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ASSISTED REPRODUCTION TECHNOLOGIES

The outcome of different post-thawed culture period in frozen-thawed embryo transfer cycle

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Abstract
Purpose To study the influence of post-thawed culture (2–4 h and 20–24 h) on the outcome of frozen-thawed embryo transfer (FET) cycle.
Methods In this retrospective study, a total of 1,353 patients were undergoing the FET treatment at the reproductive medical center between June 2010 and July 2012. 3,398 frozen-thawed embryos were divided in two study groups, depending on their post-thawed culture period: short culture (2–4 h) group and long culture (20–24 h) group. Groups were compared including clinical pregnancy rate, implantation rate, spontaneous abortion rate, ectopic pregnancy rate, multiple pregnancy rate, live birth rate and birth weight.
Results When embryos including at least one grade I embryo after thawed transferred, the clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate, live birth rate and birth weight were similar in the short culture group compared with these in the long culture group.
Conclusions The outcomes of the two approaches (short culture and long culture) are no different in FET cycles.

Keywords Frozen-thawed embryo transfer · Post-thawed culture · Clinical pregnancy rate · Implantation rate · Live birth rate

Capsule The clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate, live birth rate and birth weight were similar in the short culture (2-4 h) group and the long culture (20-24 h) group.

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Introduction

Following the first report of a live birth after transfer of cryopreserved embryos [1], cryopreservation of embryos is widely used in assisted reproduction technology (ART) [2], such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), and has become a fundamental part of ART. The advantages of frozen-thawed embryo transfer (FET) are a less invasive procedure for patients, lower costs than ART treatment, and ease of application within a short space of time, and decreasing rates of multiple pregnancies and of ovarian hyperstimulation syndrome (OHSS), and produced greater cumulative pregnancy rates per oocyte retrieval cycle, and it is of greater importance for patients who are not suitable for a fresh embryo transfer cycle due to endometrial or other factors [3, 4]. Nevertheless, compared to fresh IVF cycles, FET tends to show lower pregnancy rates.

Classical cryopreservation methods involve the acquisition of expensive apparatus (i.e. programmable rate freezers). The technique of vitrification is now applied to human IVF protocols. This technique has many advantages, such as it is quick, simple and expensive apparatus is not necessary [5]. The survival and intact cell rates in the vitrification group were significantly higher compared with those in the slow freezed group, and the implantation, clinical pregnancy and live birth rates of the vitrification group were similar to the fresh and significant higher than slow freezed group [6]. Data suggests that the blastocyst survival rate after vitrification is higher than that using slow freezing techniques [7].

Several embryologic factors have been described to be determinant for the post-thawed survival and/or viability,

including embryo morphology before freezing, blastomere loss after thawed associated with toxic influences of damaged blastomeres on sibling cells in the embryo and resumption of mitosis after a post-thawed culture period [2].

The ability to select embryos with high developmental potential not only improves the clinical pregnancy rate, but also decreases the multiple pregnancy rate. Currently, the two most common procedures to select embryos for FET differ in the duration of the post-thawed culture: one relies upon the observation of blastomere survival after thawing, requiring a short culture; the other is dependent on the observation of blastomere proliferation, requiring a long culture, generally overnight. Which procedure is best remains unclear as few studies have compared the large series of embryos over a long time period. In this study, a retrospective analysis compares the outcome of the two protocols of embryo selection for FET.

Materials and methods

Patients

This retrospective study included patients undergoing FET treatment at the reproductive medical center of Nanfang Hospital of Southern Medical University in Guangzhou between June 2010 and July 2012. The patients who transferred 2–3 embryos, at least one embryo was in grade I after thawed, were selected for this study. Irrespective of the number of FET cycles derived from each ovarian pick-up (OPU), only embryos thawed for the first FET of each OPU were eligible for the study. This study was a retrospective analysis of the data from our center and was approved by the Ethics Review Board of Nanfang Hospital of Southern Medical University.

