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Revisiting serum beta-human chorionic gonadotropin concentrations as a predictor for dizygotic twinning after in vitro fertilization

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Summary

Purpose of investigation: To determine a cut-off value for beta-human chorionic gonadotropin (β-hCG) concentrations to predict dizygotic twinning after in vitro fertilization (IVF) and double embryo transfer (DET). *Materials and Methods:* This retrospective cohort study included 233 women who conceived after DET at IVF center, Hacettepe University Faculty of Medicine. Patients with serum β-hCG concentration ≥ 25 IU/l assayed on day 14 after oocyte retrieval were included into the study. *Results:* Lower serum β-hCG concentrations were observed in non-viable pregnancy when compared to their viable counterparts. In addition, twins exhibited higher β-hCG concentrations than singletons did. Receiver operator characteristic (ROC) curve analysis showed a significant relationship between serum β-hCG concentrations and the occurrence of twin pregnancy (area under the curve = 0.85, 95% confidence interval = 0.79–0.91, $p < 0.001$). For twin pregnancy, when β-hCG ≥ 175 IU/l, sensitivity was 77.3%, specificity was 80.0%, positive predictive value (PPV) was 48.2%, and negative predictive value (NPV) was 93.8%. *Conclusion:* β-hCG ≥ 175 IU/l might be used as a new cut-off value for early prediction of viable dizygotic twins following IVF-DET treatment cycles.

Key words: Beta-human chorionic gonadotropin; In vitro fertilization; Double embryo transfer; Dizygotic twinning.

Introduction

Markers that have been sought to distinguish between viable and non-viable pregnancies before live intrauterine pregnancy can be verified by transvaginal ultrasonography. A single determination of serum human chorionic gonadotropin (hCG) concentration has been found to be predictive of pregnancy outcomes in several different studies [1-7]. These studies have postulated that hCG level is a reliable and highly predictive tool for various pregnancy outcomes. Few researchers have also investigated the predictive value of hCG for discriminating multiple pregnancies. However, they either used intact hCG instead of β-hCG or included single to four ET cycles in their analysis when interpreting the multiplicity [8, 9]. As worldwide single embryo transfer (SET) policy is now being seriously considered, transfer of three or more embryos has subsequently been restricted to double embryo transfer (DET) which should also be based on prognostic indicators of the patient. Therefore, the results of previous studies might not be suitable for the present authors' current practice with DET, since the measures of β-hCG validity (sensitivity, specificity, positive and negative predictive values) would change by the prevalence of twinning in non-DET cycles.

The present authors' primary objective was to use day-14 (after oocyte retrieval) β-hCG concentrations, to predict viable twin pregnancy following DET. Their secondary aim was to investigate in vitro fertilization (IVF) treatment characteristics in relation to dizygotic twinning.

Materials and Methods

The records of the subjects who conceived following assisted reproductive technology at the IVF unit of Hacettepe University Hospital, from January 2005 to July 2014, were analyzed for this study. A total of 236 ET cycles fulfilled the present inclusion criteria which were: (1) cycles arising from DET, (2) serum β-hCG concentration ≥ 25I U/l assayed on day 14 after oocyte retrieval, and (3) data regarding the outcome was available. Furthermore, triplet pregnancies with mono-chorionic component (n=2) and fetuses with major fetal anomalies that resulted in termination of pregnancy (n=1) were excluded from the study.

This study was exempt from institutional review board review because of its retrospective, non-interventional nature: no patients were contacted and no identifying patient information was used for purposes of this study. Patients underwent IVF according to standard stimulation protocols, which involved pituitary down-regulation with gonadotropin-releasing hormone (GnRH) agonist administered in the mid-luteal phase of the prior cycle (long protocol) or diluted GnRH agonist on days 2-4 of the cycle (micro-

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dose protocol). Alternatively, GnRH antagonist short protocols started on the fifth day of stimulation. Controlled ovarian stimulation was achieved with hCG and/or recombinant follicle-stimulating hormone. The response to stimulation was monitored with serum E2 and transvaginal ultrasound. HCG was administered to stimulate the final stages of follicular development when follicles reached maturity, defined by two to four leading follicles reaching > 18 mm. Transvaginal follicle aspiration was performed 36 hours after hCG administration.

