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ASSISTED REPRODUCTION TECHNOLOGIES

Maternal age and initial β-hCG levels predict pregnancy outcome after single vitrified-warmed blastocyst transfer

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Abstract
Purpose We retrospectively examined a large cohort of females who underwent single blastocyst transfer to determine if initial β-human chorionic gonadotrophin (β-hCG) levels on day 7 after single vitrified-warmed blastocyst transfer (SVBT) could be used to predict pregnancy outcome.
Methods The treatment cycles that gave rise to the early pregnancies included in this study were performed from 2004 to 2011 in a private infertility center. In SVBT cycles, embryos were transferred during a natural cycle or after endometrial preparation with exogenous estrogen and progesterone. A total of 11,458 cycles with β-hCG levels ≥1.0 UI/ml on day 7 after SVBT were evaluated. The proportion of live births per positive β-hCG cycle was established for 10 β-hCG ranges in 3 different age groups (Group A: 21–34 years old; Group B: 35–39 years old; Group C: 40–44 years old).
Results The proportion of live births gradually increased from 1.5 to 93.7 %, 0.8 to 87.9 %, and 0.6 to 76.2 % in Groups A, B, and C, respectively. For each range of β-hCG levels, the proportion of live births was higher for the younger age group, which reflected the increased risk of early pregnancy loss with advancing female age.
Conclusions β-hCG levels on day 7 after SVBT, in conjunction with maternal age, may be used to predict pregnancy outcomes.

Capsule Maternal age and initial β-hCG levels on day 7 after single vitrified-warmed blastocyst transfer can predict pregnancy outcome and help determine the appropriate pregnancy follow-up.

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Keywords Single vitrified-warmed blastocyst transfer · Initial β-hCG level · Maternal age · Pregnancy outcome

Introduction

In patients undergoing assisted reproduction treatment, the ability to predict outcome earlier, directly following in vitro fertilization (IVF), can decrease patients' anxiety and help to determine appropriate follow-up guidelines during the first trimester. Some hormones, such as progesterone, estradiol, relaxin, and other factors, are used to predict pregnancy outcomes [1, 2]; however, the exact role of these markers and their clinical utility has not yet been established. Previous studies have reported the usefulness of measuring serum beta human chorionic gonadotropin (β-hCG), which is a subunit of hCG [3–6]. β-hCG is a pregnancy-associated molecule produced by implanted blastocysts and trophoblast cells, and it plays an important role during the implantation period and early pregnancy. β-hCG is associated with autocrine and paracrine regulation of the endometrium, corpus luteum sustention, progesterone production, embryonic implantation, and placental syncytium formation, along with other functions [7–10]. The β-hCG level in urine or serum is used as a common indicator of pregnancy in most IVF centers. The serial measurement of β-hCG concentrations is also used to differentiate normally progressing pregnancies from multiple gestations, ectopic pregnancies (EP), or spontaneous abortions [11, 12]. However, following cycles of IVF and embryo transfer, serum β-hCG concentration is not measured until 9–12 days after blastocyst transfer or 12–14 days after cleavage-stage embryo transfer because of the limited sensitivity of β-hCG assays and the potential for false-positive results due to residual β-hCG from the injections used to induce oocyte maturation [13, 14]. Thus, there is this waiting

period before β-hCG measurements are obtained and the course of action may then be determined.

Recently, some studies reported that serum β-hCG levels measured at an earlier time point could be used to predict pregnancy outcome [15, 16]. These studies demonstrated that early serum β-hCG levels 5 days after blastocyst transfer can predict pregnancy rates, including spontaneous abortions and EP. However, in these studies, single fresh blastocyst or more fresh blastocysts were transferred to each female during the same cycle of oocyte retrieval in which hCG was injected for oocyte maturation. Urine and serum β-hCG levels are dependent on the number of implanting/implanted embryos, and β-hCG levels in females with multiple pregnancies are higher than those in females with singleton pregnancies [12]; exogenous hCG could be detected in serum 7 to 11 days after the injection [17]. Therefore, in these studies, the predictions of pregnancy rates with serum levels of early β-hCG after blastocyst transfer were not accurate because of the transfer of multiple embryos and the injection of exogenous hCG for oocyte retrieval, and it was also unclear if early β-hCG levels were associated with live birth rates after embryo transfer. Furthermore, because maternal aging negatively affects embryonic implantation and fetal development, prediction of pregnancy outcome should be based on both β-hCG levels and maternal age. In fact, previous reports have demonstrated that β-hCG levels on days 14 and 16 may predict ongoing pregnancy with maternal age [18].

