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ASSISTED REPRODUCTION TECHNOLOGIES

Use of the natural cycle and vitrification thawed blastocyst transfer results in better in-vitro fertilization outcomes

Cycle regimens of vitrification thawed blastocyst transfer

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Abstract
Purpose To compare the IVF outcomes of vitrification-thawed blastocyst transfer cycles utilizing different endometrial preparation methods.
Methods We retrospectively assessed IVF outcomes in 611 patients (648 cycles) who underwent blastocyst frozen embryo transfer (FET) between January 2007 and December 2009. All embryos had been cryopreserved by a vitrification method following a previous IVF cycle. Patients were prepared for transfer by using either the natural cycle (n=310/Group 1), the natural cycle with ovulation induction employing human chorionic gonadotropin (n=134/Group 2), or a hormonally manipulated artificial cycle with estrogen and progesterone supplementation (n=204/Group 3).
Results Multivariate logistic regression analysis showed a significant difference in clinical pregnancy rate between Groups 3 (30.4%) and 1 (41.9%) (odds ratio [OR], 0.567; 95% confidence interval [CI], 0.379–0.847, P=0.006) whereas the difference between Groups 2 and 1 was not significant (41.8% *vs*. 41.9%; OR, 0.683; 95% CI, 0.435–1.073; P=0.098). Other significant variables affecting clinical pregnancy rate were the number of embryos transferred, the grade of transferred embryos, and maximal endometrial thickness.
Conclusion The results showed that, using vitrification-thawed blastocyst transfer, employment of natural cycles with or without hCG treatment was associated with better outcomes than was the use of hormonally manipulated cycles.

Keywords Cycle regimen · Vitrification · Embryo transfer · Blastocyst · In vitro fertilization

Capsule During vitrified-thawed blastocyst transfer, the use of natural cycles with or without hCG treatment resulted in more favorable IVF outcomes than were noted when hormonally manipulated cycles were employed.

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Introduction

The increasingly frequent policy of restricting the number of embryos transferred to avoid high-order multiple pregnancies has emphasized the importance of frozen-thawed embryo transfer (FTET). Intensive efforts have been made to improve FTET outcomes, and, because of the development of successful cryopreservation techniques such as vitrification, and improved long-term in vitro culture systems, the survival rates of frozen-thawed embryos, and the number of pregnancies arising therefrom, have increased substantially [1–3].

Vitrification, a rapid freezing procedure using a high concentration of cryoprotectant, can prevent ice crystal formation, limit cellular damage, and enable embryos treated in this manner to retain a reproductive potential very similar to that of fresh embryos [4]. Although improved embryo quality has enhanced pregnancy rates, little is known about endometrial receptivity. No consen-

sus has yet been reached on optimal cycle regimen for endometrial preparation in FTET. Thus, various cycle regimens are in use worldwide, and most incorporate a mixture of FTET protocols. Moreover, insufficient evidence is available to favor the use of any particular transfer schedule over another [5].

Cycle regimens used for endometrial preparation in FTET are commonly classified into three groups: natural cycles with or without ovulation induction using human chorionic gonadotropin (hCG); artificial cycles, in which the endometrium is artificially prepared using estrogen and progesterone, with or without use of a gonadotropin-releasing hormone agonist; and stimulated cycles, in which follicular development is supported by follicle-stimulating drugs. Each of these regimens can be modified by addition of progesterone for luteal support or by changing the dosages of medications used.

The objectives of this retrospective analysis were to compare the outcomes of different endometrial preparation methods and to determine factors affecting pregnancy rates after the transfer of vitrified/warmed blastocysts. Other potential factors affecting frozen/thawed embryo transfer outcomes were also explored.

