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Original Article

Frozen two day 3 embryos and subsequently produced blastocysts by vitrification: advantages for IVF/ICSI patients at high risk of ovarian hyperstimulation syndrome

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Abstract: Ovarian hyperstimulation syndrome (OHSS) may occur during IVF/ICSI treatment, when fresh embryos transfer is required to be cancelled to avoid OHSS aggravation caused by pregnancy-induced elevation of HCG levels, and all produced embryos should be frozen. However, freezing of day 3 cleavage-stage embryo has shown a low success rate. The purpose of this study was to evaluate the advantages of freezing two day 3 cleavage-stage embryos and subsequently produced blastocysts by vitrification for IVF/ICSI patients at high risk of ovarian hyperstimulation syndrome (OHSS). A retrospective study was conducted among women undergoing IVF/ICSI treatment in a medical university teaching hospital. The clinical pregnancy rate (CPR) and implantation rate (IR) was evaluated in the subjects receiving vitrification and warming of embryos with the CryoLoop system that were at high risk of OHSS. The number of high-quality day 3 embryo per cycle, number of blastocyst per cycle, proportion of blastocysts-freezing cycle, number of day 5 or 6 frozen blastocyst per cycle were significantly higher in the 276 IVF/ICSI patients at high risk of OHSS than controls ($P< 0.05$). The CPRs for one/two thawed blastocysts transfer and two thawed day 3 embryos transfer were 56.07% and 25.00% per cycle ($P< 0.05$), and 62.50% and 27.38% per patient, respectively. A significant difference was detected in the IR of day 3 thawed embryos transfer between the two groups ($P< 0.05$). The strategy of freezing two day 3 embryos and transfer of subsequently produced blastocysts may have more advantage for patients at high risk of OHSS, which produces more high-quality blastocysts, and vitrified-warmed blastocysts transfer in thawed cycles significantly reduces the embryo replacement cycles, and results in excellent implantation and successful pregnancy in a short period of time.

Keywords: Ovarian hyperstimulation syndrome, cryopreservation, embryo transfer, clinical pregnancy, vitrification

Introduction

Ovarian hyperstimulation syndrome (OHSS) is a potentially life-threatening iatrogenic complication, which is caused by the use of hormonal therapy to artificially induce multiple follicular growths during *in vitro*fertilization (IVF) treatment. Extensive use of gonadotropin-releasing hormone (GnRH) in a down-regulation protocol for ovarian stimulation (OS) increases the risk of developing OHSS in infertile patients undergoing assisted fertilization treatment [1]. The prevalence of OHSS is reported to varies between 3%-6% (moderate OHSS) and 0.1%-2% (severe OHSS) after OS for assisted reproductive technology (ART), while a mild form of OHSS is detected in 20%-23% of IVF cycles [2]. Severe OHSS is a dangerous iatrogenic complication in assisted reproduction, which seriously threatens infertile patients' lives and causes huge disease and economic burdens [3]. In addition, there are deaths reported in patients due to OHSS-associated complications; however, none of

previously reported techniques have shown consistent effectiveness for prevention of the syndrome, except cancellation of the treatment cycle before administration of human chorionic gonadotropin (HCG) [4]. It is therefore frustrating and costly for the infertile couples, and the risk of OHSS cannot be excluded even if a second OS is performed.

The development of OHSS, notably late-onset form, is considered to be linked to the increased production of endogenous HCG at early pregnancy. It is reported that the risk of OHSS is two to five folds greater if pregnancy occurs, in both IVF and ovarian induction cycle [5]. Elective cryopreservation of all embryos and delayed embryo transfer (ET) are considered effective to reduce the risk of developing pregnancy-induced late-onset OHSS [6]. In addition, cryopreservation of all embryos and avoiding fresh ET are reported to minimize the exposure to endogenous HCG, which prevents the occurrence of pregnancy-associated late-onset OHSS [7], and provides infertile patients a possibility of pregnancy with thawed embryos. The stage favoring the storage of embryos has been extensively investigated; however, previously reported elective cryopreservation of all cleavage-stage embryos may reduce the pregnancy and live-birth rates in women at risk of OHSS in relative to transfer of fresh embryos [8]. Since vitrification has been introduced, it has replaced conventional slow-freezing approaches to freeze blastocyst-stage embryos, which results in a higher survival rate of frozen embryos and successful pregnancy of frozen-thawed ET. Recently, frozen-thawed blastocyst transfer has shown a higher implantation rate (IR) and clinical pregnancy rate (CPR) as compared to fresh/frozen-thawed ET [9], which improves greatly the pregnancy outcomes of human assisted reproduction [10-12].

Patients at high risk of OHSS are found to be relatively young, and have more available embryos, are extremely suitable for blastocyst transfer [13]. Therefore, frozen-thawed blastocyst transfer is recommended to achieve rapid pregnancy if transfer of fresh embryos is canceled for prevention of late-onset OHSS. However, acquisition of high-quality blastocysts is not always successful in all patients. For patients undergoing cryopreservation of all embryos, ifall day 3 cleavage-stage embryos are used for culturing blastocystes and then frozen, patients may lose the likelihood of ET if no qualified blastocysts produce. The major purpose of this retrospective study was to evaluate the clinical value and pregnancy outcomes of freezing two day 3 cleavage-stage embryos and transferring subsequently produced blastocysts by vitrification for IVF/ICSI patients at high risk of OHSS.

Subjects and methods

Ethical statements

This study was approved by the ethics review committee of Nanfang Hospital (permission number: nfkjk2011089) and Fujian Provincial Maternity & Children's Health Hospital (permission number: FJFYBJY2011-047). Signed informed consent was obtained from all participants following a detailed description of the purpose and potential benefits of the study.