In the current study, we examined whether initial β-hCG levels 7 days after single vitrified-warmed blastocyst transfer (SVBT) without exogenous hCG presence were associated with pregnancy outcome and maternal age.

Materials and methods

Patients and study design

The present retrospective study was reviewed and approved by the independent Institutional Review Board of our institution. A written informed consent, which informed patients that de-identified data could be used for retrospective analysis, was obtained from all patients undergoing IVF treatment at our center. During 2004–2011, 21,882 SVBT cycles (12,891 patients) were performed, and 14,642 SVBT cycles (10,597 patients) fulfilled the following inclusion criteria: (a) serum β-hCG ≥1.0 mIU/ml on day 7 after blastocyst transfer; (b) maternal age at egg retrieval between 21 and 44 years (average: 37.0±3.9 years); and (c) available data on pregnancy outcome. We excluded females with multiple pregnancies. We analyzed the medical records of the female patients who conceived with the help of assisted reproductive technology during this study period. The main causes of infertility were anovulation (0.1 %), endometriosis (1.4 %), male factor infertility (17.2 %), tubal factor infertility (19.7 %), unexplained infertility (54.9 %), and combined infertility (5.6 %), among others (1.1 %).

The main purpose of this study was to investigate the relationship between pregnancy outcome and β-hCG levels on day 7 after SVBT. A secondary aim was to investigate the relationship between EP, biochemical pregnancy loss (BPL), and β-hCG levels on day 7 after SVBT.

Minimal ovarian stimulation, oocyte retrieval, and fertilization procedures

All patients underwent a clomiphene-based minimal ovarian stimulation protocol or drug-free natural cycle IVF treatment [19, 20]. Oocyte retrieval was performed without any anesthesia, using a fine 21–22 gauge needle (Kitazato, Japan). Follicular flushing was not used during oocyte retrieval. Conventional insemination (49.0 % of patients) was performed approximately 3 h after retrieval, and intracytoplasmic sperm injection was performed 5 h after retrieval (51.0 % of patients). P1/cleavage stage medium (Irvine Scientific, USA) or human tubal fluid (HTF; Irvine Scientific, USA) with 10 % serum substitute supplement (Irvine Scientific, USA) was used as the culture medium after insemination.

Embryo culture, blastocyst monitoring, and vitrification

Fertilization assessment was performed 16–20 h after insemination. Normally fertilized zygotes with 2 pronuclei were cultured individually in a drop of 20 μL of Quinn's Advantage Protein Plus cleavage medium (SAGE, USA) from days 1 to 3. The embryos were transferred to Quinn's Advantage Protein Plus blastocyst medium (SAGE, USA) on day 3 and cultured until day 5 to 7. All embryos were cultured at 37 °C under the gas phase of 5 % O_2, 5 % CO_2, and 90 % N_2 with 100 % humidity in water jacket small multigas incubators or dry desktop incubators (Astec, Japan).

Hatching blastocysts and blastocysts that reached an inner diameter >160 μm by day 5 to 6 were vitrified immediately according to the Cryotop method. If the developing embryo did not fulfill the desired criteria, it was cultured further, with a maximum culture period up to day 7. For day 7 blastocysts, vitrification criteria were hatched blastocysts or those that reached an inner diameter >180 μm.

Post-thawing embryo culture, embryo transfer procedure, and outcome measures

Single vitrified-warmed blastocysts were transferred on day 4.5 to 5 after ovulation during a spontaneous natural cycle or hormone replacement cycle, as described in a previous report [19]. After thawing, surviving blastocysts were cultured for 30 min to 2 h until blastocoel re-expansion was confirmed.

Only blastocysts that remained the same size or grew compared to the size before vitrification were transferred, while degenerating blastocysts were discarded. The single blastocyst transfer was performed under vaginal ultrasonography guidance using a specially designed soft silicone inner catheter (Kitazato, Japan) by placing a single blastocyst, suspended in minimal medium volume, in the upper part of the uterine cavity.

