Assisted Hatching at the Time of Warming Improves Pregnancy and Implantation Outcomes for Vitrified Human Expanded Blastocyst Transfer

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Abstract: *Vitrification has been focused on as a promising approach for human blastocyst cryopreservation, but few reports are available on the effect of assisted hatching (AH) in conjunction with human vitrified blastocyst transfers. Therefore, in this study, AH with acidic Tyrode was performed at the time of warming of vitrified blastocysts before transfer in order to improve the implantation and pregnancy rates. In the AH group, 13 clinical pregnancies (54.2%) and 15 implantations (36.6%) out of 41 blastocysts transferred were obtained. In the non-AH group, 1 clinical pregnancy (12.5%) and 1 implantation (6.7%) out of 15 blastocysts transferred were obtained. AH on the vitrified blastocysts after warming improved the implantation rate significantly (P<0.03). The pregnancy rate was also increased statistically in the AH group (P<0.05). The results suggest that the vitrification procedure may cause hardening of the zona pellucida and AH of vitrified blastocysts would be useful for clinical application.*

Key words: *Assisted hatching, Blastocyst, Cryopreservation, Human, Vitrification*

Introduction

With the introduction of the sequential culture system, blastocyst culture is being adopted by many IVF clinics as a means to increase the pregnancy rates and also minimize multiple gestations [1–3]. Therefore, a reliable procedure for cryopreservation of supernumerary blastocysts is needed. Recently, many human pregnancies that originate from an ultra-rapid vitrification technique are achieved after cryopreserving the blastocysts with the cryotop [4], the cryoloop [5–7], electron microscope grids [8, 9] or the hemi-straw [10]. In addition, the survival rate of expanded blastocysts after vitrification increases when, to reduce ice crystal formation, the blastocoele is artificially shrunk by pipetting, with [11, 12] a glass micro-needle [13] or two 29-gauge needles [9].

On the other hand, the freezing-thawing procedure may cause hardening of the zona pellucida and some investigators report the usefulness of assisted hatching (AH) for frozen-thawed cleavage stage embryos [14, 15]. Nevertheless, few reports are available on the effect of AH in conjunction with human vitrified blastocyst transfers [10]. Therefore, we retrospectively evaluated the pregnancy and implantation rates after the transfer of vitrified blastocysts in conjunction with or without an AH protocol to assess the efficacy of AH in human vitrified blastocyst transfer.

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Materials and Methods

Patients and IVF

All patients who entered this vitrified blastocyst transfer program, had undergone consecutive embryo transfer [16] with fresh embryos but failed to become pregnant and had had previous multiple failures of conventional day 2 or day 3 embryo transfers. The day of oocyte retrieval was considered as day 0.

Women were treated with GnRH analogue buserelin acetate (MOCHIDA, Tokyo, Japan) and hMG (Nikken, Tokyo, Japan) in either a long- or a short-treatment protocol. They were administered hCG (TEIZO, Tokyo,

Japan) when dominant follicles reached a diameter of 18 mm. Oocytes were collected 35 h after hCG (TEIZO) administration by a vaginal ultrasound-guided procedure. The oocytes were inseminated by either conventional IVF or ICSI, and incubated in HTF medium (Irvine Scientific, CA) containing 10% (v/v) serum substitute supplement (SSS; Irvine) at 37°C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2. Fertilization was confirmed at 15–18 h after insemination by the presence of two pronuclei.

Embryo culture and grading of blastocysts

Fertilized oocytes were washed well and cultured in Blast Assist Medium 1 (Medicult, Jyllinge, Denmark) until day 3, and then placed in Blast Assist Medium 2 (Medicult) until day 6. In all cases, consecutive embryo transfer was performed, in which one or two cleaved embryos were transferred on day 2 or day 3 (first step of embryo transfer) and one or two blastocysts were transferred on day 5 or day 6 (second step of embryo transfer) [16]. After a consecutive embryo transfer, surplus embryos that developed to the expanded blastocyst stage (diameter ≥160 μm) were cryopreserved on day 5 or day 6. For expanded blastocysts the development of the inner cell mass (ICM) and trophectoderm can be assessed. The ICM grading is as follows: A. tightly packed, many cells; B. loosely grouped, several cells; C. very few cells [17]. The trophectoderm grading is as follows: A. many cells forming a tightly knit epithelium; B. few cells; C. very few cells forming a loose epithelium [17]. Only expanded blastocysts scoring BB or higher were vitrified.

Preparation of vitrification solutions

The expanded blastocysts were vitrified by the method developed by Kuwayama [18] with a cryotop that consists of polyethylene laminate film (20 mm × 0.7 mm × 0.1 mm, L × W × T; Kitazato Supply, Inc., Shizuoka, Japan).

