Effect of vitrification versus slow freezing of human day 3 embryos on β -hCG levels

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Abstract

Purpose The study was designed to investigate the effect of vitrification and slow freezing for the cryopreservation of human day 3 embryos on serum β -hCG levels in pregnancies established after frozen embryo transfer (FET).

Methods Of the 1384 FET cycles initiated, 1131 embryo transfers met study criteria and assigned to one of two groups: 797 slow-freezing embryo transfers or 334 vitrified embryo transfers. Median values of β -hCG and outcome of all pregnancies were compared between the two groups. Predictive values of serum β -hCG on day 12 after embryo transfer for establishing ongoing pregnancy and pregnancy failure were determined by receiver operating characteristic (ROC) curve analysis.

Results In the slow-freezing group, 383 ongoing pregnancies occurred (48.1 %), and transfers of vitrified embryos resulted in 154 pregnancies (46.1 %). Median β -hCG values (279.2 IU/L) for ongoing pregnancies after transfer of vitrified embryos were significantly lower than that of slow frozen embryos (320.5 IU/L). The median values of β -hCG for singleton in the two groups was statistically significant ($P<0.05$). For slow-freezing embryo transfers, the cut-off value of β -hCG in predicting ongoing pregnancy was 147 IU/L (sensitivity 88.3 %, specificity 80.7 %). For vitrified embryo transfers, the value was 135 IU/L (sensitivity 84.4 %, specificity 76.3 %).

Conclusions Day 12 β -hCG levels after FET are significantly affected by the methods of embryo cryopreservation for

ongoing pregnancies. Furthermore, when using β -hCG cut-off value to assess pregnancy outcome, the cryopreservation methods should be taken into account.

Keywords Vitrification · Slow-freezing · Human chorionic gonadotrophin · Cut-off value

Introduction

Human chorionic gonadotropin (hCG) is a glycoprotein hormone, secreted by both the cytotrophoblast and syncytiotrophoblast [1]. It is present in the maternal serum as early as 6~7 days after fertilization [1]. Since hCG is the first observable signal of trophoblast proliferation in maternal blood, it is used for the early detection of pregnancy [2]. Earlier studies have demonstrated that β -hCG measurements in early pregnancy have proven to be predictive of pregnancy outcome [3–5]. However, when considering the diagnostic efficacy of β -hCG values, one must consider that advances in assisted reproduction techniques might have possible effects on initial serum β -hCG concentrations [6, 7].

In this study, two basic techniques have been employed for the cryopreservation of human day 3 embryos: slow-freezing and vitrification. Slow freezing was the dominant method of cryopreservation in human assisted reproduction laboratory for many years. Recent studies have reported increasingly successful clinical results with vitrification [8–11]. Vitrification is an ultra-rapid method of cooling cells into a glass-like state. In contrast to slow-freezing techniques, vitrification procedure requires an extremely high cooling rate and much higher concentrations of cryoprotectant. It is unclear whether these changes could affect the ability of the embryo to secrete hCG after implantation.

Therefore, the purpose of the present study is to investigate the effects of vitrification and slow freezing for the

Capsule This investigation shows that day 12 β -hCG levels after FET are significantly affected by the methods of embryo cryopreservation for ongoing pregnancies.

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cryopreservation of human day 3 embryos on serum β -hCG levels in pregnancies established after FET. As far as we know, no previous studies have reported the possibility that the cryopreservation methods may affect embryo developmental potential *in vivo* as assessed by the concentration of β -hCG. It is our hope that this report will be useful for clinical practice and as an aid in counseling IVF patients on the chances of successful or failed pregnancy in the future.

Materials and methods

Design of the study

The retrospective study was performed in the Reproduction Centre, Sir Run Run Shaw Hospital, China between January 2012 and April 2013. Between January 2012 and May 2012, cleavage-embryos were conventionally cryopreserved by slow freezing. From June 2012, vitrification gradually replaced slow freezing. Patients were included only when they underwent the “freeze-all” embryo strategy on the day 3 after oocyte retrieval. Exclusion criteria included patients involving rescuing fertilization by ICSI, mixed fresh/thawed embryo transfer, mixed cleavage/blastocyst embryo transfer and those in which embryos were simultaneously slowly frozen and vitrified as described below.

