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Preliminary study for pregnancy rate according to developmental stages of vitrified-warmed blastocyst

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Summary

Purpose: To examine the pregnancy rate according to developmental stages of vitrified-warmed human blastocyst at the time of embryo transfer (ET). *Materials and Methods:* Retrospectively, the outcomes of total 140 elective frozen thawed blastocyst transfers were analyzed regarding the embryo developmental stage of the thawed blastocysts at the time of transfer. Group 1 included only expanding blastocysts, group 2 included an expanding blastocyst and a hatching blastocyst, and group 3 included only hatching blastocysts. *Results:* Implantation rate, clinical pregnancy rate, and ongoing pregnancy rate were significantly higher in group 3 than group 1 and 2. Implantation rates were 32.6% (group 1), 56.6% (group 2), and 77.2% (group 3) ($p < 0.05$). Clinical pregnancy rates were 25.6% (group 1), 45.3% (group 2) and 70.5% (group 3) ($p < 0.05$). Ongoing pregnancy rates were 20.9% (group 1), 39.6% (group 2), and 47.7% (group 3) ($p < 0.05$). *Conclusions:* Pregnancy rate was higher in transfer of hatching blastocysts than in expanding blastocysts.

Key Words: Assisted hatching; Frozen-thawed embryo transfer; Hatching blastocyst; Pregnancy rate.

Introduction

As the cryopreservation technique has been developed, the outcomes of in vitro fertilization program are improved. Cryopreservation of surplus embryos became ordinary procedure in most IVF clinics. Even more, some authors have suggested elective cryopreservation for better IVF outcomes in case of enough number of oocytes retrieved [1-3].

Despite development of cryopreservation technique, there are few or no studies about the time interval between warming of vitrified blastocysts and transferring of embryos. Blastocyst development in the time of embryo transfer (ET) may be in the expanding stage or in the hatching stage. This difference of developmental stages may have influence on the pregnancy rate.

In fresh cycle, there is one study about the relationship of developmental speed and clinical outcomes in blastocyst transfer [4]. In this study, rapid developing embryos showed better outcomes than slow developing embryos. However in frozen-thawed cycle, not much is known about the effects of blastocyst developmental stages on pregnancy rate.

In this study, the authors compared the pregnancy rate according to the developmental stages of vitrified-warmed blastocysts in elective cryopreservation cycle.

Materials and Methods

Patients & COS protocol

A total of 140 women aged less than 35 were included in this study from July 2008 to August 2013. Inclusion criteria of patients were defined as follows; 1) basal follicle-stimulating hormone (FSH) level less than 10 mIU/ml, 2) normal uterine cavity, and 3) adequate sperm for IVF or ICSI. Basic characteristics are described in Table 1.

Blastocyst culture and selection

After standard IVF or ICSI, fertilization was assessed 15-18 hours after insemination by the presence of two pronuclei. All embryos were cultured in sequential media G1/G2 in 6% CO_2, 5% O_2, and 89% N_2 environment [5]. All blastocysts were evaluated using Gardner and Schoolcraft's scoring system [6]. In this study, blastocysts more than grade 3BB were selected for cryopreservation and ET.

Vitrification and thawing

Artificial shrinkages of all blastocysts more than grade 3BB were performed by aspirating blastocoels fluid with ICSI pipettes [7]. After artificial shrinkage, the blastocysts were equilibrated in G10, G10E20 for five minutes, respectively. After an approximate volume of 0.3 μl of vitrification solution (G25E25) containing blastocysts (maximum two) was loaded on the self-manufactured "capped-pulled straw"; capped-pulled straw was seeded on LN2 vapour for 30 seconds, and then plunged into liquid nitrogen [7]. Thawing was performed in one ml of 0.5, 0.25, and 0.125 mol/l sucrose and DPBS with 20% SSS for three minutes, respectively. After washing three times in 37°C DPBS with 10% SSS, AH was performed.