Subjects

A total of 2,178 patients undergoing IVF orICSI treatment during the period from March 1, 2012 through July 11, 2014 were enrolled in this study, and a total of 2,595 IVF or ICSI cycles were performed in Fujian Provincial Maternity & Children's Health Hospital, including 1,859 IVF and 736 ICSI cycles. All patients received a standard down-regulation protocol for OS with GnRH and follicle-stimulating hormone (FSH). Oocytes were retrieved 36 hours after HCG administration, and inseminated with husband's sperm using a conventional IVF or ICSI technique. Fertilization was assessed 14 to 18 hours after insemination, and the zygotes were cultured for additional 3 days. Two high-quality day 3 cleavage-stage embryos were selected for ET or freezing by vitrification if ET was not applicable, and day 3 surplus embryos were cultured for 2 days to the blastocyst stage. Day 5 or 6 blastocyst-stage embryos were vitrified if subsequently produced. For patients at high risk of OHSS, the scheduled fresh ET was cancelled if they had complaints of pelvic pain or discomfort, or had their serum estradiol levels of > 4,500 pg/ml on the day of HCG administration and >15 follicles in an intermediate (< 15 mm) to large size (>16 mm) (1). Two day 3 cleavage-stage embryos were vitrified, and the surplus embryos were cultured to day 5 or 6 cleavage-stage blastocysts and vitrified. In the present study, a total

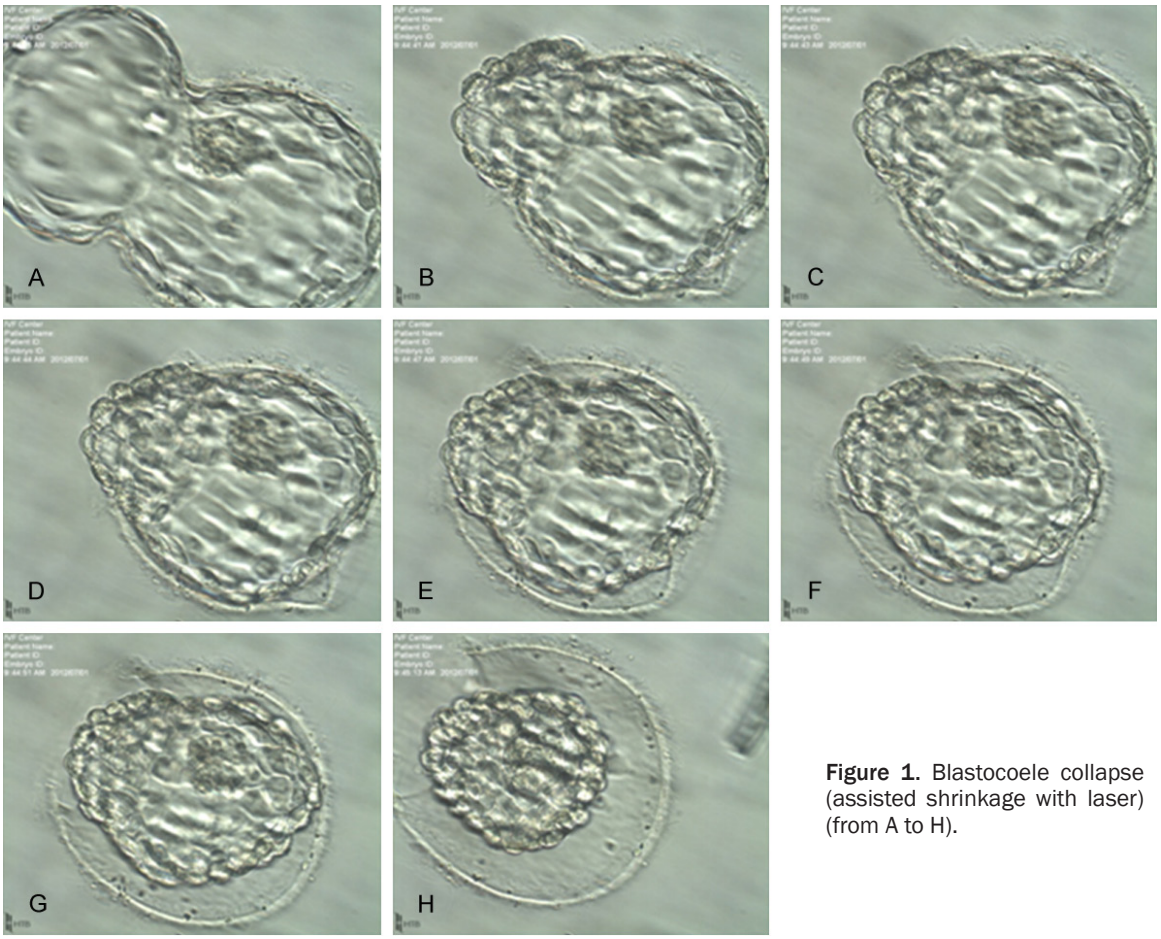

Figure 1. Blastocoele collapse (assisted shrinkage with laser) (from A to H).

of 280 cycles of fresh ET were cancelled in 276 patients due to the risk of developing OHSS, and they agreed to undergo oocytes retrieval with cryopreservation of their embryos.

Cleavage-stage embryo and blastocyst freezing by vitrification

Embryos were vitrified with a Cryoloop procedure. All freezing and warming procedures were performed on a pad at 37°C. Embryos were vitrified in a two-step procedure in human tubal fluid (HTF)-MOPS (SAGE IVF Inc.; Trumbull, CT, USA) supplemented with 5 mg/ml human serum albumin (HSA) containing dimethyl sulfoxide (DMSO)and ethylene glycol (EG). Before blastocysts vitrification, a ZILOS-tk laser (Hamilton Thorne Biosciences; Beverly, MA, USA) blastocoele puncture was performed to create an opening in the zona and a small defect in the trophectoderm causing the blastocoele to leak until collapse (**Figure 1**).

For vitrification, cleavage-stage embryos (for 2 or 3 minutes) and collapsed blastocysts (for 1 minute) were equilibrated in a mixture of 7.5% DMSO and 7.5% EG (v/v) solution at 37°C after twice initial washing in HTF-MOPS. Embryos were then placed into the vitrification solution composed of 15% EG, 15% DMSO and 10 mg/ml Ficoll in a 0.65 M sucrose solution for 40 seconds at 37°C. The Cryoloop consisted of a 2-mm nylon loop at the end of a stainless steel rod (Hampton Research; Laguna Niguel, CA, USA). By dipping the loop in the second vitrification solution, a film was created. The surface tension, due to the low volume and Ficoll, kept this film intact while embryos were placed onto the loop using a pulled glass pipette. The loop containing the embryos was then plunged directly into LN2 inside a cryovial and stored until warming.