Dydrogesterone (30 mg/day orally) was routinely administered during the early luteal phase after the transfer procedure. For cases in which endogenous progesterone production from the placenta was found to be insufficient, intramuscular or intravaginal progesterone was also administered until the ninth week of pregnancy. During the first trimester, pregnancies were followed every 5 days by serum β-hCG, estradiol, and progesterone measurements and ultrasound until approximately 9 weeks of ongoing gestation, at which point patients were referred to their treating obstetrician for subsequent care.

Serum β-hCG levels were measured on day 7 after SVBT using the Enzyme Immunoassay System (Tosoh Bioscience LLC, USA), which was confirmed not to react with follicle-stimulating hormone, luteinizing hormone, thyroid stimulating hormone, or prolactin. Before measuring serum β-hCG levels, we performed a 6-point calibration. Serum β-hCG levels were measured using the same system throughout this study.

Patients were divided into the following 10 groups according to serum hCG level: 1.0–4.9, 5.0–9.9, 10–19, 20–29, 30–39, 40–49, 50–59, 60–69, 70–79, and ≥80 mIU/ml. Clinical pregnancy (CP; with a confirmed gestational sac at 5 weeks of pregnancy) and live birth (LB; live birth at ≥22 weeks of pregnancy) per each embryo transfer procedure were calculated with respect to each serum hCG range and maternal age: Group A (21–34 years old, n=3,832); Group B (35–39 years old, n=6,521); and Group C (40–44 years old, n=4,238). CP was followed with ultrasound until approximately 9 weeks of gestation, and LB was established in writing by females and/or obstetricians. EP was defined by an increase of serum β-hCG levels and observation of a gestational sac outside of the uterus. BPL was defined by a temporal increase of serum β-hCG levels (>20 mIU/ml) without a gestational sac.

The positive predictive value (PPV), negative predictive value (NPV), and Youden's index of CP and LB were calculated for each serum β-hCG levels (5, 10, 20, 30, 40, 50, and 60 mIU/ml) in each age group to determine the cut-off value. The level of β-hCG on day 7 in patients who conceived with a BPL or EP was compared to that of patients who conceived a normal pregnancy. We also examined whether maternal age affected β-hCG levels in BPL or EP patients.

Statistical analysis was performed using SPSS statistics software version 20 (SAS Institute, USA), R software (The R Foundation) and 2-way Contingency Table Analysis [21]. The Mann–Whitney U-test, Kruskal-Wallis test for nonparametric data, and nominal variables were analyzed by the Cochran-Armitage test. A p value <0.05 was considered statistically significant.

Results

Pregnancy outcomes are outlined in Table 1. The association between CP rate (CPR)/LB rate (LBR) and serum β-hCG levels on day 7 after SVBT was evaluated according to maternal age (Table 2). A positive correlation was observed between pregnancy outcome and serum β-hCG levels. In all age groups, CPR and LBR increased with rising serum β-hCG levels (p<0.0001). No correlation was observed between maternal age and serum β-hCG levels. However, among females with β-hCG levels <50 mIU/ml, the CPR of Group C was significantly lower than that of Groups A and B (p<0.05). Although the CPR of Group C was comparable to that of other groups among females with β-hCG levels ≥50 mIU/ml, the LBR of Group C was significantly lower than that of Groups A and B among females with β-hCG levels ≥10 mIU/ml (p<0.05). These results indicate that CPR and LBR can be estimated using serum β-hCG levels and maternal age, especially with Groups A and B.