Partial zona dissection as artificial hatching

Artificial hatching was performed using ICSI pipette after thawing procedure (Figure 1). Briefly, frozen-thawed blastocyst was held by holding pipette to 9 o'clock direction and then zona pellucida (ZP) was completely penetrated by injection pipette from 3 o'clock direction to 9 o'clock direction. As holding and ICSI

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Table 1. — *Patient characteristics and COS protocol.*

		Group 1 (n=43)	Group 2 (n=53)	Group 3 (n=44)	*p*-value
Age (years)		33.2 ± 3.7	32.7 ± 2.9	32.6 ± 3.9	NS
BMI (kg/m^2)		24.1 ± 4.4	22.2 ± 3.1	21.3 ± 2.3	0.028§,
Infertility duration (years)		6.1 ± 3.1	4.6 ± 3.3	4.5 ± 3.3	NS
FSH (mIU/ml)		7.6 ± 3.1	7.4 ± 3.1	8.1 ± 3.6	NS
LH (mIU/ml)		7.7 ± 7.8	4.9 ± 2.2	5.5 ± 3.3	0.027*, 0.024□
Male age (years)		38.6 ± 6.0	36.2 ± 3.2	40.9 ± 8.2	0.028*, 0.031§, 0.000□
COS protocol	Long	29	27	33	
	Ultralong	1	6	0	
	Antagonist	12	20	11	
	Ultrashort	1			
Total FSH dose (IU)		2544.2 ± 871.4	2745.3 ± 1609.8	2950.6 ± 1120.6	0.170*, 0.007§

*: group 1 *vs*. group 2; §: group 1 *vs*. group 3; □: group 2 *vs*. group 3.

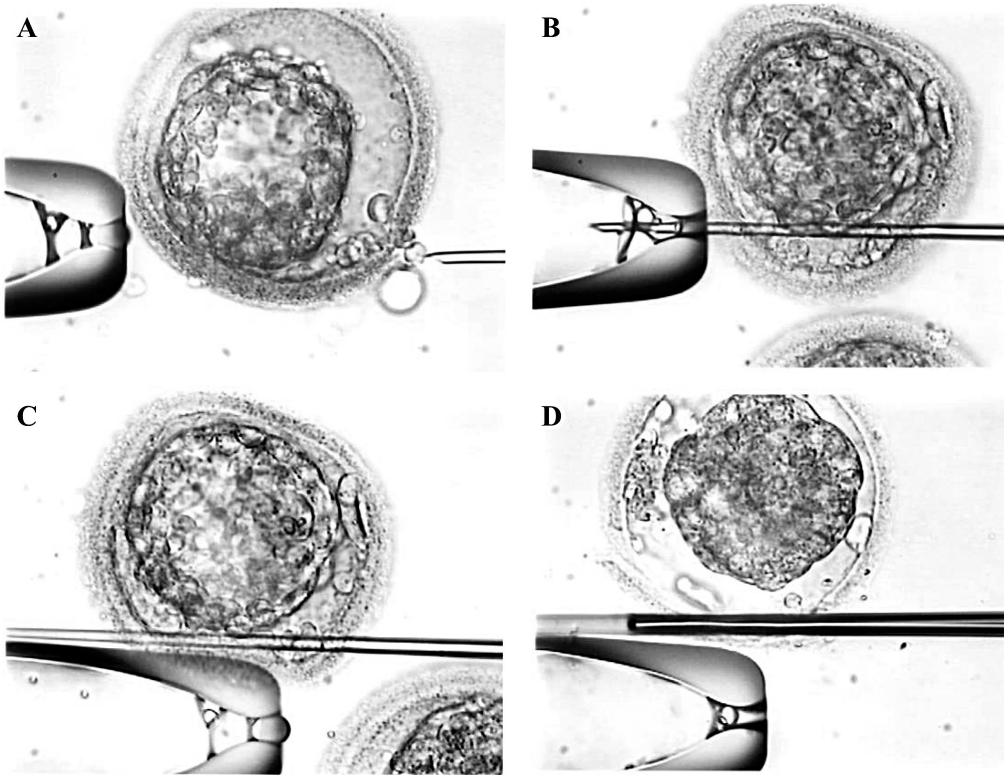

Figure 1. — Zona dissection using ICSI pipettes just after thawing. (A) Holding a blastocyst to 9 o'clock direction. (B) Inserted injection pipette from 3 o'clock direction. Penetration through perivitelline space to 9 o'clock direction. (C) Rubbing and flicking ZP using holding and injection pipettes. (D) Dissected ZP.