Vitrified cleavage-stage embryo and blastocyst thawing

Vitrified embryosand blastocysts were warmed in a series of 37°C pre-warmed sucrose solu-

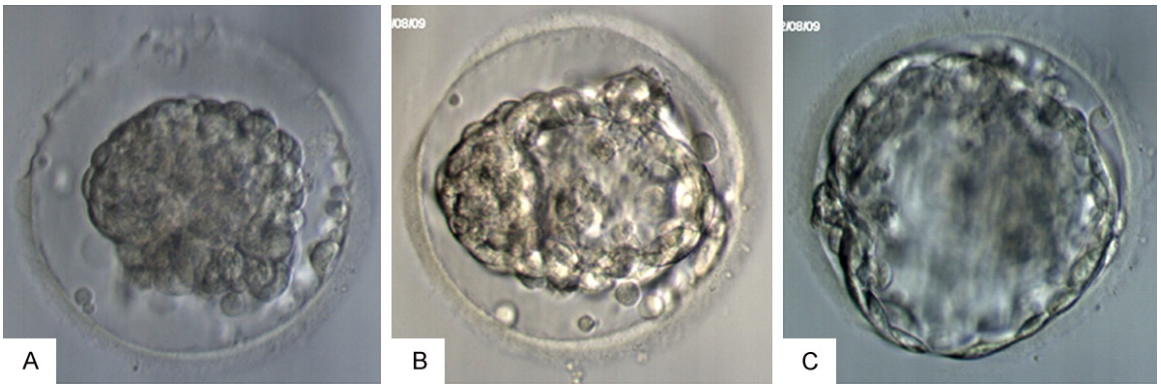

Figure 2. Blastocyst-stage embryos on day 5. A. 0 hours after warming, completed assisted hatching; B. 0.5 hours after warming, small blastocoele; C. 2 hours after warming, re-expansion.

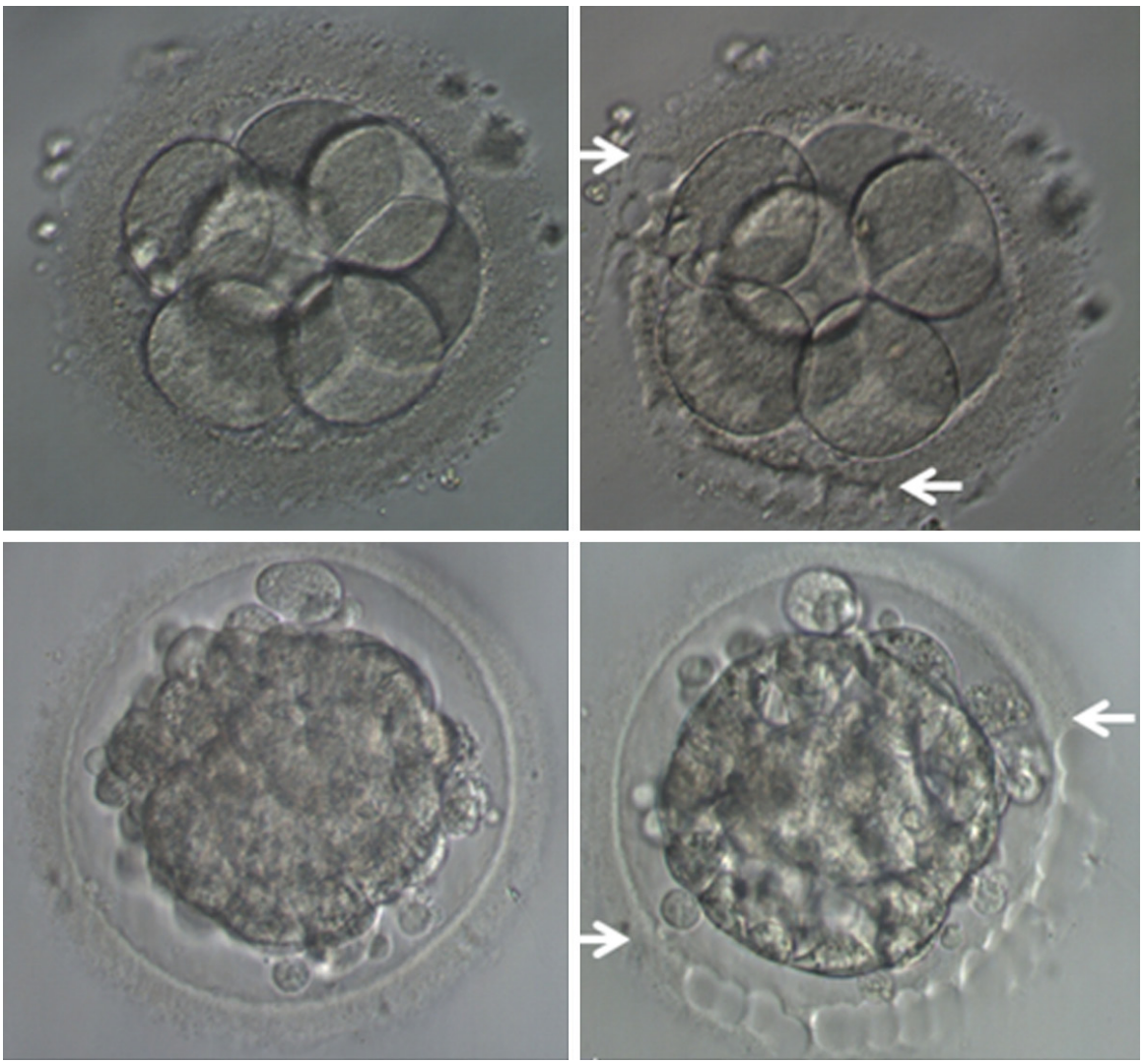

Figure 3. Assisted hatching: An opening (indicated by arrow) is created in the zona using laser pulse.

tions. Warming was performed in two steps. The nylon loop containing the embryos was removed from the vial and dipped into HTF-MOPS containing with 0.25 M sucrose at 37°C.