IVF/ICSI procedures

The protocols for ovarian stimulation in IVF were followed [8]. Follicles were aspirated transvaginally under ultrasound guidance at 36 h post hCG injected. Cumulus-oocyte-complexes (COCs) were handled on heated stages in G-IVF medium (10136, Vitrolife, Vitrolife inc., Göteborg, Sweden) warmed to 37 °C. For IVF, the COCs were mixed with sperm at 39–40 h post hCG injected. For ICSI, the cumulus cells were removed enzymatically and the sperm was injected at 40–42 h post hCG injected. After fertilization, zygotes, day 2 and day 3 embryos were cultured in G1-plus medium (10128, Vitrolife).

Cleaving embryos were scored and graded every day for blastomere number, cleavage plane and degree of fragmentation. Day 3 embryo was scored based on the following: grade I: number of cells ≥6 with equal size blastomeres, regular morphology, integrated zona pellucida, no vacuoles, and fragments <5 %; grade II: number of cells <6, or number of blastomeres ≥6 with cells of equal size or roughly equal size, particles in cytoplasm, fragments between 5 % and 20 %; grade III: embryo with blastomeres of distinctly unequal size, irregular morphology, distinct particles in cytoplasm, fragments between 20 % and 50 %; grade IV: embryo abnormal development, severely unequal cell size, significant cytoplasmic particles, a great quantity of vacuoles, fragments >50 %. Higher grade embryos were selected for fresh embryo transfer. Surplus grade I and grade II embryos were cryopreserved.

Embryo freezing and thawing

Vitrification solution A was G-MOPS (10129, Vitrolife) containing 20 % HSA (10064, Vitrolife), solution B was solution A contained 7.5 % EG (33068, Sigma, Sigma-Aldrich Co. LLC., St. Louis, MO, USA) and 7.5 % DMSO (D2650, Sigma), and solution C was solution A contained 15 % EG, 15 % DMSO, and 1.0 mol/L sucrose (S1888, Sigma). Embryos were rinsed in solution A for 1 min, and then the embryos were transferred into solution B and stored for 5 min. Finally, they were transferred into solution C and placed onto cryotip within 1 min, and then frozen in liquid nitrogen.

The cryotip with the embryo was removed from the liquid nitrogen, and they were placed in 1.0 mol/L sucrose thawing solution and 0.5 mol/L sucrose solution for 1 min and 3 min, respectively. Next, they were put in G-MOPS solution with 20 % HSA for 5 min. Each embryo was individually transferred into a drop with 25 μL G1-plus. They were checked for blastomere survival under an inverted microscope and incubated until transfer. Short post-thawed incubation (group A) consisted of a culture for a 2–4 h period before transfer. Long post-thawed incubation (group B) consisted of an overnight culture for a 20–24 h period and was observed again to confirm that they had further development before transfer.

Freezed-thawed embryos transfer

The warmed embryos were transferred in natural cycles, stimulated cycles (gonadotrophin or clomiphene citrate) or hormonal replacement cycles. Endometrial thickness ≥7 mm was considered mandatory for proceeding with embryo thawed. A maximum of 3 vitrified-warmed embryos were transferred under ultrasound guidance using a Wallace Embryo Replacement Catheter (1816N, Wallace, Smiths Medical Int., Kent, UK). Luteal phase support was achieved with progesterone, which was continued daily for at least 2 weeks after embryo transfer. Serum hCG concentrations were measured 14 days after embryo transfer. Clinical pregnancy and implantation rates were determined by the detection of sacs by ultrasound at 6 weeks after embryo transfer.

Follow-up

All pregnant women were followed up until 2 months after parturition. All delivered infants were evaluated for complications during pregnancy or at delivery, gestational age, gender, birth weight and defects.

Main outcome measures

A clinical pregnancy was defined as ultrasonographic visualization of an intrauterine gestational sac with fetal heartbeat. A spontaneous abortion was defined as a clinical pregnancy lost before 20 weeks of gestation. Implantation rate was defined as the number of observed intrauterine gestational sacs divided by the number of embryos transferred. An ectopic pregnancy had been diagnosed laparoscopically or by ultrasound. The live birth rate was the ratio of live births to embryos transferred.

Statistical analysis

All data are presented as mean ± standard deviation or percentage. Statistical evaluation was performed by using a chi-square test or *t*-test, with SPSS software (SPSS, Chicago, IL, USA). Differences with $P<0.05$ were considered as significant.