Of the 1384 frozen-thaw cycles initiated during the study interval, 253 were excluded from analysis, 148 for blastocyst culture after embryo thawing, 79 for unavailable β -hCG values on day 12 post-embryo transfer, and 26 for cycle cancellation because of thawed embryo death. Of the remaining 1131 embryo transfers performed, 797 were a slow-freezing embryo transfer and 334 were a vitrified embryo transfer. This study had the approval of institutional review board.

Ovarian stimulation

Patients included in the study were treated by routine controlled ovarian hyperstimulation (COH) as described by Xue et al. [12]. Briefly, the ovaries were stimulated with follicle-stimulating hormone (FSH) after mid-luteal pituitary down-regulation with a gonadotrophin releasing hormone (GnRH) agonist. Follicular development was monitored by ultrasound scanning. HCG was administered when at least three follicles measured >18 mm diameter. Transvaginal oocyte retrieval was performed 36 h after hCG injection.

Fertilization and embryo culture

Oocytes were fertilized using either conventional IVF or ICSI and then cultured in fertilization media (G-IVF, Vitrolife Sweden AB, Sweden) containing 10 % serum substitute supplement (SSS, Irvine Scientific, Santa Ana, CA) and

incubated. Normal fertilization was assessed and confirmed by the presence of two pronuclei and second polar body at 16–18 h after insemination. The embryos were washed and cultured in cleavage medium (G-1, Vitrolife Sweden AB, Sweden) with 12 % SSS and incubated at 6 % CO₂, 37 °C incubators for 48 h before being frozen. Embryonic development was assessed on Day 3 after oocyte retrieval with an inverted microscope using routine examination of: [1] the number of blastomeres; [2] the degree of cytoplasmic fragments; [3] the uniformity of blastomeres. Embryos morphology was scored as: grade 1, equal blastomeres with no obvious fragmentation; grade 2, <20 % fragmentation and/or unequal blastomeres; grade 3: 20–50 % cytoplasm fragmentation; grade 4: >50 % cytoplasm fragmentation. An embryo with 6–12 blastomeres and Grades 1 and 2 was defined as good quality. The criteria for cryopreservation were at least four blastomeres, fragments <50 % and no multinucleate blastomeres.

Slow freezing/thawing of day 3 embryos

Slow freezing was performed as described by Edgar et al. [13]. Briefly, embryos were equilibrated in 1.5 M propanediol (PROH) (Freezing Kit, Irvine Scientific, Santa Ana, CA) at room temperature for 10 min, then transferred to 1.5 M PROH/0.1 M sucrose and loaded into 0.25 ml plastic straws (CBS High Security Straws, Cryo-Bios, France) and then sealed. Straws were put in a controlled-rate freezer (Kryo-360, Planer Product Ltd, UK). The temperature was slowly reduced to −7 °C at a rate of 2 °C/min. Ice nucleation was induced manually by liquid nitrogen-cooled forceps. The straws were slowly cooled to −30 °C at a rate of −0.3 °C/min and then rapidly to −120 °C at a rate of −30 °C/min. straws were then plunged into liquid nitrogen and stored until thawing.

Embryos were thawed at room temperature. Straws were moved rapidly from liquid nitrogen to air for 30 s followed by immersion in water at 30 °C for 30 s. Embryos were then placed into each of the following solutions: 1.0 M PROH/0.2 M sucrose for 5 min, 0.5 M PROH/0.2 M sucrose for 5 min, and 0.2 M sucrose for 10 min. Thawed embryos were incubated in fresh medium (G2, Vitrolife Sweden AB, Sweden) with 12 % serum substitute supplement (SSS, Irvine Scientific, Santa Ana, CA) until transfer.

Vitrification and warming protocols

For vitrification, day 3 embryos were cryopreserved using the method described previously [14]. Embryos were suspended in equilibration solution containing 7.5 % ethylene glycol (EG)+7.5 % dimethylsulfoxide (DMSO) for 5 min at room temperature, then transferred to vitrification solution containing 15 % EG+15 % DMSO+0.5 M sucrose for 40–60 s. Then

embryos were loaded on the Cryotop strip (Kitazato Supply Co, Fujinomiya, Japan) and plunged into liquid nitrogen immediately.