As the base medium, Dulbecco's phosphate buffered saline solution (PBS 1 × Irvine) plus 20% (v/v) SSS (Irvine) was used. The equilibration solution contained 7.5% (v/v) ethylene glycol (EG) (Sigma Chemical Co., MO) and 7.5% (v/v) dimethyl sulfoxide (DMSO) (Kanto Chemical Co., Tokyo, Japan). The vitrification solution was composed of 15% (v/v) EG, 15% (v/v) DMSO and 0.5 mol/l of sucrose (Nacalai Tesque, Inc., Kyoto, Japan). Both cryoprotectant solutions were warmed briefly in an incubator at 37°C, and blastocysts were handled on the stage warmer of a dissecting microscope at 38°C.

Artificial shrinkage of expanded blastocysts and vitrification

Before starting the vitrification procedure, artificial shrinkage of all expanded blastocysts was performed in the equilibration solution. First, pipetting of the expanded blastocyst was conducted with a fine hand-drawn glass pipette slightly smaller in diameter (~140 μm) than the expanded blastocyst. After confirmation of slight shrinkage of the blastocoele, pipetting was performed with a pipette slightly smaller in diameter than the first one (~100–120 μm). This procedure was repeated 2–3 times until the blastocoele collapsed completely. After blastocoele contraction, the blastocysts were equilibrated in the equilibration solution for another 2 min before exposure to the vitrification solution. The blastocysts were then incubated in the vitrification solution and loaded, within 45 s, onto the tip of the cryotop with ~1 μl of cryoprotectant solution. Then the cryotop was immediately plunged into liquid nitrogen.

Warming and artificial AH procedure

Before warming blastocysts, 1.0 mol/l sucrose solution, 0.5 mol/l sucrose solution, and the base medium were warmed briefly in an incubator at 37°C. The warming procedure was done as follows. The cryotop tip with the blastocysts was plunged directly into 1.0 mol/l sucrose solution for 1 min. The blastocysts were then transferred to a 0.5 mol/l sucrose solution for 3 min and washed twice in the base medium for 5 min. All steps were completed on the stage warmer of a dissecting microscope at 38°C. As soon as warming of the blastocysts had been completed, AH was performed while the warmed blastocysts remained collapsed (within 5 min after completing the warming procedure) in Sperm Washing Medium (Irvine). After immobilization of the blastocyst on a holding pipette (HUMAGEN, VA) at 9 o'clock, acidic Tyrode's solution (pH 2.1–2.5; Irvine) was expelled from the assisted hatching pipette (HUMAGEN) toward a hemisphere of the embryo to dissolve only the outer surface of the zona pellucida (large zona thinning). After large zona thinning, the assisted hatching pipette was pressed against the thinning inner zona pellucida at 3 o'clock and a 35- to 40-μm hole was drilled by suction with the assisted hatching pipette. Subsequently, the blastocysts were rinsed and cultured in Blastocyst medium (Irvine) containing 10% SSS (Irvine) for further culture until transfer. The post-warming survival of blastocysts was observed 3 h after warming under a microscope, and re-expanded blastocysts were judged to have survived.

Table 1. Patient characteristics

	AH	non-AH	P value
No. of patients	24	7	
Female age (range of age)	35.3 ± 3.9 (30–45)	34.1 ± 4.0 (27–39)	NS
Prior cycles (range of prior cycles)	3.3 ± 1.7 (2–7)	2.9 ± 1.2 (2–5)	NS

NS: not significant.

Table 2. Embryo development

	AH	non-AH	P value
Number of MII oocytes	11.2 ± 5.7	14.6 ± 4.9	NS
Fertilization (% of total MII oocytes)	77	76	NS
Cleavage (%)	99	99	NS
Day 5 blastocysts (number)	2.6 ± 2.0	2.9 ± 2.5	NS
Day 5 blastocysts (%)	34.8	30.8	NS
Total blastocyst (days 5 and 6)	4.4 ± 2.5	4.7 ± 2.8	NS
Total blastocysts (%)	58.0	50.8	NS

NS: not significant.

Table 3. Results of human expanded blastocyst vitrification in relation to assisted hatching (AH) from the zona pellucida

	AH	non-AH	P value
No. of cycles for vitrification	24	7	
No. of cycles for warming	24	8	
No. of expanded blastocysts vitrified	41	15	
No. of blastocysts which survived (%)	41 (100)	15 (100)	NS
No. of hatching blastocysts (%)	25 (61.0)	0 (0)	P<0.01
No. of embryos transferred	41	15	
Average no. of blastocysts transferred	1.7 ± 0.6	1.9 ± 0.8	NS
No. of embryos implanted (%)	15 (36.6)	1 (6.7)	P<0.03
No. of cycles transferred	24	8	
No. of clinical pregnancies (%)	13 (54.2)	1 (12.5)	P<0.05

NS: not significant.