Table 1. Characteristics of patients in OHSS high risk group and control group

Parameter	OHSS high risk group (n=276)	Control group (n=1902)	*P* value
No. of cycles	280	2315	
Mean age (yr)*	30.06±3.84	32.38±4.71	0.000
Mean spouse age (yr)*	32.70±4.45	34.65±5.21	0.000
Body mass index (kg/m^2)	20.92±2.40	21.25±2.73	0.120
Duration of infertility (yr)*	4.18±2.71	5.17±3.55	0.000
Type of sterility (%)			
Primary sterility*	153 (54.64)	1029 (53.13)	0.002
Secondary sterility*	127 (45.36)	1286 (55.55)	0.002
Cause of infertility (%)			
Tubal factor*	119 (42.50)	1230 (54.47)	0.001
Endometriosis*	6 (2.14)	163 (7.04)	0.003
Ovulation failure*	10 (3.57)	24 (1.04)	0.001
PCOS*	29 (10.35)	48 (2.07)	< 0.0001
Male factor	74 (26.43)	523 (22.59)	0.172
Unexplained infertility	42 (15.00)	327 (14.13)	0.760
Basal serum sexual hormone level			
FSH (mIU/ml)*	5.39±1.38	6.54±2.73	0.000
LH (mIU/ml)*	4.63±3.35	3.76±2.50	0.000
E_2 (pmol/L)*	16.42±9.40	17.68±12.05	0.000
PRL (ng/ml)*	41.60±21.28	41.90±29.79	0.092
T (nmol/ml)	0.39±0.15127	0.35±0.23	0.869
FSH/LH*	1.57±1.73	2.40±3.68	0.005
Basal AFC			
Left ovary*	11.47±4.04	7.79±3.65	0.000
Right ovary*	12.95±4.25	8.40±4.05	0.000
Type of fertilization (%)			
IVF	192 (68.57)	1667 (72.01)	0.256
ICSI	88 (31.43)	648 (27.99)	0.256

Note: Values are presented as number, number (%) or mean ± SD. *indicates a significant difference between the OHSS high risk group and control group.

After 1 minute in the first solution, the embryos were moved into HTF-MOPS containing with 0.125 M sucrose for 3 minutes. Embryos were then transferred to HTF-MOPS at 37°C for 5 minutes before being moved to wash drops of G1 or G2, depending on developmental stages, and placed into medium for embryo-assisted hatching (AH). After AH, the embryos were finally incubated in medium for 1 to 2 hours (**Figure 2**) prior to ET.

Embryo AH

AH was performed on all vitrified day 3 embryos and blastocysts with a laser treatment soon after warming. The zona pellucid (ZP) surrounding day 3 embryos was thinned to more than two-thirds of its initial thickness and a distance covered more than one-third of ZP circumference, and more than one-third of blastocysts ZP was burned up far from the collapsed blastocysts (**Figure 3**).

Endometrial preparation

Endometrial preparation was performed in natural and artificial cycles. For the artificial cycles, exogenous estrogen (E_2) and progestogen were administered to prime the endometrium following conventional ultrasound examination at days 2 to 4 of menstruation. Endometrial preparation was started with E_2 administration at an oral daily dose of 2 to 15 mg according to previous endometrium thickness. If the thickness was 8 mm or greater, progesterone was injected at a daily dose of 20 mg for two days, 40 mg a day for another two days, and 60 mg a day for the fifth day. Thawed ET was planned 3, 5 or 6 days, respectively, after detection of the endogenous luteinizing hormone (LH) surge or administration of HCG in natural cycles and the start of progesterone injection in artificial cycles. The embryos were warmed on the day of ET with a post-thawing culture of 1 to 2 hours.

ET and pregnancy validation

One or two warmed embryos were moved into 37°C fresh Embryo Glue medium (Vitrolife; Goteborg, Sweden) at the time of ET. ET was performed with ultrasound-guided soft catheters (Cook Ireland Ltd.; Limerick, Ireland). After ET, all patients were given luteal support with injection of 60 mg progesterone (Xianju Pharmaceutical Co., Ltd.; Hangzhou, China).

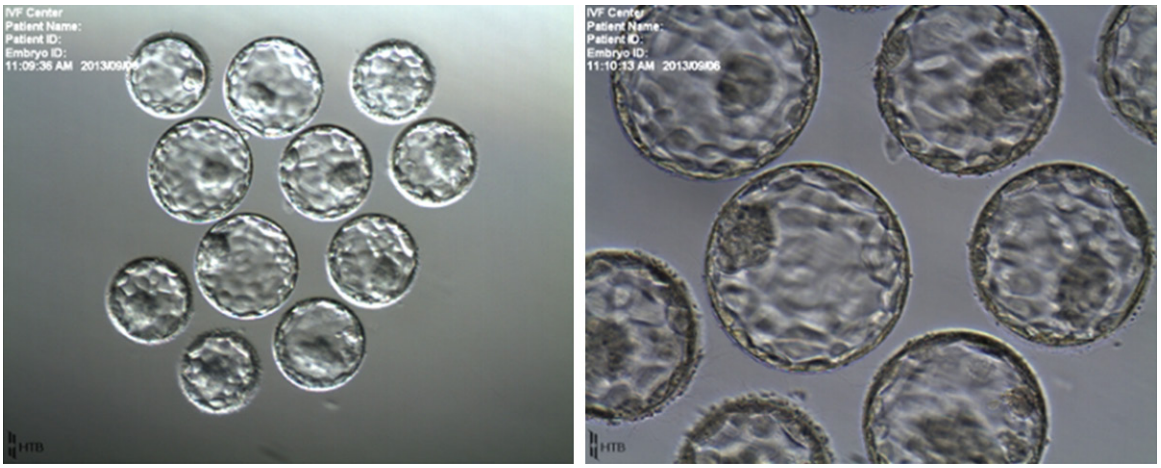

Figure 4. High-quality blastocysts (day 5) produced from patients at high risk of OHSS.

Pregnancy was confirmed by measuring HCG level 10 days after blastocyst transfers. Clinical pregnancy was defined as presence of a gestational sac(s) with or without a fetal heartbeat, revealed by B-mode ultrasonography 30 days after ET. The number of sacs was taken as the number of implantations.

Statistical analysis

All data were tested for normal distribution prior to the selection of statistics. Differences of the means of numerical variables were tested for statistical significance using a Student's *t* test, while categorical variables were compared using Fisher's exact test or chi-square tests. Pearson correlation analysis was performed to indicate the strength and direction of a relationship between two random variables, and receiver operating characteristic (ROC) curves analysis was used to determine the cutoff value. All statistical analyses were performed using the statistical software SPSS version 17.0 (SPSS Inc.; Chicago, IL, USA), and a *P*-value < 0.05 was considered statistically significant.