Materials and methods

Patients

We retrospectively reviewed all frozen-thawed blastocyst transfer cycles using vitrification performed between January 2007 and December 2009 at the fertility center of CHA University. All embryos had been cryopreserved by vitrification during a previous cycle. We included only those cycles in which a patient showed regular menstruation without any intrauterine pathology, and we evaluated only patients with no history of recurrent implantation failure or recurrent abortion. After excluding patients who required artificial cycles because of irregular or absent normal cycles, an endometrial preparation protocol for each patient was chosen primarily by the attending physician. Although many factors were considered when a protocol was decided upon, the two main considerations were convenience and cost. In all, 648 cycles (611 patients) were classified into three groups based on the endometrial preparation method used. Group 1 consisted of patients with natural cycles showing spontaneous LH surges (n=310); Group 2 those with natural cycles who received an hCG trigger (n=134); and Group 3 patients with artificial cycles stimulated by estrogen and progesterone supplementation (n=204).

This study was approved by the institutional review board of the CHA Fertility Center, which waived patient consent for this retrospective analysis.

Blastocyst freezing and thawing

Our vitrification and thawing procedures have been described previously [1]. Briefly, a base solution of HEPES-buffered human tubal fluid medium (SAGE IVF, Pasadena, CA) with 20% (w/v) human serum albumin (HSA, SAGE IVF) was prepared, and 1.5 mol/L ethylene glycol (EG; Sigma Chemical Co., St. Louis, MO) was subsequently added. To this preparation, 5.5 mol/L EG and 1.0 mol/L sucrose (Sigma) were also added, yielding the final vitrification solution. Warming solutions were prepared by adding 1.0, 0.5, 0.25, 0.125, and 0 mol/L sucrose to the base solution. Blastoceles were artificially reduced in size using laser-assisted hatching and micro-needle puncture to improve survival after warming. Supernumerary blastocysts were collapsed, placed in 1.0 mL amounts of pre-warmed equilibration solution, and incubated at room temperature for 5 min. The blastocysts were next immersed in vitrification solution for 20 s at room temperature. One to two blastocysts, depending on quality, were loaded onto an electron microscope (EM) gold grid (Tedpella, Redding, CA) using a fine pipette, and excess vitrification solution was removed with underlying paper. The grid was immediately plunged into a small container in Vit-Master™ (IMT, Tattenhall, UK) filled with slush nitrogen (SN_2) and placed in a pre-cooled customized grid holder. The solution was warmed for 18–24 h before ET. Rationale for transfer of vitrification-thawed embryos 18–20 h after warming was based on our unpublished data, which revealed better cycle outcomes than did culturing for 2–3 h or 12 h. Extension of culture time after thawing permitted full recovery, and excellent viability, of thawed blastocysts, thus increasing clinical pregnancy rates.

To warm the blastocysts, the grid holding the cells were removed from the holder using fine forceps under LN_2 and transferred immediately to wells of Falcon 2-well culture dishes (Beckton Dickinson, Franklin Lakes, NJ) containing 1.0 mL amounts of warming solution with 1.0 mmol/L sucrose at 37°C. Cells were sequentially transferred, at 2.5 min intervals, to warming solutions containing 0.5, 0.25, 0.125, and 0 mol/L, also at 37°C. After the final warming step, blastocysts were detached from the grid by pipetting and placed into 0.5 mL amounts of pre-equilibrated G2 medium. Blastocysts were graded by size, and by development of the inner cell mass and trophoectoderm. Only good quality cells (trophoectoderm of less than grade 3) were transferred [6].

Endometrial preparation

Modified natural cycle Patients in group 1 (with a natural cycle and a spontaneous LH surge) were closely monitored for evidence of surge and dominant follicle collapse, using serum assays and transvaginal ultrasound. Thawed embryos were transferred after ovulation was observed, usually on day 5, although, in several patients (n=39), transfer occurred on day 4, mostly for personal patient reasons.

For patients in group 2 (natural cycle with an hCG trigger), ovulation was induced using 10,000 IU of urinary hCG (IVF-C; LG Chemical, Seoul, South Korea) when the dominant follicle was >20 mm in diameter and an endometrium >8 mm in thickness was detected by ultrasound. In both groups, progesterone (600 mg/d; Utrogestan; Hanhwa Pharmaceuticals, Seoul, South Korea) was administered vaginally for luteal support starting on the ET day.