For warming, the Cryotop strip was directly inserted into thaw medium with 1.0 M sucrose in HEPES-based medium plus 20 % SSS for 1 min at 37 °C. Warmed embryos were transferred to diluent medium with 0.5 M sucrose for 3 min and then incubated in each of the following solutions for 5 min: 0.25 M sucrose and sucrose-free HEPES. Embryos were subsequently placed in G2 medium containing 12 % SSS and cultured for 2 h prior to transfer.

Preparation for FET

Three main types of clinical protocols were used for endometrial preparation: natural, hormone replacement or low dose human menopausal gonadotrophin (HMG)-stimulated cycles [15]. For women with regular menstrual cycles, the frozen-thawed embryos were replaced in natural cycles. Embryo transfer was performed 3 days after ovulation, or four and a half days after the luteinizing hormone (LH) peak.

A hormone replacement treatment (HRT) or a low-dose HMG-stimulated protocol was used for women with irregular ovulation or anovulatory cycles. For hormone replacement cycles, we maintained the same dosage of oral estradiol valerate tablets (Progynova; Schering AG, Berlin, Germany) about 6–8 mg/d for 14–28 days. If the double thickness of the endometrium exceeded 8 mm, injections of 80 mg/d of progesterone injection were administered for 3 days before embryo transfer and the original dosage was maintained after transfer. A serum estradiol level close to 300 ng/L was also necessary for ET to proceed.

For low dose HMG-stimulated cycles, mild stimulation was initiated with a low dose of HMG (Menotrophin for Injection, Livzon Pharmaceutical Group, Inc., Guangdong, China) on days 7–8 of the menstrual cycle. Dose was adjusted according to follicular development as monitored by ultrasound. Injections of 40 mg/d of progesterone were administered for 2 days after HCG injection. Embryo transfer was performed about four and a half days after the HCG injection and 80 mg/d of progesterone was injected for luteal support after transfer.

β -hCG testing and pregnancy outcome

Venous blood was collected routinely on day 12 after embryo transfer. Serum β -hCG concentration was measured using chemiluminescence immunoassay (Beckman Access 2 Immunoassay System, Beckman Coulter Corporation). The detection limit is 0.5 IU/L and the measurement range goes up to 20 000 IU/L.

Pregnancy was assessed through serum hCG levels rising above 5 IU/L by the twelfth day after transfer. The number of gestational sacs and fetal heart beats was determined on the

basis of two ultrasonic examinations, the first at 5 to 6 weeks and the second at 11 to 12 weeks after embryo transfer. Patients with intrauterine fetal death, monochorionic twins, spontaneous (vanishing twins) and selective reduction of twin pregnancies were excluded from the study.

Pregnancy outcomes were grouped into six categories: biochemical pregnancies, singletons, twins, triplet gestations, ectopic pregnancies and miscarriages (first trimester spontaneous abortion). Biochemical pregnancy was defined as a temporary rise of serum HCG without signs of intrauterine pregnancy. Ongoing pregnancy for this study was defined as gestations progressing beyond 12 weeks. Non-viable pregnancies constituted biochemical pregnancies, ectopic pregnancies and miscarriages.

Statistical analysis

Analyses were performed using the SPSS 17.0 statistical package (SPSS, Inc., Chicago, IL). Receiver operating characteristic (ROC) curve analysis was performed to determine optimal cut-off values with a corresponding sensitivity and specificity. To assess β -hCG cut-off values predictive of viable pregnancies for each group, ROC curves were performed using a graphic representation of sensitivity (or true-positive rate) vs. 1 minus specificity (or false-positive rate). The percentages for area under the curve (AUC) and 95 % confidence intervals were generated for each ROC curve. The cut-off point with the optimal sensitivity and specificity was identified by the software. In addition, serum β -hCG values of 200 IU/L, 300 IU/L, 400 IU/L, and 500 IU/L were chosen to evaluate the predictive values for ongoing pregnancies based on published reports [16, 17].

Mann–Whitney *U*-test (two-tailed testing) was conducted to compare the differences between the slow-freezing and vitrified embryo transfer samples. The Chi square test or Fisher exact test was performed to compare differences in pregnancies outcome between the two groups. A *P* value of <0.05 was considered statistically significant.