Results

Demographic and clinical characteristics

Of the 2,178 subjects recruited, 276 cases at high risk of OHSS were assigned to the OHSS high-risk group (n=276), and the others served as controls (n=1,902), and they were given 280 and 2,315 cycles of IVF/ICSI treatment, respectively. There were significant differences in age, duration of infertility, type of sterility, causes of infertility, baseline levels of FSH, LH, prolactin (PRL) and E_2, baseline FSH/LH and baseline ovary antral follicle count (AFC) between the two groups ($P< 0.01$) (**Table 1**). A total of 3,356 embryos from 2,178 patients were vitrified, including 561 day 3 embryos at six to eight cell stage, 1,693 day 5 blastocysts (**Figure 4**) and 1,102 day 6 blastocysts in fresh cycles, with a 51.37% rate of blastocyst formation. Significant differences were detected in the dose of the starting gonadotropin, dose of total gonadotropin per cycle, duration of gonadotropin administration per cycle, serum levels of LH, E_2, progesterone and PRL on the day of HCG administration, and the number of oocytes retrieved per cycle between the two groups ($P< 0.01$), and there were significant differences seen in the number of high-quality day 3 embryo per cycle, number of blastocyst per cycle, proportion of blastocysts-freezing cycle, number of day 5 or 6 frozen blastocysts per cycle ($P< 0.01$) (**Table 2**). However, significant difference of cleavage rate was only found in IVF patients and significant difference of blastocyst formation rate was observed only in ICSI patients between the two groups ($P< 0.01$) (**Table 2**).

Comparison of pregnancy outcomes between groups

A total of 887 frozen-thawed ET cycles were performed among 814 subjects, including 276 cases in the OHSS high-risk group and 538 cases in the control group, and totally

Table 2. Cycle characteristics of patients in OHSS high risk group and control group

Parameter	OHSS high risk group (n=276)	Control group (n=1902)	*P* value
No. of cycles	280	2315	
Dose of the starting gonadotropin (IU)*	182.77±97.97	205.01±41.79	0.000
Dose of total gonadotropin per cycle (IU)*	1932.96±527.00	2211.55±684.59	0.000
Days of gonadotropin per cycle (d)*	5.95±2.55	6.90±3.09	0.000
Serum sexual hormone level on hCG administration day			
LH (mIU/ml)*	0.70±0.35	1.12±0.84	0.000
E_2 (pmol/L)*	8492.40±3781.55	3679.80±2463.06	0.000
P (nmol/L)*	1.24±0.67	0.95±1.11	0.000
PRL (ng/ml)*	62.91±34.68	47.55±31.43	0.000
Total number of oocytes retrieved	6132	21038	
No. of oocytes retrieved per cycle*	21.90±6.40	9.09±5.18	0.000
Clinical outcomes of IVF			
No. of IVF cycles	191	1665	
Fertilization rate (%)	3396/4151 (81.81)	12500/15043 (83.10)	0.055
Cleavage rate (%)*	3056/3396 (89.99)	11052/12500 (88.42)	0.011
Top quality day 3 embryo rate (%)	1593/3056 (52.13)	5953/11052 (53.86)	0.092
No. of top quality day 3 embryo per cycle*	8.34±4.72	3.57±3.00	0.000
Blastocyst formation rate (%)	624/1522 (41.00)	1668/4335 (38.48)	0.089
No. of blastocyst per cycle*	3.27±2.64	1.13±1.51	0.000
No. of top quality blastocyst per cycle*	1.90±1.95	0.62±1.06	0.000
% of blastocysts freezing cycle*	135/191 (70.68)	578/1667 (34.67)	< 0.0001
No of frozen day 5 blastocyst per cycle*	1.51±1.88	0.52±1.02	0.000
No of frozen day 6 blastocyst per cycle*	0.97±1.15	0.33±0.71	0.000
Clinical outcomes of ICSI			
No. of ICSI cycles	89	650	
Fertilization rate (%)	1387/1643 (84.42)	4211/5052 (83.35)	0.329
Cleavage rate (%)	1233/1387 (88.90)	3791/4211 (90.03)	0.250
Top quality day 3 embryo rate (%)*	674/1233 (54.66)	2209/3791 (58.27)	0.028
No. of top quality day 3 embryo per cycle*	7.57±4.32	3.40±3.02	0.000
Blastocyst formation rate (%)*	338/587 (57.58)	785/1599 (49.09)	0.0005
No. of blastocyst per cycle*	3.80±2.65	1.21±1.71	0.000
No. of top quality blastocyst per cycle*	2.54±2.22	0.72±1.26	0.000
% of blastocysts freezing cycle*	69/89 (77.53)	229/650 (35.23)	< 0.0001
No. of frozen day 5 blastocyst per cycle*	1.97±1.87	0.57±1.10	0.000
No. of frozen day 6 blastocyst per cycle*	1.17±1.51	0.41±0.83	0.000

Note: Values are presented as number, number (%) or mean ± SD. *indicated a significant difference between the OHSS high risk group and control group.

1,551 vitrified embryos were thawed and transferred, including 431 day 3 embryos and 1,120 blastocysts. The pregnancy rate was 34.50% per transfer cycle following frozen-thawed embryo transfer, with a cumulative rate of 43.86% per patient and an IR of 30.34%. There were significant differences in the CPR per transfer cycle for both the day 3 cleavage-stage thawed ET cycle (25.00% vs. 13.24%) and blastocysts transfer cycles (56.07% vs. 44.04%) between the two groups ($P < 0.05$). In addition, a significant difference was found in the CPR per patient only if thawed blastocysts were transferred (62.50% vs. 46.67%, $P< 0.05$), and in the IR only in the day 3 cleavage-stage thawed ET cycles between two groups. However, no significant differences were seen in multiple pregnancy rates, abortion rates and

Table 3. Comparison of clinical outcomes in frozen-thawed embryo transfer cycles between the OHSS high risk group and control group