The PPV, NPV, and Youden's index of CP and LB are presented in Tables 3 and 4. For Groups A and B, which included all patients younger than 40 years, the highest Youden's Index was 30 mIU/ml for prediction of CP and LB. In Group C, which included all patients aged 40 years and older, the highest Youden's Index

Table 1 Pregnancy outcome following SVBT

		% (/transfer)
No. of transfers (cycle)	21,882	
Patient age at egg retrieval, years	37.4±3.9	
Patient age at embryo transfer, years	37.8±3.8	
Not pregnant (β-hCG MAX<20 mIU/ml)	10,425	47.6 %
Biochemical pregnancy loss	1,522	7.0 %
Ectopic pregnancy	52	0.2 %
Clinical pregnancy (GS+)	9,883	45.2 %
First trimester abortion	1,318	6.0 %
Ongoing pregnancy (FHB+)	8,566	39.1 %
Second trimester abortion	1,162	5.3 %
Artificial abortion	23	0.1 %
Still birth	39	0.2 %
Live birth	7,342	33.6 %

Patient age is presented as the Mean±standard deviation

SVBT single vitrified-warmed blastocyst transfer, *β-hCG* β-human chorionic gonadotropin, *GS* gestational sac, *FHB* fetal heart beat

Table 2 Clinical pregnancy and live birth rates stratified by β-hCG and maternal age

	Patients β-hCG range	β-hCG levels on day 7 after single vitrified-warmed blastocyst transfer (mIU/ml)										p[a]
		1.0–4.9	5.0–9.9	10.0–19.9	20.0–29.9	30.0–39.9	40.0–49.9	50.0–59.9	60.0–69.9	70.0–79.9	≥80	
CPR	Group A	1.8 % (5/271)	18.4 % (40/217)	41.8 % (155/371)	67.2 % (270/402)	83.7 % (340/406)	93.7 % (402/429)	97.4 % (455/467)	98.1 % (411/419)	98.4 % (305/310)	98.5 % (532/540)	$p<0.0001$
	Group B	2.4 % (15/622)	10.7 % (45/422)	34.3 % (232/676)	64.1 % (442/690)	84.9 % (567/668)	91.5 % (653/714)	95.9 % (662/690)	97.9 % (607/620)	97.8 % (491/502)	99.0 % (908/917)	$p<0.0001$
	Group C	1.2 % (8/663)	7.0 % (32/457)	26.5 % (150/567)	55.4 % (242/437)	76.5 % (326/426)	87.5 % (344/393)	94.0 % (359/382)	96.7 % (293/303)	94.6 % (212/224)	98.2 % (379/386)	$p<0.0001$
LBR	Group A	1.5 % (4/271)	10.6 % (23/217)	26.1 % (97/371)	50.0 % (201/402)	64.5 % (262/406)	79.3 % (340/429)	84.8 % (396/467)	87.1 % (365/419)	87.4 % (271/310)	93.7 % (506/540)	$p<0.0001$
	Group B	0.8 % (5/622)	3.6 % (15/422)	17.2 % (116/676)	39.0 % (269/690)	59.4 % (397/668)	66.8 % (477/714)	75.7 % (522/690)	82.6 % (512/620)	84.3 % (423/502)	87.9 % (806/917)	$p<0.0001$
	Group C	0.6 % (4/663)	1.5 % (7/457)	9.5 % (54/567)	20.8 % (91/437)	30.5 % (130/426)	47.6 % (187/393)	58.1 % (222/382)	66.3 % (201/303)	64.3 % (144/224)	76.2 % (294/386)	$p<0.0001$

Group A: 21–34 years old; Group B: 35–39 years old; Group C: 40–44 years old

B-hCG β-human chorionic gonadotropin, *CPR* clinical pregnancy rate, *LBR* live birth rate

[a] Cochran–Armitage test for trend

Table 3 Prediction of clinical pregnancy by β-hCG levels on day 7 after SVBT

	Patients group	Cut-off value						
		5.0mIU/ml	10mIU/ml	20mIU/ml	30mIU/ml	40mIU/ml	50mIU/ml	60mIU/ml
PPV	Group A	0.817	0.858	0.913	0.951	0.972	0.981	0.983
	Group B	0.781	0.833	0.902	0.946	0.965	0.978	0.984
	Group C	0.654	0.739	0.845	0.905	0.940	0.960	0.968
NPV	Group A	0.982	0.908	0.767	0.627	0.514	0.422	0.349
	Group B	0.976	0.943	0.830	0.695	0.577	0.485	0.416
	Group C	0.988	0.964	0.887	0.797	0.703	0.626	0.561
Youden's Index	Group A	0.288	0.468	0.650	0.701	0.657	0.548	0.405
	Group B	0.316	0.505	0.689	0.724	0.654	0.545	0.417
	Group C	0.343	0.553	0.710	0.710	0.623	0.503	0.362

Group A: 21–34 years old; Group B: 35–39 years old; Group C: 40–44 years old

B-hCG β-human chorionic gonadotropin, *SVBT* single vitrified-warmed blastocyst transfer, *PPV* positive predictive value, *NPV* negative predictive value

values were 20 and 30 mIU/ml for prediction of CP and 40 mIU/ml for prediction of LB.