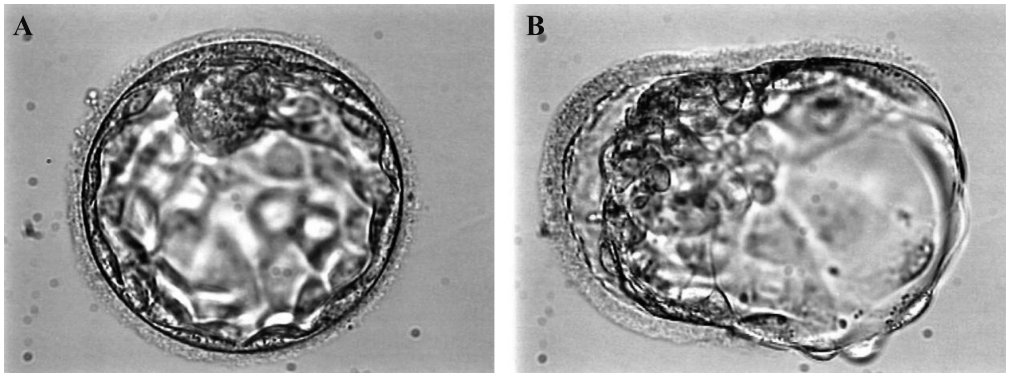

Figure 2. — (A) Expanding blastocyst and (B) hatching blastocyst at five hours after thawing in the same patient.

Table 2. — *Clinical outcomes according to blastocysts status.*

	Group 1 (n=43)	Group 2 (n=53)	Group 3 (n=44)	*p*-value
Transferred embryo number	2.2 ± 0.8	2.4 ± 0.5	1.8 ± 0.9	0.000
Mean endometrial thickness (mm)	9.4 ± 0.8	9.6 ± 0.7	9.4 ± 0.5	NS
Implantation rate* (%)	14 (32.6)	30 (56.6)	34 (77.2)	0.000
Clinical pregnancy rate* (%)	11 (25.6)	24 (45.3)	31 (70.5)	0.000
Ongoing pregnancy rate* (%)	9 (20.9)	21 (39.6)	21 (47.7)	0.028
Miscarriage (n)	2	3	10	0.000

* per embryo transfer cycles.

pipette was split, zona dissection was created.

Endometrial preparation and blastocyst transfer

Leuprolide acetate 0.5 mg/day was administrated from the preceding menstrual cycle day 21 and after menstruation, sequentially increasing doses of oral estradiol valerate was administrated. For luteal support, oral estradiol valerate two mg twice a day and vaginal progesterone once a day were administered from cycle day 15. Frozen-thawed blastocysts were transferred on cycle day 20 or five days after the start of luteal support.

Outcome measures and statistical analyses

If the blastocyst was hatching out from the zona more than 30% of the volume of blastocyst, it was defined as hatching blastocyst, and if the ZP was intact, the authors defined it as expanding blastocyst. Retrospectively, Group 1 included only expanding blastocysts, group 2 consisted of one expanding and one hatching blastocyst, and group 3 was composed of only hatching blastocysts (Figure 2).

Implantation was defined as the condition with serum β-hCG which is higher than ten mIU/ml on 12 days after ET. Clinical pregnancy was defined as the presence of gestational sac with cardiac activity on pregnancy six weeks per ET cycle. Ongoing pregnancy was defined as the presence of gestational sac with cardiac activity on pregnancy 12 weeks per ET cycle. Clinical outcomes of frozen-thawed blastocyst transfers were analyzed by Student's *t*-tests and chi-square test. A value of *p* less than 0.05 was considered to be statistically significant. Data are expressed as mean ± standard deviation unless otherwise specified.

Results

Table 1 shows the patient characteristics and COS protocol. There is no significant difference in age, infertility duration, and basal FSH among three groups. In group 3 BMI was significantly lower than group 1. Male age and total FSH dose were significantly higher in group 3 than in groups 1 and 2.

Table 2 shows the clinical outcomes. Endometrial thickness was not different in all groups and numbers of transferred embryo were significantly lower in group 3. The average numbers of transferred embryos were 2.2 ± 0.8 in group 1, 2.4 ± 0.5 in group 2, and 1.8 ± 0.9 in group 3, respectively. However implantation , clinical pregnancy, and ongoing pregnancy rates were significantly higher in group 3 than groups 1 and 2. Implantation rates were 32.6% in group 1, 56.6% in group 2, and 77.2% in group 3. Clinical pregnancy rates were 25.6% in group 1, 45.3% in group 2, and 70.5% in group 3. Ongoing pregnancy rates were 20.9% in group 1, 39.6% in group 2, and 47.7% in group 3. However miscarriage was higher in group 3 than in groups 1 and 2.