Parameter	OHSS high risk group (n=276)	Control group (n=538)	*P* value
No. of cycles	306	581	
Age (yr)*	30.77±3.79	33.41±4.50	0.000
Spouse age (yr)*	33.49±4.62	35.96±5.11	0.000
Endometrial preparation (%)			
Nature cycles*	142 (46.41)	398 (70.07)	< 0.0001
Artificial cycles*	164 (53.59)	170 (29.93)	< 0.0001
Type of endometrium (%)			
Type A	254 (83.01)	481 (84.68)	0.583
Type B	52 (16.99)	87 (15.32)	0.583
Endotrium thickness (mm)*	9.97±1.85	9.33±2.34	0.000
Clinical outcomes of thawed cleavage-stage embryos transfer			
No. of cycles	92	136	
Embryo survival rate (%)	177/179 (98.88)	247/252 (98.02)	0.705
No. of embryo transfer per cycle*	1.95±0.23	1.84±0.37	0.014
Implantation rate (%)*	28/177 (15.82)	19/247 (7.69)	0.013
Clinical pregnancy rate per cycle (%)*	23/92 (25.00)	18/136 (13.24)	0.036
Clinical pregnancy rate per patient (%)	23/84 (27.38)	18/118 (15.25)	0.053
Multiple pregnancy rate (%)	4/23 (17.39)	1/18 (5.56)	0.363
Abortion rate (%)	2/23 (8.70)	4/18 (22.22)	0.377
Ectopic pregnancy rate (%)	0/23 (0.00)	0/18 (0.00)	
Clinical outcomes of thawed blastocysts transfer			
No. of cycles	214	445	
Embryo survival rate (%)	385/387 (99.48)	730/733 (99.59)	1.000
No of embryo transfer per patient*	1.80±0.40	1.65±0.48	0.000
Implantation rate (%)	154/385 (40.00)	266/730 (36.44)	0.270
Clinical pregnancy rate per cycle (%)*	120/214 (56.07)	196/445 (44.04)	0.005
Clinical pregnancy rate per patient (%)*	120/192 (62.50)	196/420 (46.67)	0.000
Multiple pregnancy rate (%)	37/120 (30.83)	71/196 (36.22)	0.391
Abortion rate (%)	21/120 (17.50)	23/196 (11.73)	0.204
Ectopic pregnancy rate (%)	2/120 (1.67)	1/196 (0.51)	0.560

Note: Values are presented as number, number (%) or mean ± SD. *indicated a significant difference in frozen-thawed embryo transfer cycles between the OHSS high risk group and control group.

ectopic pregnancy rates either transferred with day 3 thawed embryos or blastocysts (**Table 3**).

Correlation between blastocyst formation and its affecting factors

Among the patients at high risk of OHSS (n=276), there was strong association of the number of blastocyst formation per cycle with its affecting factors, including number of follicles aspirated per cycle, number of oocytes retrieved per cycle, fertilization rate, cleavage rate, number of high-quality day 3 embryos per cycle, and number of embryos for blastocyst culture per cycle (**Table 4**; $P< 0.01$). In addition, ROC curves were plotted for prediction of blastocyst cryopreservation by vitrification (**Figure 5**), and the areas under ROC (AUC) and the cut-off values of aforementioned factors were shown in **Table 5**.

Comparison of CPR achieved with different ARTs

Among the subjects at high risk of OHSS, 560 two day 3 embryos and 753 blastocysts were vitrified in 280 IVF/ICSI cycles. Both two day 3 embryos and blastocysts were successfully fro-

Table 4. Correlation between blastocyst formation and its affecting factors in patients at high risk of OHSS (n=276)

Characteristics	Pearson correlation coefficient	*P* value
No. of follicles aspirated per cycle	0.345	0.000
No. of oocytes retrieved per cycle	0.435	0.000
Fertilization rate (%)	0.350	0.000
Cleavage rate (%)	0.269	0.000
No. of quality day 3 embryos per cycle	0.726	0.000
No. of embryos for blastocyst culture per cycle	0.708	0.000

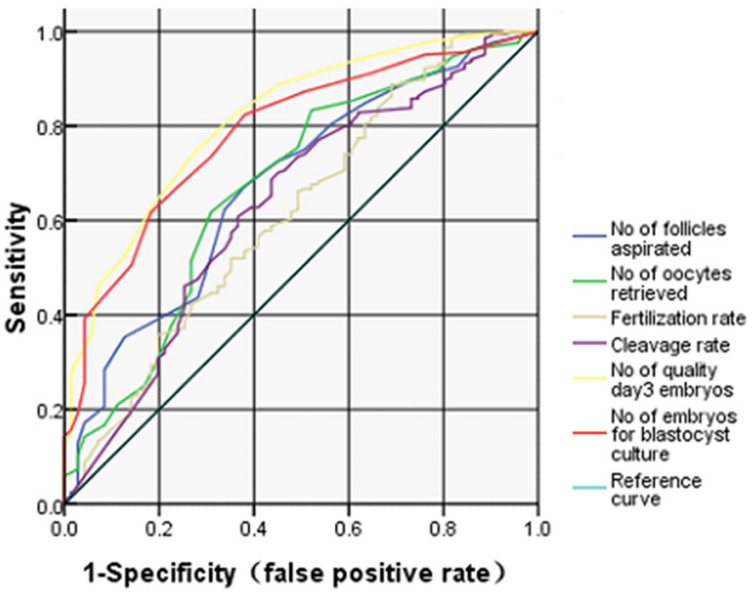

Figure 5. Receiver operating characteristic (ROC) curve for prediction of blastocyst cryopreservation by vitrification in patients at high risk of OHSS.

zen in 204 (72.86%) and 76 cycles (27.14%) per patient, and only two day 3 embryos were vitrified per patient since none of blastocysts was produced. After freezing of all embryos, patients in the OHSS high-risk group developed OHSS in 72 cycles, with a 25.71% incidence of OHSS per cycle (1.79% for mild form, 14.29% for moderate form, and 9.63% for severe form, respectively). Of the 306 warming cycles, the CPRs were 56.07% per cycle and 62.50% per patient for one or two thawed blastocysts transfer, and were 25.00% per cycle and 27.38% per patient for two day 3 thawed ET, respectively, with a 53.56% cumulative CPR per patient seen.

Discussion

Urgently elective cryopreservation of all embryos remains the best option for prevention of OHSS for patients at high risk of OHSS, notably pregnancy-induced late-onset OHSS. As an iatrogenic severe complication during assisted reproduction treatment, OHSS may occur at either the luteal phase or early pregnancy, with an incidence estimated to be 20% to 33% for mild form, 3% to 6% for moderate form, and 0.1% to 2.0% for severe form [2, 14, 15]. In the current study, a 2.77% overall incidence of OHSS was observed in total subjects, with 0.19% for mild, 1.54% for moderate and 1.04% for severe forms seen, respectively; however, the prevalence increased to 25.71% in the high-risk population for OHSS, with 1.79% for mild, 14.29% for moderate and 9.63% for severe forms, respectively.In a multi-center study conducted in Israel, the incidence of severe OHSS showed an increase tendency with year in patients undergoing IVF, from 0.06% in 1987 to 0.24% in 1996 [16]. OHSS is considered as the most feared complication of IVF-related OS for both patients and doctors, and its severe form may lead to hospitalization, disability and death in patients undergoing IVF, which should be paid much attention. Timely assessment of the risk of developing severe OHSS, cancellation of fresh ET and elective cryopreservation of all embryos are of great significance to reduce the risk of developing severe OHSS.

