rate between the laser pulse and sucrose groups (59.7 vs. 49.3%, Fig. 2) was observed. Efficiency of dehydration (relative reduction in mean diameter; 0.77 and 0.86, respectively, Fig. 1) was comparable between both treatment groups. Since the calculated S/V ratios in collapsed blastocysts in both treatment groups were ≥ 1.0 than the S/V ratio of non-collapsed controls (1.30 and 1.16, respectively), accelerated dehydration and permeation of cryoprotective additives were expected in these blastocysts [25], and thus, the potential risk of ice crystal formation in the blastocoelic cavity may have been reduced. Treatment with hyperosmotic sucrose solutions induces not only

shrinkage of blastocoelic cavity but also dehydration of individual embryonic cells. Therefore, further investigation may be required for the optimal combination of sucrose concentration and exposure period. One possible advantage of the sucrose treatment would be the easy handling of several blastocysts in a single operation, without expensive equipment such as a laser pulse generator [15] and/or complicated operation protocol for artificial shrinkage [14, 16, 17]. In the Japanese abstract presented at the 2006 Annual Meeting of Japan Society of Fertilization and Implantation by Hayashi et al., all seven blastocysts collapsed in 0.5 M sucrose solution had survived the vitrification procedure, and resulted in five pregnancies after blastocyst transfer (without control data for comparison).

Based on retrospective analyses, beneficial effect of artificial shrinkage on implantation may be explained by improving the developmental kinetics of vitrified blastocysts during post-warm culture (Table 2), rather than the morphological grading of vitrified surviving blastocysts

Table 3 Direct comparison of implantation rate and clinical pregnancy rate derived from vitrified-warmed human blastocysts with collapsed blastocoelic cavity between this study and previous publications [14, 15]

Investigators	Artificial shrinkage	Implantation ¹	Clinical pregnancy* ²
This study	Laser pulse	77/129 (59.7)	77/129 (59.7)
	Sucrose	37/75 (49.3)	37/75 (49.3)
	Non-collapsed control	13/38 (34.2)	13/38 (34.2)
Vanderzwalmen et al. [14]	Micro-needle	9/49 (18.4)	8/35 (22.9)
	Non-collapsed control	1/14 (7.1)	1/7 (14.3)
Mukaida et al. [15]	Laser pulse	18/37 (48.6)	16/26 (61.5)
	Micro-needle	191/411 (46.5)	144/240 (60.0)
	Non-collapsed control	N.A. ³	29/85 (34.1)

¹ Implantation: No. embryos implanted/No. embryos transferred (%).

² Clinical pregnancy: No. patients pregnant/No. patients received embryos (%).

³ N.A. Data not available.

after warming (Table 1). The fact that post-warm blastocysts with faster developmental kinetics resulted in higher implantation outcomes has been reported in humans [21, 26, 27] and sheep [28]. The criterion of fast-developing embryos employed in the present study ($\geq 50\%$ increase in diameter at 2-h culture) has been adopted from Shu et al. [21]. The significantly higher implantation rates of the fast- vs. slow-developing blastocysts were found both in the laser pulse (68.8 vs. 50.8%) and sucrose (65.7 vs. 35.0%) groups. Absence of significant difference in non-treated control group (41.7 vs. 30.8%) may be due to the limited number of blastocysts transferred. Na^+/K^+ -ATPase (pump) in plasma membrane is responsible for the expansion of blastocoelic cavity, and $>60\%$ of total ATP produced during blastocyst development is utilized for this ATPase [29]. If the energy generation -the mitochondrial function- of non-collapsed, vitrified-warmed blastocysts was indeed inferior to that from their in collapsed counterparts, the slower expansion of the blastocoelic cavity observed during the post-warm culture of non-treated blastocysts may be justified. The absence of correlation between the morphological grading and the implantation was noted especially in the laser pulse group (Table 1). The survivors in this group were appeared to carry less ICM and/or TE cells but their implantation rate was not low (64.1%, 25/39), remaining the possible reasons unclear.

In conclusion, artificial shrinkage of human blastocysts prior to Cryotop vitrification is associated with increased probability of fast-developing embryos, resulting in the higher clinical pregnancy rates. While both treatments by applying a laser pulse and exposing to hyperosmotic sucrose solutions were effective in inducing artificial shrinkage, the latter (sucrose treatment) may be preferable in view of technical challenges and cost.

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