Results

Between June 2010 and July 2012, 1,353 FET cycles from a total of 2,624 were analyzed, 787 in the short culture group (2–4 h of post-thawed culture) and 566 in the long culture group (20–24 h of post-thawed culture) (Fig. 1). The patients' age and the factors of infertility were in Table 1. Nobody was lost to follow-up.

The clinical outcomes of the FET cycles were summarized in Table 1. The clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate, live birth rate and birth weight were similar in the short culture group compared with these in the long culture group ($P>0.05$).

In order to account for the age affecting on the clinical outcome, we divided the patients in three groups: ≤35, 35–38 and >38, according to the patients' age. In each age range, the clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate, live birth rate and birth weight were similar in the short culture group compared with these in the long culture group ($P>0.05$) (Table 2).

To detect the number of transferred embryos impacting on the clinical outcome, the patients were divided to two groups: two embryos transferred and three embryos transferred. In two embryos transferred groups, the clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate, and birth weight were similar in the short culture group compared with these in the long culture group ($P>0.05$), but the live birth rate was higher in the long culture group than that in the short culture group ($P=0.022$) (Table 3). In three embryos transferred groups, the clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate, live birth rate and birth weight were similar in the short culture group compared with these in the long culture group ($P>0.05$) (Table 3).

Discussion

In this retrospective study, the results demonstrates that the clinical pregnancy rate, implantation rate, multiple pregnancy

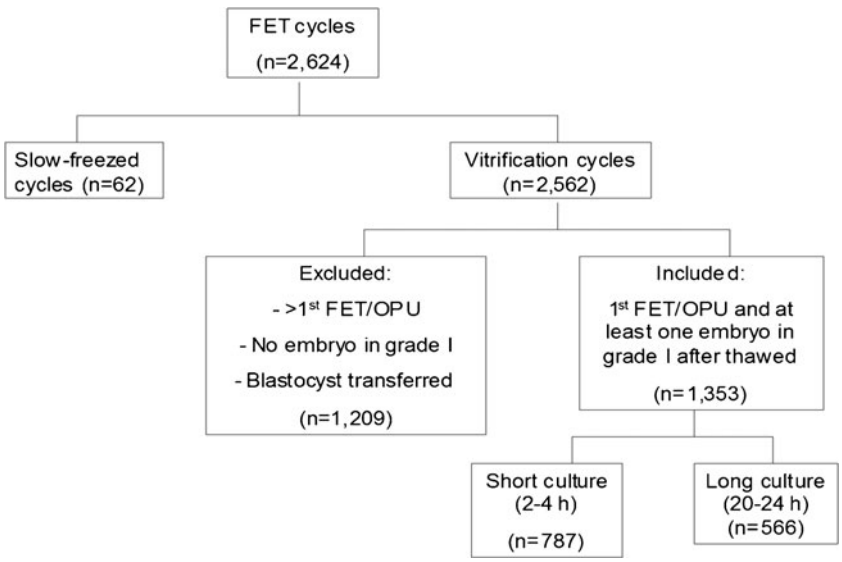

Fig. 1 Flow chart summarizing thawed embryo accountability of the study

Table 1 Demographic and clinical characteristics of FETs in the two different post-thawed culture period groups

	Group A Short culture group (2–4 h)	Group B Long culture group (20–24 h)	*P*-value
Maternal age at OPU, mean ± SD (range)	32.2±4.7 (22–49)	31.7±4.4 (21–45)	
No. transferred embryos, mean ± SD	2.5±0.5	2.5±0.5	
Factor of infertility			
Female (%)	441/787 (56.0)	331/566 (58.5)	
Male (%)	126/787 (16.0)	86/566 (15.2)	
Both (%)	213/787 (27.1)	141/566 (24.9)	
Unexplained (%)	7/787 (0.9)	8/566 (1.4)	
Clinical pregnancy rate (%)	349/787 (44.3)	265/566 (46.8)	0.367
Implantation rate (%)	476/1,969 (24.2)	371/1,422 (26.1)	0.204
Multiple pregnancy rate (%)	118/349 (33.8)	91/265 (34.3)	0.891
Abortion rate (%)	65/349 (18.6)	40/265 (15.1)	0.250
Ectopic pregnancy rate (%)	24/349 (6.8)	15/265 (5.7)	0.540
Live birth rate (%)	334/1,969 (17.0)	269/1,422 (18.9)	0.142
Birth weight (g), mean ± SD (range)	2,861.9±654.6 (1,050–4,700)	2,868.8±549.1 (1,200–4,470)	0.886

rate, abortion rate, ectopic pregnancy rate, live birth rate and birth weight were similar in the short culture group compared with these in the long culture group.