It has been demonstrated that young age, low body weight, high ovarian response to controlled OH (COH), high E_2 level, rapid elevation of E_2 level, size and number of induced follicles, number of oocytes retrieved, and development of polycystic ovarian syndrome (PCOS) are risk factors of OHSS [5]. The results of this study showed that the subjects at high risk of OHSS were found to have significantly lower age, lower body mass index (BMI), higher E_2 level, more AFC and oocytes retrieved, and higher

Table 5. Prediction of blastocyst cryopreservation in patients at high risk of OHSS (n=276)

Characteristics	AUC	SE	Sig.	95% CI	Cut-off point
No. of follicles aspirated per cycle	0.676	0.037	0.000	0.603, 0.748	24.50
No. of oocytes retrieved per cycle	0.673	0.039	0.000	0.597, 0.749	17.50
Fertilization rate (%)	0.618	0.040	0.003	0.539, 0.697	64.15
Cleavage rate (%)	0.634	0.040	0.001	0.555, 0.712	88.68
No. of quality day 3 embryos per cycle	0.816	0.029	0.000	0.760, 0.872	6.50
No. of embryos for blastocyst culture per cycle	0.787	0.030	0.000	0.728, 0.846	4.50

incidence of PCOS in relative to controls ($P< 0.01$), which further validated that these factors are associated with the high risk of developing OHSS.

Since there are currently no approaches available to eliminate severe OHSS during ART, prevention or cycle cancellation remains the best strategy. Cryopreservation of all obtained embryos has been successfully employed to reduce the late onset of severe OHSS, and avoiding fresh ET is found effective to prevent the development of late-onset OHSS in high-risk population [4]. Pregnancy is found to increase the incidence of OHSS. It is reported that the risk of developing OHSS increases by 2- to 5-fold after pregnancy in either IVF or ovulation induction cycle [16]. However, cryopreservation of all obtained embryos during IVF may reduce the risk of developing OHSS and achieve satisfactory pregnancy outcomes [4]. In high-risk OHSS patients with E_2 level > 4,086 pg/ml and > 50 follicles, a 1.8% OHSS incidence was observed following cryopreservation of all embryos, while the incidence was 1.8% in the remaining patients undergoing fresh ET; in addition, the pregnancy rate was significantly higher in patients undergoing cryopreservation of all embryos than that obtained with "normal" fresh ET followed by freezing of remaining embryos, and was similar to that following fresh ET in the same center [17]. A similar study was designed in a lower-risk OHSS population with > 2,724 pg/ml E_2 levels of and/or > 20 oocytes, the incidence of moderate OHSS was 4.3% and 0.5% in patients with and without cryopreservation of all embryos, and the pregnancy rate after frozen-thawed ET was 32.6%, with a 65% cumulative CPR and a 22.9% IR seen [18]. Queenan and colleagues [19] reported 13% incidence of both moderate and severe OHSS, 58% pregnancy rate of frozen-thawed ET and 67% cumulative delivery rate in 15 patients who had > 4,500 pg/ml E_2 levels and > 25 follicles and had cryopreservation of all embryos. In a prospective randomized study comparing the pregnancy outcomes of pronuclear embryo cryopreservation vs. fresh ET, comparable pregnancy rates (46.3% vs. 48.3%) and OHSS incidence (0 vs. 6%) were observed between the two groups, and a significantly lower rate of transfer cancellation was seen in patients undergoing cryopreservation of pronuclear embryos as compared to those with fresh ET [20]. However, a reduction was detected in the CPR after cryopreservation in relative to controls, while OHSS incidence was comparable in both subject groups [21]. In the present study, a low incidence of OHSS (2.7%) was observed in total population, and the overall CPR was 34.50% per ET cycle after 887 cycles of frozen-thawed embryo replacement if cryopreservation of two day 3 embryos and subsequently produced blastocysts was performed in patients at high risk of OHSS with > 4,500 pg/ml E_2 level and >15 oocytes, with a 43.86% cumulative CPR and a 30.34% IR per patient. In addition, the pregnancy rate was 56.07% per transfer after 214 cycles of verified-warmed blastocyst transfer in the high-risk OHSS group, with a cumulative CPR rate of 62.50% per patient and an IR of 40%, which was significantly higher than that obtained with "normal" frozen embryo transfer and was even higher than that following fresh ET in our hospital. Our findings indicate the great advantages of successfully vitrified blastocysts for high-risk OHSS patients.

The developmental stage of embryos favored for preservation has been extensively examined in patients at high risk of OHSS. Pronuclear-stage oocytes are thought to produce pregnancy rates comparable to fresh IVF, and cryopreservation is recommended at the pronuclear stage [22]. Transfer of 2.7 embryos, which developed from frozen-warmed pronuclear-stage oocytes, was reported to achieve a 28.5%

CPR per ET [23]. Vitrification is considered effective to cryopreserve human cleavage-stage embryos in IVF patients at high risk of OHSS [4], and a 3-year follow-up study revealed improved vitrification using Cryoleaf for cryopreservation of day 3 embryos, with 36.4%, 25.8%, and 36.3% CPRs in the fresh-cycle, slow-freezing, and vitrification groups, respectively [24]. In addition, Han AR et al. [25] reported a 44.2% CPR of frozen ET for cleavage stage, 43.1% for blastocysts, and 31.8% for post-thaw extended-culture blastocysts, indicating that blastocyst transfer may not achieve a better pregnancy outcome than cleavage-stage ET. In a retrospective study to compare the clinical outcome of fresh vs. vitrified-warmed blastocyst transfer, the IR and CPR of vitrified-warmed blastocyst transfer cycles (37.0% and 55.1%) were significantly higher than those (25.2% and 36.4%) of fresh blastocyst transfer cycles [11]. In another retrospective case-control study designed to compare the clinical outcomes between fresh blastocyst and vitrified-thawed day 5 blastocyst transfer, transfer of 111 vitrified-thawed blastocysts in 59 cycles resulted in 59.3% CPR, 43.2% IR, 47.5% ongoing pregnancy rate, and 60.7% and 39.3% rates for singleton and twin pregnancies, and no significant differences were observed in CPR, IR, and ongoing pregnancy rate between the fresh blastocyst transfer group and the vitrified-thawed blastocyst transfer group [10]. Such a finding is inconsistent with the guidance on prevention of OHSS for the clinician, which reports that the success rate of establishing a pregnancy using frozen-thawed embryos is generally lower than using fresh embryos [26].