Table 5 compares the β-hCG level on day 7 after SVBT among pregnancy outcomes. B-hCG levels were significantly lower for BPL and EP compared to CP ($p<0.05$). On the other hand, β-hCG level of LB was significantly higher than CP ($p<0.05$). By non-parametric analysis, LB and BPL occurrence were not significantly different among age groups; however, EP occurrence was significantly different among age groups ($p<0.05$). Therefore, β-hCG levels of EP patients were compared among age groups. We found that β-hCG levels were significantly lower in Group A compared to Group B and Group C ($p<0.05$). Moreover, the β-hCG levels of EP patient's ≥35 years old were significantly higher than those of EP patient's ≤34 years old ($p<0.05$).

Discussion

Here we have demonstrated that clinicians must consider not only β-hCG levels but also maternal age when discussing the probability of pregnancy and a live birth with patients. The results of our large retrospective study are comparable to those of a previous report [22]; however, our data included earlier measurement of β-hCG levels and used only SVBT outcomes. Therefore, our data will become increasingly important as many hospitals begin employing SVBT more often than other techniques. To our knowledge, this study is the first report employing SVBT-specific initial β-hCG levels and the first to demonstrate the relationship between these β-hCG levels and pregnancy outcome.

Previous studies included multiple embryo transfers and injected exogenous hCG for oocyte retrieval (12, 17).

Table 4 Prediction of live birth by β-hCG levels on day 7 after SVBT

	Patients group	Cut-off value						
		5.0mIU/ml	10mIU/ml	20mIU/ml	30mIU/ml	40mIU/ml	50mIU/ml	60mIU/ml
PPV	Group A	0.691	0.729	0.787	0.832	0.867	0.886	0.900
	Group B	0.600	0.643	0.709	0.763	0.796	0.829	0.854
	Group C	0.372	0.424	0.497	0.557	0.621	0.665	0.700
NPV	Group A	0.985	0.945	0.856	0.742	0.648	0.558	0.484
	Group B	0.992	0.981	0.921	0.832	0.739	0.663	0.598
	Group C	0.994	0.990	0.961	0.927	0.888	0.839	0.791
Youden's Index	Group A	0.194	0.326	0.487	0.553	0.552	0.479	0.370
	Group B	0.206	0.338	0.493	0.559	0.538	0.482	0.391
	Group C	0.224	0.374	0.510	0.561	0.565	0.496	0.385

Group A: 21–34 years old; Group B: 35–39 years old; Group C: 40–44 years old

B-hCG β-human chorionic gonadotropin, *SVBT* single vitrified-warmed blastocyst transfer, *PPV* positive predictive value, *NPV* negative predictive value

Table 5 Comparison of β-hCG levels among pregnancy outcomes on day 7 after SVBT

Pregnancy outcome (n)	Mean±SD (range)				*p* value (compared with CP)	**p* value
	All	Group A	Group B	Group C		
Live birth (7,342)	61.1±27.6 (1.3–210.2)	59.9±27.2 (3.2–186.6)	61.8±27.8 (2.7–198.0)	61.3±27.6 (1.3–210.2)	<0.0001	0.072
Biochemical pregnancy loss (1,522)	28.7±17.9 (2.1–84.6)	29.0±21.4 (2.1–84.6)	28.2±16.7 (7.4–83.5)	29.2±16.7 (2.1–72.8)	<0.0001	0.832
Ectopic pregnancy (52)	14.2±12.9 (1.6–55.5)	6.6±6.5 (1.6–23.1)[a]	14.2±11.9 (1.6–42.9)[b]	18.9±15.3 (1.9–55.5)[b]	<0.0001	0.012
Clinical pregnancy (9,883)	57.0±27.6 (1.1–156.0)	57.4±27.3 (3.0–186.6)[a]	57.9±28.0 (1.1–198.0)[a]	54.5±27.3 (1.3–210.2)[b]	–	<0.001

Data marked with superscript a and b in each row were significantly different from each other ($p<0.05$)

* Kruskal-Wallis test for nonparametric data

Group A: 21–34 years old; Group B: 35–39 years old; Group C: 40–44 years old

B-hCG β-human chorionic gonadotropin, *SD* standard deviation, *CP* clinical pregnancy

Therefore, it was unclear if the early hCG level resulting from only single implanted egg may also be associated with pregnancy outcome after embryo transfer.