Hormonally manipulated artificial cycle Patients in Group 3 (artificial cycle) were commenced on 4–6 mg/day oral estradiol valerate (E2) (Progynova; Schering Korea, Seoul, South Korea), beginning on cycle day 3. In each hormonally manipulated cycle, the standard initial dosage of estradiol valerate was 4 mg. However, patients with reduced ovarian reserves (basal FSH>10 IU/ml and basal E2 <70 pg/ml) received 6 mg of estradiol valerate. Patients who showed inadequate endometrial development during embryo transfer in a previous fresh cycle were also candidates for higher initial doses of estradiol. Patients returned on cycle day 14 for transvaginal ultrasound measurement of endometrial thickness. If the endometrial lining was ≥8 mm in thickness, patients were commenced on vaginal progesterone (600 mg/d; Utrogestan; Hanhwa Pharmaceuticals, Seoul, South Korea). If the endometrial thickness was <8 mm, the dose of E2 was increased to 8 mg/d. Thawed embryos were transferred 5 days after commencement of progesterone administration.

Cycle outcome

Pregnancy was defined as a positive serum β-hCG test 12 days after ET. Clinical pregnancy was defined by the presence on ultransonography of a fetal heartbeat approximately 4 weeks after a positive pregnancy test. Implantation rate was defined as the number of gestational sacs on ultrasound per total number of transferred embryos. Ongoing pregnancy was defined as pregnancy beyond 14 weeks of gestation, and miscarriage was defined as the spontaneous cessation of clinical pregnancy.

Statistical analysis

All statistical calculations were performed using Microsoft Excel version 7 (Microsoft Corporation, New York, NY) and SPSS (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL). Differences among groups were assessed by one-way analysis of variance, Chi-square tests, and Student's t-tests, as appropriate. A P value <0.05 was considered statistically significant. Multivariate analysis was used to determine the association between clinical characteristics and the occurrence of pregnancy.

Results

Patients in the three groups had similar demographic characteristics and reproductive histories (Table 1). The cycles resulting in surplus embryo freezing were statistically comparable (Table 2). The implantation (34.9% *vs*. 24.3%, P=0.003), clinical pregnancy (41.9% *vs*. 30.4%, P=0.008), and ongoing pregnancy (38.1% *vs*. 27.5%, P=0.013) rates were significantly higher in Group 1 than in Group 3. However, Groups 1and 2 showed comparable implantation levels (34.9% *vs*. 33.3%, P = NS), clinical pregnancy (41.9% *vs*. 41.8%, P = NS) and ongoing pregnancy (38.1% *vs*. 38.1%, P = NS) rates . Among Group 1, clinical pregnancy rates for embryos transferred on days 4 and 5 were comparable (41.0% *vs*. 42.1%, P=0.902). There were no significant differences among the three groups in terms of miscarriage (8.1% *vs*. 5.2% *vs*. 6.4% for Groups 1–3, respectively) or multiple pregnancy (26.2% *vs*. 28.6% *vs*. 16.1%) rates, including two pairs of monozygotic twins in Group 1 (Table 2). Multivariate analysis of other factors possibly confounding pregnancy outcomes yielded similar results (Table 3). Compared with Group 1 patients, those in Groups 2 and 3 had odds ratios for clinical pregnancy of 0.683 (95% confidence interval [CI], 0.435–1.073; P=0.098) and 0.569 (95% CI, 0.379–0.847; P=0.006), respectively, and the odds ratios for ongoing pregnancy were 0.694 (95% CI, 0.439–1.095; P=0.117) and 0.574 (95% CI, 0.381–0.864; P=0. 008), respectively. Multivariate analysis showed that the number of embryos transferred (OR, 1.874; 95% CI, 1.333–2.634; P<0.001), grade of transferred embryos (OR, 0.489; 95% CI, 0.335–0.712; P<0.001), and maximal endometrial thickness (OR, 1.282; 95% CI, 1.169–1.405; P<0.001) (Table 3), showed significant independent effects on clinical pregnancy rates. Age at transfer significantly affected the ongoing pregnancy rate (OR, 0.938; 95% CI, 0.890–0.988; P=0.016), but had no effect on clinical pregnancy rate.