Discussion

In human embryo implantation, embryo quality, endometrial receptivity, and embryo-endometrial synchrony are important factors [8]. Recently human IVF program have shown remarkable improvements, but there are still some problems to be solved like ZP hardening or embryo-endometrial asynchrony. Some researchers suggest AH or elective frozen-thawed ET as alternative solution for these problem [9, 10].

This study included only elective frozen-thawed ET cycles. In the authors' clinic, this is routine approach for normal responders. Elective frozen-thawed ET has some benefits such as OHSS-free by using GnRH agonist trigger in GnRH antagonist cycle, and synchrony of embryo development with endometrial proliferation [11]. Until now, there is no comparative research of implantation and pregnancy rate between hatching and expanding embryos in human IVF program.

During blastocyst thawing process, blastocyst goes through the serial stages from shrunken stage to expanding, hatching, and hatched stage. In this study, the authors transferred the embryos at four to six hours later after thawing. Four to six hours later, the blastocyst stages are quite different from expanding stage to just before hatched stage. The present authors supposed that the difference of developmental stage might affect the pregnancy rate of frozen-thawed ET cycle. Hatching blastocyst was defined as more than 30% of trophoblast extrusion from Zp. In group 3 which transferred only hatching blastocysts, the implantation rate, clinical pregnancy, and ongoing pregnancy rates were significantly higher than other groups. Several factors may be involved in this results. Firstly, the patients' basal characteristics such as BMI and male age were statistically different in this study. However, the averages of BMI were under 25 in all groups and male age was significantly higher in group 3, which showed higher pregnancy rate.

For these reasons, despite the statistical differences of patients' basal characteristics, the authors thought the results may have significance. Secondly, developmental stage of each embryo might affect the results. In groups 2 and 3 including hatching blastocysts showed higher pregnancy rate than the group 1, including only expanding blastocysts. Especially in this study, all embryos were electively frozen and all transfers were conducted in artificial endometrial preparation cycle. So this constant condition heightens the possibility that developmental stage of blastocyst affects the pregnancy rate. The developmental stages could be affected by embryo quality before cryopreservation and culture duration after warming.

Elgindy *et al.* compared with clinical outcomes in human fresh ET depending on embryo expanding day; Day 5 expanding embryo and Day 5 non-expanding, but Day 6 expanding embryo. The rate of implantation and pregnancy was significantly low in Day 5 non-expanding but Day 6 expanding group. Furthermore, in Day 5 non-expanding group, the number of embryo that developed to blastocysts were lesser and embryo vitrification rate and live birth rate were significantly lower [4]. Also, a study for post-warming survival rate of frozen-thawed goat blastocyst showed that the survival rate of hatching blastocyst was meaningfully higher than the survival rate of expanding blastocyst after thawing 20 hours [12]. This higher survival rate could cause a better implantation rate. These studies suggest that lower quality embryo develops slowly and developmental stage of embryo is affected by the embryo quality itself. However, this theory cannot be applied instantly to the comparison between hatching and expanding stages of warmed blastocysts.

Culture duration after warming also may be an important factor for development of blastocyst. Perhaps every embryo has different quality so that each embryo needs a different length of time to go through the hatching process. Time duration after warming can affect the pregnancy rate by the difference of developmental stage of blastocyst.

The present authors transferred embryos after four to six hours later after warming process under elective frozen-thawed ET with artificial endometrial preparation. If they cultured blastocyst in a longer time, the more expanding blastocysts proceeded to hatching stage. In this case, holding blastocyst transfer to hatching stage might become an optional trial for increasing implantation rate.

The present study has several limitations. First, small number of cases was analyzed. Also, in group 3, the miscarriage rate was higher than other groups. The value of higher pregnancy rates is counterbalanced by this higher miscarriage rate partly. Despite these limitations, the pregnancy rate was improved significantly in the hatching embryos. The authors believe that this finding needs to be confirmed with more sufficient cases.

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