One limitation of this study is that patients' characteristics, such as body mass index (BMI) and basic hormone levels, the dose of the drugs in the ovarian stimulation procedure, the number of oocytes from the OPU and the way to insemination are not mentioned. In addition, abortion and birth weight are self-reported by patients. Although the conclusions from this study are based on retrospective data and have the inherent limitations of retrospective studies, there are strengths in its design that afford strong confidence on the results. First, the study was based on a large sample of FET cycles. Second, in order to eliminate the effects of the embryo quality on the clinical outcome in this study, we selected the cycle that has at least one embryo in grade I after thawed in short culture group and the cycle that has at least one embryo in grade I after thawed and at least one embryo observed to resume mitosis in long culture group. Third, the quality of data is high, without missing data or a single case lost to follow up. As this study aims to focus on embryo developmental potential following two different post-thawed culture durations, implantation rate and live birth rate per embryo were considered to be more accurate and informative outcome measures.

Following the first reports of human pregnancies after the transfer of freezed-thawed embryos, cryopreservation has become an increasingly important therapeutic strategy in reproductive medicine. In 2008, FET cycles accounted for almost 20 % of all assisted reproductive technology treatment cycles in Europe [9]. However, the clinical pregnancy rate in flash cycles is higher than that in FET cycles. The main reason may be that the freezing-thawing process could induce the damage of embryo developmental potential. The process has been attributed to damage the intracellular organelles and oxydative stress induced by crystallization [10].

High-quality embryos maintain high pregnancy rates [11, 12]. Looking at clinical data where single implantation frequently results from the transfer of two or more embryos of comparable morphology, it is clear that a large proportion of failed implantations must be ascribed to the embryo. There are two approaches to select the embryos for transfer in FET cycles. One relies upon the observation of blastomere survival after thawed, requiring a shorter culture. The major key factor contributing to the success of FET is thought to be the survival of embryos after thawed. Several studies reported that a lower development potential for damaged embryos compared to embryos with all blastomeres intact [13, 14]. The other relies upon the observation of blastomere proliferation, requiring a longer culture, generally overnight. Day 2 slow-frozen-thawed embryos with mitosis resumption after an additional culture period have better implantation ability than uncleaved embryos ($P<0.05$), but day 3 frozen-thawed embryos gave better results than day 2 embryos with overnight culture after thawed ($P<0.05$) [2]. Other studies got the same results [15–17]. In this study, the results indicated that the clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate, live birth rate and birth weight were similar in the short culture group compared with these in the long culture group. In the other studies day 2 and day 3 embryos were used slow-freezing way for cryopreservation, and in this study the vitrified approach was used for cryopreservation embryos and only day 3 embryos were selected for the research. Moreover, the embryo quality may be higher in this study than that in the other studies. A recent

Table 2 The influence of patients' age in the two different post-thawed culture period groups