Embryo transfer was scheduled on day 5 after ovulation in the spontaneous cycles irrespective of whether they had been day 5 or day 6 at the time of vitrification. The time from warming to transfer ranged from 3 to 5 h, and 32 transfers were performed. One to three surviving blastocysts were transferred into the patient's uterus. Pregnancy was first assessed by urinary hCG 9 days after blastocyst transfer, and then clinical pregnancy was confirmed by the presence of fetal heart activity 30 days after blastocyst transfer.

Statistical analysis

The data obtained were examined for differences by means of Student's group t-tests and Fisher's exact probability test as appropriate. Results are expressed as the mean ± SD. $P<0.05$ was considered as statistically significant.

Results

There were no differences in parameters evaluated regarding patient characteristics and IVF outcome in the AH and non-AH groups (Tables 1 and 2). The percentage of patients having ICSI was similar in both groups (63% in the AH group vs. 57% in the non-AH group). Table 3 shows the results of human expanded blastocyst vitrification in relation to AH from the zona pellucida. A total of 56 were vitrified in 31 vitrification cycles. In the AH group, 21 (51%) were vitrified on day 5, and 20 (49%) on day 6. In the non-AH group, 8

(53%) were vitrified on day 5, and 7 (47%) on day 6. After 32 cycles of warming for blastocyst transfer, 56 (100%) of the embryos survived. Of these, AH was performed on 41 (24 warming cycles). Out of 41 embryos from the AH group, 25 (61.0%) started the hatching process 3 h after warming. In the non-AH group no embryos (0%) out of the 15 started the hatching process after 3 h warming ($P<0.01$). In addition, although there were no differences in the survival rate and the mean number of blastocysts transferred, we observed statistically significant differences between the two groups in the implantation and pregnancy rates. The implantation rate in terms of fetuses per transferred embryo in the AH and non-AH groups were 36.6 and 6.7% respectively ($P<0.03$). The overall pregnancy rates per warming cycle were 54.2 and 12.5% for the two groups respectively ($P<0.05$).

So far, 8 healthy babies have been born. Seven children resulted from the AH group and 1 in which AH was not performed.

Discussion

This study shows that significantly higher pregnancy and implantation rates can be attained with the transfer of vitrified expanded blastocysts in conjunction with AH. These results are in agreement with those reported by Vanderzwalmen *et al.* [10].

In this study, we had drilled the zona pellucida with acid. This procedure is difficult for fresh expanded blastocysts because trophectoderm attaches to the zona pellucida. Therefore, it is possible that the trophectoderm is damaged as soon as the zona pellucida is drilled by AH, but in our vitrification system, we used artificial shrinkage of the blastocoele which in our clinical experience has proved to be effective for expanded and hatched stage blastocysts [12]. Consequently, the AH procedure was easy to perform for vitrified blastocysts because the blastocoele was still shrunken by artificial shrinkage and there was a perivitelline space within 5 min after the warming procedure was completed. During AH procedure, the blastocysts were exposed to acidic Tyrode's solution for a while, and so might be damaged as a result of chemical toxicity, but the present data on the AH group suggest that an AH procedure has little detrimental effect on vitrified blastocysts at the time of warming.

The lower pregnancy and implantation rates observed after transfer of non AH expanded blastocysts in the present study, suggests unsuccessful in-utero hatching, rendering implantation difficult or impossible. Hatching deficiencies can result from zona hardening, which might occur after *in vitro* culture of human embryos [19, 20] or after cryopreservation [14, 15]. Therefore, if cryopreservation can interfere with the spontaneous hatching process, AH may be beneficial for the clinical outcome of cryopreserved embryo transfer. It is shown that AH may be beneficial for frozen-thawed cleavage stage embryos [14, 15] or for vitrified-warmed blastocyst stage embryos [10]. In addition, the resistance to enzymatic removal of the zona pellucida of morulae and blastocysts by pronase increases after vitrification as compared with the unfrozen embryo [10]. Moreover, 3 h after warming, a significant increase in the percentage of warmed blastocysts in hatching process was observed in the AH group compared with the non-AH group in the present study. Consequently, hardening of the zona pellucida *in vitro* may also be due to the vitrification protocol.

The only suggested adverse outcome of AH could be an increased rate of monozygotic twinning [21]. So far, we have not observed monozygotic twins after the AH procedure. This might be due to the fact that the drilling a hole after large zona thinning creates a sizeable opening, reducing abnormal blastocyst escape.

In conclusion, AH significantly improved the pregnancy and implantation rates after vitrified blastocyst transfer. The results suggest that the vitrification procedure may cause hardening of the zona pellucida, and AH for vitrified blastocysts would be useful for clinical application.

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