Table 1 Patient demographic and clinical characteristics in groups of women undergoing one of three different cycle regimens for FTET

	Natural (n=310)	Natural + hCG (n=134)	HT (n=204)	P value
Age (years)	34.2±3.7	33.7±3.3	33.7±3.7	NS
BMI (kg/m^2)	20.7±2.8	20.5±3.5	20.7±2.4	NS
Infertility duration (years)	3.4±2.1	3.9±3.5	3.1±2.9	NS
Indications for treatment (%)				NS
Tubal factor/peritoneal	133 (42.9)	67(50)	99(48.5)	
Male factor	79 (25.4)	35 (26.1)	51(25)	
Ovulatory	21(6.8)	8(5.9)	18(8.8)	
Unexplained	77(24.8)	24 (17.9)	36(17.6)	
Basal FSH (mIU/ml)	6.9±2.4	6.7±2.1	6.5±2.6	NS
Basal E2 (pg/ml)	28.5±18.6	26.4±17.8	23.9±17.7	NS

NS not statistically significant (P>0.05)

Values are reported as means±standard deviations, or as numbers

Discussion

Although the optimal endometrial preparation method for FTET remains unclear, several retrospective studies have suggested that better outcomes may result from transfer of day-3 frozen embryos using natural cycles [7–9]. Appropriate concentrations of E2 may act on the endometrium and afford better synchronization between the endometrium and the embryo [8]. Reduced serum estrogen concentrations in patients undergoing natural cycles were found to result in greater endometrial thickness, suggesting that higher E2 concentrations during hormone replacement therapy may

Table 2 Comparison of cycle outcome among cycle regimens used for FTET

	Natural (n=310)	Natural + hCG (n=134)	HT (n=204)	P value
Fresh cycle variables				
Total dose of gonadotropin (IU)[a]	1776.6±524.8	1766.0±450.0	1801.1±498.7	NS
Peak E2 level (pg/ml)[a]	2046.7±976.9	2102.6±1008.7	2186.6±1058.2	NS
No. of oocytes retrieved[a]	13.9±6.0	14.8±6.2	15.1±7.4	NS
No. of 2PN embryos[a]	10.2±4.3	10.9±5.0	10.9±5.3	NS
No. of frozen blastocysts[a]	3.3±2.3	4.0±3.6	3.6±3.1	NS
FTET cycle variables and outcome				
No. of embryos transferred[a]	1.7±0.5	1.8±0.6	1.7±0.6	NS
Grade of transferred embryos[a]	2.4±0.5	2.3±0.5	2.4±0.5	NS
Maximal endometrial thickness achieved (mm)[b]	9.7±2.0[d]	10.5±1.9[d,e]	9.4±2.0[e]	d : <0.001 e : <0.001
Implantation rate (%)[c]	34.9[d] (181/518)	33.3 (80/240)	24.3[d](85/350)	d : 0.003
Clinical pregnancy rate (%)[c]	41.9[d] (130/310)	41.8[e] (56/134)	30.4[d,e] (62/204)	d : 0.008 e : 0.032
Ongoing pregnancy rate (%)[c]	38.1[d] (118/310)	38.1[e] (51/134)	27.5[d,e] (56/204)	d : 0.013 e : 0.04
Multiple pregnancy rate (%)[c]	26.2 (34/130)	28.6 (16/80)	16.1 (10/62)	NS
Miscarriage rate (%)[c]	8.1(12/130)	5.2 (6/56)	6.4(5/62)	NS
Biochemical pregnancy rate (%)[c]	2.6 (33/310)	2.2 (10/310)	4.4 (22/204)	NS

NS = not statistically significant (P>0.05)

Values within rows with the same superscript were significantly different (P<0.05)