The results of the present study confirmed this finding. If fresh ET was cancelled for prevention of late-onset OHSS, all produced blastocysts were vitrified after freezing of two day 3-stage embryos, and similar excellent clinical outcomes of vitrified-thawed blastocyst transfer were observed in subjects at high risk of OHSS. Our findings showed a 56.07% CPR per cycle and 62.50% per patient, and 40% PR, and the CPR of thawed blastocysts transfer was significantly higher than that of normal patients and vitrified-thawed cleavage-stage ET. The results demonstrate that cryopreservation of two day 3 embryos and the surplus blastocysts may benefit high-risk OHSS patients for preventing the development of late-onset OHSS, and the IR and CPR are not reduced if vitrified-thawed blastocysts are transferred. The results may be explained by the following factors. Firstly, vitrification was employed instead of slow-freezing for blastocyst cryopreservation, which enhanced the post-thaw survival and development of blastocysts and improved the potential of blastocyst transfer. Kuć et al. [27] also supported our findings, which reported a higher CPR (50.4%) of day 5 or 6 verified-thawed blastocyst transfer than that (25.9%) of slow-freezing blastocyst transfer ($P< 0.05$). Vitrification is considered to avoid the damage to frozen cells caused by formation of ice crystals [28], and the cryoprotectant formula have been modified to be less toxic and more efficient. According to the results reported by Hong et al. [9], we cryopreserved embryos at 37°C, which accelerated the permeation of cryoprotectant into embryos and reduced the cell exposure to cryoprotectant, resulting in a low toxicity. In addition, Cryoloop was employed as a carrier for cryopreservation, which allows less liquid volume, larger area to contact liquid nitrogen, and great acceleration in temperature-lowering speed [29]. Secondly, verified-thawed blastocyst transfer was used to achieve twice embryo self-optimizations. The first selection aims to shift from cleavage-stage embryos to blastocyst-stage embryos, which eliminates the embryos with developmental arrest in cleavage stage, while the second is a process of thaw and survival of frozen blastocysts, which may eliminate the blastocysts that are not tolerate to cryopreservation and have poor ability. Therefore, verified-thawed blastocyst transfer may greatly improve the pregnancy rate of frozen ET [30, 31]. Thirdly, verified-thawed blastocyst transfer mostly closely approximates normal pregnancy process, when the greatest synchronization is observed between embryo and endometrial development. It has been proved that endometrial receptivity and embryo-endometrium synchronization are of great importance in frozen-thawed ET [10, 32]. The higher implantation and clinical pregnancy rates observed for vitrified-warmed blastocyst transfer cycles may arise from better endometrial receptivity and enhanced synchronization between embryo and endometrial development. In high-risk OHSS patients, a high E_2 level (> 5,000 pg/ml) on the day of HCG administration was found to affect embryo implantation, resulting in a decreased CPR [13]. It is there-

fore speculated that vitrified-warmed blastocyst transfer is of great advantage for subjects at high risk of OHSS if fresh ET is cancelled to reduce pregnancy-induced late-onset severe OHSS, which has a better clinical outcomes.

However, blastocyst culture is not always successful. The success of blastocyst culture depends on the development of day 3 embryos. It was reported that the blastocyst formation rate was 76% for day 3 eight-cell embryos, and 54% for day 3 six-cell embryos [33]. In the current study, two day 3 cleavage-stage embryos were vitrified and all surplus embryos were cultured to blastocysts in patients at high risk of developing OHSS, and the blastocyst formation rate was 45.62%, with 41% for IVF patients and 54.66% for ICSI patients, respectively. Our findings showed that two day 3 embryos and blastocysts were successfully frozen in 204 cycles (72.86%), and two day 3 embryos were vitrified in only 76 cycles (27.14%). Factors affecting the formation of high-quality blastocysts have been identified in patients at high risk of OHSS [13, 34], which include age, gonadotrophin dose, insemination method, and number of day 3 eight-cell embryos. The results of the present study showed that age, duration of infertility, causes of infertility, baseline ovary AFC, gonadotrophin dose,number of oocytes retrieved per cycle, and number of high-quality day 3 embryos per cycle affected the number of high-quality blastocysts produced and frozen in patients at high risk of OHSS. In addition, strong associations of blastocyst formation number per cycle were detected with the number of follicles aspirated per cycle, number of oocytes retrieved per cycle, fertilization rate, cleavage rate, number of high-quality day 3 embryos per cycle, number of embryos for blastocyst culture per cycle ($P< 0.01$). ROC curves revealed that the blastocyst culture and cryopreservation may be successful in patients at high risk of OHSS if they had > 24.50 follicles aspirated per cycle, > 17.50 oocytes retrieved per cycle, > 64.15% fertilization rate, > 88.68% cleavage rate, > 6.5 high-quality day 3 embryos per cycle, and > 4.5 embryos for blastocyst culture per cycle. It was reported that blastocyst culture was appropriate if at least four day 3 eight-cell embryos were present [35], whereas Levitas et al. [36] suggested that the presence of more than two day 3 eight-cell embryos was required for blastocyst culture. These reports support our findings. In this study, the number of high-quality day 3 embryos per cycle included the total number of embryos with more than six cells. Therefore, the high-risk OHSS patients with these characteristics may be more suitable for blastocyst culture derived from the surplus embryos after freezing of two day 3 embryos if fresh ET is not available.

In conclusion, the results of the present study demonstrates that urgent cryopreservation of all embryos is required to minimize the incidence of pregnancy-induced late-onset OHSS in patients at high risk of OHSS, and the strategy of freezing two day 3 embryos and subsequently produced blastocysts may have more advantages for patients at that risk for effective and safe production of blastocysts with higher quality. Vitrified-warmed blastocyst transfer in thawing cycles may remarkably reduce ET cycles in patients at high risk of OHSS, and result in satisfactory IR and CPR and successful pregnancy within a short period of time. Further studies are required to develop *in vitro* embryo culture system for yielding blastocysts with higher quality; more appreciate cryopreservation protocols for increasing embryo survival and better screening methods to identify developmental competence after warming.

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Disclosure of conflict of interest

None.

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