Results

A total of 797 slow freezing and 334 vitrified transfer cycles were included in this study. The clinical parameters for slow-freezing and vitrification groups were summarized in Table 1. The results indicate that the overall survival rate and the proportion of embryos with 100 % intact blastomeres were significantly higher with vitrification than that with slow-freezing (*P*=0.000 for both). The number of IVF and IVF/ICSI cycles were significantly higher in the slow-freezing group (*P*=0.025 and *P*=0.004,

Table 1 Comparison between slow-freezing and vitrification after embryo thaw and transfer

Variable	Slow-freezing	vitrification	<i>P</i> values
Embryo transfer cycles	797	334	
Natural cycles (%)	38 (4.8)	10 (3.0)	0.177
HRT cycles (%)	669 (83.9)	289 (86.5)	0.270
HMG-stimulated cycles (%)	90 (11.3)	35 (10.5)	0.691
Age (mean $\pm$ SD)	31.2 $\pm$ 4.6	31.7 $\pm$ 4.9	0.217
Primary infertility (%)	327 (41.0)	118 (35.3)	0.073
Secondary infertility (%)	470 (59.0)	216 (64.7)	
Warmed embryos per patient (mean $\pm$ SD)	2.1 $\pm$ 0.7	2.2 $\pm$ 0.8	0.210
Embryos from IVF (%)	526 (66.0)	197 (59.0)	0.025 ^a
Embryos from ICSI (%)	229 (28.7)	103 (30.8)	0.405
Embryos from mixed IVF-ICSI (%)	42 (5.3)	34 (10.2)	0.004 ^b
Number of blastomeres in fresh embryo			
6–12 cells (%)	1376 (82.9)	608 (84.0)	0.514
<6 and >12 cells (%)	284 (17.1)	116 (16.0)	
Embryo survival rate (%)	1512/1660 (91.1)	702/724 (97.0)	0.000 ^b
Blastomere survival rate (%)			
100 %	1105 (66.6)	645 (89.1)	0.000 ^b
75–<100 %	292 (17.6)	31 (4.3)	0.000 ^b
50–<75 %	115 (6.9)	26 (3.6)	0.001 ^b
<50 %	148 (8.9)	22 (3.0)	0.000 ^b
Embryos transferred (mean $\pm$ SD)	1.8 $\pm$ 0.4	1.8 $\pm$ 0.4	0.126
Good-quality embryos transferred (mean $\pm$ SD)	1.3 $\pm$ 0.8	1.2 $\pm$ 0.8	0.120
Implantation rate	37.6 %	36.3 %	0.588
Ongoing pregnancies (%)	383 (48.1)	154 (46.1)	0.550
Singletons (%)	254 (31.9)	103 (30.8)	0.974
Twins (%)	128 (16.1)	51 (15.3)	0.740
Triplets (%)	1 (0.1)	0 (0)	
Biochemical pregnancies (%)	69 (8.7)	21 (6.3)	0.188
Ectopic pregnancies (%)	11 (1.4)	7 (2.1)	0.435
Miscarriages (%)	34 (4.3)	10 (3.0)	0.400

^a Significantly different from other values with the same superscript in the same row ($P<.05$)

^b Significantly different from other values with the same superscript in the same row ($P<.01$)

respectively); whereas the ICSI cycles in both groups were similar. Moreover, the percentage of different endometrial preparation protocols, the mean female age, the percentage of patients with primary infertility, number of blastomeres in fresh embryo, the number of warmed embryos per warmed cycle, the number of embryos transferred per transfer cycle, and the number of good-quality embryos per transfer cycle were all similar between the two groups. Similarly, there was no significant differences in implantation rate, the percentage of ongoing pregnancy ($P=0.550$), biochemical pregnancy ($P=0.188$), ectopic pregnancy ($P=0.435$) and miscarriage ($P=0.400$).

Median β -hCG values on day 12 post-embryo transfer are shown in Table 2. Comparisons of median values between the slow-freezing and vitrification groups suggest discriminatory

values or reveal difference between the two groups. The values of β -hCG for ongoing pregnancies and singleton pregnancies in the vitrification group were significantly lower than that of slow-freezing embryo transfer group (both $P<0.05$). There were no significant differences in twin pregnancy ($P=0.081$), biochemical pregnancy ($P=0.314$), ectopic pregnancy ($P=0.717$) and miscarriage ($P=0.889$) between the two groups.