	Group A			Group B			*P*-value (group A vs group B)		
Maternal age at OPU	≤35	35–38	>38	≤35	35–38	>38	≤35	35–38	>38
Clinical pregnancy rate (%)	288/612 (47.1)	33/85 (38.8)	28/90 (31.1)	229/441 (51.9)	20/74 (27.0)	16/51 (31.4)	0.119	0.116	0.974
Implantation rate (%)	398/1,496 (26.6)	41/230 (17.8)	39/243 (16.0)	326/1,085 (30.0)	26/198 (13.1)	20/139 (14.4)	0.055	0.183	0.666
Multiple pregnancy rate (%)	100/288 (34.7)	7/33 (21.1)	11/28 (39.3)	80/229 (34.9)	5/20 (25.0)	4/16 (25.0)	0.960	0.749	0.336
Abortion rate (%)	47/288 (16.3)	12/33 (36.4)	6/28 (21.4)	31/229 (13.5)	5/20 (25.0)	4/16 (25.0)	0.380	0.390	0.786
Ectopic pregnancy rate (%)	20/288 (6.9)	0/33 (0)	4/28 (14.3)	14/229 (6.1)	0/20 (0)	0/16 (0)	0.705		
Live birth rate (%)	289/1,496 (19.3)	22/230 (9.6)	23/243 (9.5)	237/1,085 (21.8)	18/198 (9.1)	14/139 (10.1)	0.116	0.867	0.847
Birth weight (g), mean ± SD (range)	2,834.2±634.4 (1,050–4,700)	3,235.0±804.0 (1,100–4,700)	2,835.8±671.8 (1,810–3,900)	2,867.4±560.3 (1,200–4,470)	2,844.4±399.2 (2250–3,600)	2,923.6±553.3 (1,800–3,500)	0.521	0.071	0.746

Table 3 The influence of No. of embryos transferred in the two different post-thawed culture period groups

	Group A		Group B		*P*-value (Two embryos transfer in group A *vs* group B)	*P*-value (Three embryos transfer in group A vs group B)
	Two embryos transfer	Three embryos transfer	Two embryos transfer	Three embryos transfer		
Maternal age at OPU, mean ± SD (range)	31.2±4.5 (22–49)	33.2±4.7 (23–45)	30.9±4.0 (21–44)	32.5±4.6 (21–45)		
Clinical pregnancy rate (%)	166/387 (42.9)	183/400 (45.8)	133/274 (48.5)	132/292 (45.2)	0.151	0.887
Implantation rate (%)	224/774 (28.9)	254/1,200 (21.2)	179/548 (32.7)	193/876 (22.0)	0.147	0.636
Multiple pregnancy rate (%)	57/166 (34.3)	60/183 (32.8)	43/133 (32.3)	45/132 (34.1)	0.715	0.809
Abortion rate (%)	32/166 (19.3)	44/183 (24.0)	20/133 (15.0)	21/132 (15.9)	0.336	0.078
Ectopic pregnancy rate (%)	13/166 (7.8)	11/183 (6.0)	7/133 (5.3)	8/132 (6.1)	0.337	0.985
Live birth rate (%)	154/774 (19.9)	180/1,200 (15.0)	138/548 (25.2)	131/876 (15.0)	0.022	0.977
Birth weight (g), mean ± SD (range)	2,871.2±626.7 (1,050–4,700)	2,853.9±679.3 (1,250–4,700)	2,850.1±530.9 (1,450–4,100)	2,888.5±569.2 (1,200–4,470)	0.763	0.634

research reported that the pregnancy rate of the FET cycles that transfer of day 3 vitrified–warmed embryos with observed mitosis was higher than that of the cycles transferred the embryos without observed mitosis [18].

Owing to numerous advances in culture systems and the introduction of more refined tools for the classification of the embryo's developmental capacity, transfer of two embryos does not diminish the chance of a birth when compared with three embryos transfer and more than four embryos for transfer [19]. However, the lack of a strong predictor of embryo viability imposes severe restrictions to the general implementation of elective single embryo transfer, so the clinical pregnancy rate of single embryo transfer is lower than that of double embryo transfer [20]. In this study, when the same number of embryos transferred, the clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate and birth weight were similar in the short culture group compared with these in the long culture group, but the live birth rate in long culture group was higher than that in the short culture group when two embryos transferred ($P<0.05$).

Age is an important determinant of the success rate of infertility treatment. In this study, the clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate, live birth rate and birth weight were similar in the short culture group compared with these in the long culture group when patients' age were in the same range.

In conclusion, when embryos including at least one grade I embryo after thawed transferred, the clinical pregnancy rate, implantation rate, multiple pregnancy rate, abortion rate, ectopic pregnancy rate, live birth rate and birth weight were similar in the short culture group compared with these in the long culture group. The roles of the two approaches (short culture and long culture) are no different in FET cycles.

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