In this study, we included only single vitrified-warmed blastocysts that were transferred on day 4.5 to 5 after ovulation during a spontaneous natural cycle or hormone replacement cycle. Therefore, we could accurately detect β-hCG levels from an implanted egg without the presence of exogenous hCG, and we were able to demonstrate that β-hCG levels on day 7 after SVBT can accurately predict pregnancy outcome.

We demonstrated a positive correlation between pregnancy outcome and serum β-hCG levels. Moreover, previous studies (12, 16) demonstrated a correlation between β-hCG levels after embryo transfer and pregnancy outcomes. However, these studies evaluated the interrelationship between β-hCG levels and successful pregnancy in cleavage stage embryo transfer or multiple embryo transfer cases; β-hCG levels after SVBT were not previously analyzed. In this study, we only analyzed data from females who conceived following SVBT.

We demonstrated that β-hCG levels on day 7 after SVBT accurately predict embryo implantation, developmental fate, and delivery. Furthermore, we demonstrated a negative correlation between pregnancy outcome and maternal age, similar to a previous study [19]. However, the previous study did not demonstrate a relationship between pregnancy outcome and β-hCG level in different age groups. Although there was no correlation between maternal age and serum β-hCG levels, pregnancy outcome significantly declined with increasing maternal age. It is recognized that embryonic chromosomal abnormalities increase with advancing maternal age [23] and that the most common cause of first trimester spontaneous abortion is embryonic chromosomal abnormality [24], suggesting that the decline in LBR in Group C was caused by maternal aging.

Additionally, we determined the predictive values for CP and LB. The Youden's Index for Groups A and B, which included all patients younger than 40 years of age, indicated that 30 mIU/ml was the cut-off value for predicting CP and LB. The Youden's Index for Group C, which included all patients' ≥40 years, indicated that 20 and 30 mIU/ml was the cut-off values for prediction of CP and 40 mIU/ml was the cut-off value for prediction of LB. These results indicate that cut-off values are dependent on maternal age.

Interestingly, accurate measurement of β-hCG levels only 7 days after SVBT was related to the final pregnancy outcome. In cases with low β-hCG, the implanted embryo may have a chromosomal abnormality causing developmental delay or abnormal proliferation of trophoblast cells. Alfarawati et al. reported that morphology and aneuploidy were linked at the blastocyst stage [25]; therefore, low blastocyst quality may result in low early levels of β-hCG. We also demonstrated that β-hCG levels were significantly lower in cases of BPL and EP compared to cases of CP. When β-hCG levels on day 7 after SVBT were low, this may indicate a potential EP, a result similar to a previous report [3]. Furthermore, we detected higher levels of β-hCG in EP patients ≥35 years old than in those ≤34 years old. The reason for this result has not yet been elucidated; however, these observations may appear that when estimating of EP, we needs to considering not only β-hCG level but also maternal age.

It should be noted that cases of EP were limited in this study. Therefore, the statistical significance of our EP results may have been limited. In addition, our data analysis was performed per cycle. Therefore, the results included multiple data points from the same patients. Moreover, these data do not include both fresh embryo transfers and multiple embryo transfers. Therefore, these data are difficult to apply to those methods.

In conclusion, we demonstrated that maternal age and initial β-hCG levels on day 7 after SVBT can predict pregnancy outcome and help determine the appropriate pregnancy follow-up. Therefore, when clinicians counsel couples on the day of the pregnancy test after SVBT, unrealistic expectations of older patients can be prevented and concerns amongst younger patients can be mitigated.

Conflict of interest The authors declare that they have no conflict of interest.

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