To determine β -hCG thresholds predictive of viable pregnancy, ROC curves were used to compare sensitivity and specificity at each β -hCG value in the slow-freezing and vitrification groups. The analyses showed that an β -hCG level of 147 IU/L yielded optimal sensitivity (88.3 %) and specificity (80.7 %) for the slow-freezing group and the hCG level of 135 IU/L resulted in optimal sensitivity (81.3 %) and

Table 2 Median values of serum β -hCG on day 12 post-embryo transfer

	β -hCG value	Slow-freezing	vitrification	P-value
Ongoing pregnancies	Median	320.5 (203.0–457.5)	279.2 (164.8–402.6)	0.013 ^a
	Rang	30.5–1233.0	51.9–914.7	
Singleton	Median	259.5 (173.4–371.6)	223.8 (138.7–324.4)	0.039 ^a
	Rang	30.5–805.0	51.9–687.4	
Twin	Median	488.0 (361.5–648.4)	401.3 (267.8–642.6)	0.081
	Rang	142.0–1233.0	120.9–914.7	
Biochemical pregnancies	Median	31.0 (14.3–67.0)	45.0 (23.0–382.0)	0.314
	Rang	5.9–305.5	9.0–170.0	
Ectopic pregnancies	Median	74.1 (11.5–382.0)	96.0 (32.6–424.7)	0.717
	Rang	9.1–800.0	11.5–439.0	
Miscarriages	Median	133.6 (97.5–247.9)	147.9 (75.5–260.5)	0.889
	Rang	46.0–542.0	50.0–429.0	

Note: Data are expressed as median (interquartile range)

^a Significantly different from other values with the same superscript in the same row ($P < 0.05$)

specificity (72.0 %) for the vitrification group (Fig. 1). For slow-freezing embryo transfer, positive predictive value (PPV) of ongoing pregnancy was 93.9 % when a β -hCG value >147 IU/L. Likewise, for vitrified embryo transfer, the probability of ongoing pregnancy was 93.5 % using a β -hCG cut-off value >135 IU/L. For ongoing pregnancies in the slow freezing group, using the proposed cut-off point of 50 IU/L

and 200 IU/L, the probability of ongoing pregnancy was 85.6 % and 94.5 %, respectively. For vitrified transfer group with β -hCG values >50 IU/L and >200 IU/L, the probability of having ongoing pregnancy was 86.4 % and 94.5 %, respectively. Detailed ROC curve results and PPV for ongoing pregnancy at multiple β -hCG value reference points are presented in Table 3.

Fig. 1 ROC curves of serum β -hCG levels on day 12 post-embryo transfer to predict ongoing pregnancies. Dashed line = values of slow-freezing group. Solid line = values of vitrification group. The value of 147 IU/L for the slow-freezing group is indicated by an open circle. The value of 135 IU/L for vitrification group is indicated by a filled circle. These two circles represent the optimal sensitivity and specificity for the assay

Discussion

In this study, a total of 1384 transfer cycles were evaluated for several outcome measures. The embryo and blastomere survival rates after vitrification were significantly higher than that after slow-freezing. However, we did not find any statistically significant difference in pregnancy outcomes after vitrified embryo transfer compared with slow-freezing embryo transfer.

A number of studies have reported that β -hCG level represents trophoblastic mass and function and is predictive of pregnancy outcomes [1, 3–7], yet early β -hCG values after frozen embryo transfers have not studied. We further evaluated whether vitrification of day 3 embryos have an effect on β -hCG level of early pregnancy. In this study, we analyzed serum hCG concentrations from 497 slow freezing transfers and 192 vitrified transfers. The results showed that for ongoing pregnancies, vitrified embryo transfer was associated with lower hCG concentrations on day 12 post-embryo transfer compared with slow-freezing embryo transfer. In addition, the ROC analyses performed revealed that the optimal cut-off values for vitrified embryo transfer (135 IU/L) was somewhat lower than those for slow-freezing embryo transfer (147 IU/L).