[a] One-way ANOVA

[b] t-test

[c] X^2 test

Table 3 Multivariate analysis of FTET factors affecting clinical pregnancy

Covariate	Estimate	OR	95% CI	Std. error	P-value
Age	−0.042	0.959	0.911–1.009	0.026	0.107
No. of frozen embryos	−0.001	0.999	0.937–1.064	0.032	0.966
No. of embryos transferred	0.628	1.874	1.333–2.634	0.174	<0.001
Grade of transferred embryos	−0.716	0.489	0.335–0.712	0.192	<0.001
Maximal endometrial thickness achieved	0.248	1.282	1.169–1.405	0.047	<0.001
Natural cycle	0	1			
Natural + hCG	−0. 381	0.683	0.435–1.073	0.230	0.098
HT	−0. 568	0.567	0.379–0.847	0.205	0.006

compromise the window of implantation (WOI) [7]. In contrast, however, we observed no significant differences in endometrial thickness between patients experiencing natural and artificial cycles. Ultrasound can easily and non-invasively assess endometrial function and thereby predict endometrial receptivity. Unfortunately, however, endometrial thickness may not reflect tissue function. Endometrial histology and thickness do not correlate while the ultrasound texture of the endometrium may be of greater prognostic value than is endometrial thickness when implantation is considered [10, 11]. After adjusting for endometrial thickness in multivariate analysis, we found significant differences in pregnancy rates, supporting previous findings, showing that endometrial thickness did not necessarily reflect tissue function. This may be attributable not only to gross changes in the endometrium but also in the microenvironment, including the presence of pinopodes and variations in the concentrations of adhesion molecules and cytokines. Thus, it can be hypothesized that, expression of implantation markers is not associated with endometrial thickness. However, as no reports on this issue have appeared, the hypothesis should be evaluated in further studies. The microenvironment of the endometrium is primarily regulated by the ovarian steroid hormones, estrogen and progesterone. Indeed, the expression of potential implantation markers such as endometrial integrin β3 subunit and leukemia inhibitory factor (LIF) was significantly reduced in the presence of supraphysiologic concentrations of estrogen [12, 13]. Although several candidate molecules are under active investigation, the roles played by these factors in implantation are currently not fully known. However, abundant evidence suggests that genes highly expressed during the WOI of a natural cycle tend to be downregulated at higher estrogen concentrations [12–16]. Therefore, patients with normal ovarian function may achieve the best results using natural rather than hormonally manipulated cycles, because exogenous estrogen may alter the physiologic concentration of endogenous hormone, resulting in negative effects on the endometrium [7, 15].

Few studies have compared the use of spontaneous natural cycles and natural cycles with hCG in IVF treatment. However, a recent randomized controlled trial suggested that the former cycle type was superior, indicating that hCG administration during the late follicular phase may induce a hormonal cascade in the endometrium and negatively impact the ongoing pregnancy rate [17]. We found no difference in cycle outcome when natural cycle use was compared with the employment of the natural cycle but using an hCG ovulation trigger, in agreement with previous findings, showing that triggering of ovulation with hCG can significantly reduce the number of visits necessary to schedule ET, without adverse effects on cycle outcomes [18]. Moreover, we found that natural cycles accompanied by hCG use yielded an optimal endometrial thickness, because of the decidualizing effects of hCG on the endometrium during the WOI.

In conclusion, use of natural cycles, with or without hCG ovulation triggering, may yield the best results after vitrified/warmed blastocyst transfer to regularly ovulating women. The major drawback of our study was the retrospective design. Although previous report suggested that estrogen administration as oral tablets (2–6 mg initial dose), transdermal patches, and vaginal tablets, has yielded comparable outcomes, dose of estrogen used varied [19]. Moreover, patients undergoing natural cycles received supplementary transvaginal progesterone to offset any potential luteal phase defect, and the cycles were thus not truly natural. Nevertheless, our present study, involving a large number of cycles, is, to the best of our knowledge, the first to report that pregnancy rates are significantly higher when natural cycles rather than hormonally manipulated artificial cycles are employed for vitrified/warmed blastocyst transfer. Although natural cycles showed the best results, hormonally stimulated cycles may still be the first choice for women who have irregular menstrual cycles or poor ovarian function. Because the number of frozen embryo transfer cycles increases exponentially, there is an urgent need for well-controlled randomized trials assessing relationships between endometrial preparation methods and pregnancy outcomes, and also considering convenience and cost.

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