Table 3 Prediction of viable pregnancies by serum β -hCG levels on day 12 post-embryo transfer

	β -hCG Value	Slow-freezing			vitrification		
		Sensitivity	Specificity	PPV	Sensitivity	Specificity	PPV
	50	99.0	44.7	85.6	100	42.1	86.4
	135 ^c	91.6	77.2	93.1	84.4	76.3	93.5
	147 ^d	88.3	80.7	93.9	81.8	76.3	93.3
Note: PPV=positive predictive value	200	75.7	85.1	94.5	67.5	84.2	94.5
^c proposed optimal cut point for vitrification embryo transfers	300	53.8	90.4	94.9	45.5	89.5	94.7
^d proposed optimal cut point for slow-freezing embryo transfers	400	34.5	93.9	95.0	25.3	94.7	95.2
	500	21.7	97.4	96.6	16.2	100	100

At least two possible mechanisms might explain the results observed in this study. The first explanation is direct toxicity of the cryoprotectants. One of the major potential problems with DMSO is that it is a very potent solvent. This permeating cryoprotectant may introduce toxic compounds into the embryos [18, 19]. Recent studies have shown that vitrification of cleavage embryos resulted in severe loss of methylation in the H19/Igf2 differentially methylated domain (DMD) [20, 21] and down-regulation of the expression of Bax, Bcl2 and P53 genes [22]. A recent study revealed high concentration of vitrification solution can affect embryo morphology by increasing the percentage of blastomere fragmentation [23], which is clinically considered as an indicator of decreased embryo viability and developmental ability [24]. However, there is no direct evidence regarding possible effects of cryoprotectant on the hCG secretion. Further research is needed to confirm this explanation.”

There is also a possible explanation that vitrification protocol might have effect on the kinetics of embryo development. The amount of hCG actually reflect the number of viable cytotrophoblast and syncytiotrophoblast cells producing the hormone [1, 2]. Our results indicate that, at equivalent time intervals subsequent to fertilization, serum hCG concentrations are significantly higher in ongoing pregnancies achieved after slow-freezing embryo transfer compared with those after vitrified embryo transfer. This would imply that cytotrophoblast and syncytiotrophoblast cells resulting from vitrified embryos might proliferate at a lower rate in contrast to those from slow-freezing embryos. Results from previous studies on mouse embryo showed that vitrification reduced mitochondrial dynamics and increased reactive oxygen species (ROS) production in embryos [25–27]. Oxidative stress may trigger the apoptosis cascade in the developmental rate of embryos after thawing [26], which may be an important mechanism underlying the decreased capacity of the vitrified embryo to hCG secretion. Although we are

unable to generalize these results directly to humans because of the genetic differences between humans and animals, there are reasons to suspect that vitrification might impair subsequent embryonic development after transfer that lead to modulations in hCG secretion. Additional research is needed to test the impact of vitrification on the embryonic developmental competence. As noted in the current systematic review by Edgar and Gook [28], there is no evidence to support the conclusion that vitrification is the primary method of human cleavage-stage embryo cryopreservation.

As expected, regardless of cryopreservation method, the approximately doubled hCG level in twin pregnancies is consistent with the results of previous studies [29, 30]. Low hCG concentration is always associated with non-viable pregnancy in the first trimester [31, 32]. Additionally, in our series, the median hCG concentrations in ongoing pregnancies were about 5-fold higher in slow-freezing group and 3-fold in vitrification group compared with those in non-viable ones, which is inconsistent with the results reported previously [31]. The cut-off values for predicting ongoing pregnancy (both vitrification and slow freezing) were higher than those reported by Poikkeus et al. [31]. The differences could be partly due to the different hCG assays, and partly due to the different study designs. Subjects described in the study of Poikkeus et al. included fresh and frozen embryo transfers. In this study we considered only those patients who underwent the transfer of slow-freezing or vitrified embryos.

In conclusion, so far as is known, this is the first study to investigate the relationship between cryopreservation methods and day 12 β -hCG levels post-embryo transfer. Our study indicates that day 12 hCG levels are significantly affected by the method of embryo cryopreservation used and highly predictive of ongoing pregnancies. Discriminatory hCG cut-off values have been determined which are specific for each cryopreservation method. However, further prospective studies are needed to confirm our findings.

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