Embryos were transferred to the uterus either three days (cleavage stage) or five days (blastocyst stage) after oocyte retrieval. Embryo quality was assessed on the same day as transfer. The cleavage stage embryos were scored based on cell number and degree of fragmentation according to grading system of Hardarson (2001) [10]. The embryos with appropriate developmental stage, < 20% fragments and mild degree of uneven-sized blastomeres (grade I, grades IIA and IIB, and grade IIAB) constituted the day-3 embryos suitable for transfer. In case of extended culture, all blastocyst stage embryos were evaluated using the grading system of Gardner and Schoolcraft (1994). The blastocysts were graded according to degree of expansion and quality of inner cell mass and trophoectoderm [11]. The total number of embryos suitable for ET (either at cleavage or blastocyst stage) was calculated for each patient.

On day 14 of oocyte retrieval, regardless of the day of ET, each patient had her β-hCG level assessed. The β-hCG levels were measured by chemiluminescence immunoassay technique on an autoanalyzer. The inter-assay coefficient of variation was determined as 4.5% which was obtained from the "measurement uncertainty" results of the present laboratory.

Pregnancy was defined by serially increasing serum ß-hCG titers to at least 25 IU/l within 14 days after oocyte retrieval. All of the patients underwent transvaginal ultrasound at five to six weeks' gestation or when β-hCG exceeded 2,000 IU/L, in order to determine the location and number of pregnancies. Biochemical pregnancy was defined as transient pregnancies that spontaneously resolved before sonographic confirmation. Observation of fetal cardiac activity was performed at six to seven weeks of gestation. The non-viable pregnancy included biochemical pregnancy, ectopic pregnancy, first-trimester abortions (an-embryonic pregnancy, missed abortion, and spontaneous abortion prior to 12 weeks of gestation in both singleton and twin pregnancies) and second trimester abortions (in both singleton and twin pregnancies). Viable pregnancy was defined as one resulting in delivery of at least one live fetus at ≥ 23 weeks of gestation.

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 22.0. After determining whether the variables met the normality and homoscedasticity assumptions, non-parametric analyses were performed. Kruskal–Wallis tests were conducted to explore the impact of pregnancy outcome on serum ß-hCG concentrations, patient, and IVF treatment characteristics, Mann-Whitney U tests were conducted to analyze continuous and discrete ordinal variables, and nominal data were analyzed with χ^2 tests. All Mann–Whitney U and χ^2 tests were two-tailed, and Bonferroni's correction was used to adjust for multiple comparisons unless otherwise stated. Bonferroni's correction involves dividing the alpha level of 0.05 by the number of tests that the researcher intends to perform and using the revised alpha level as the criterion for determining significance. In the present analyses, this process resulted in a stricter alpha level of 0.05/3 = 0.0166. Receiver operator characteristic (ROC) curve analysis was used to assess the predictive value of β-hCG and maternal age on occurrence of twin births. The percentage for area under the curve (AUC) and confidence intervals (CI 95%) were generated for the ROC curve. Discrimination threshold was chosen on the basis of optimal sensitivity and specificity. Diagnostic indices (sensitivity and specificity) and positive (PPV) and negative predictive values (NPV) were calculated. Multiple logistic regression analysis was used to investigate the relationship between occurrence of twin pregnancy and various explanatory variables. A p value < 0.05 was considered statistically significant.

Table 1. — *Pregnancy outcome in patients undergoing IVF-ICSI with an initial β-hCG value > 25 IU/l.*

Pregnancy outcome	n	%
Non-viable		
Pregnancy loss (n=89)		
Biochemical pregnancy	49	21.0
Spontaneous abortion (first trimester)	33	14.2
Ectopic pregnancy	3	1.3
Spontaneous abortion (second trimester)	4	1.7
Viable		
Singleton (n=100)		
Delivered singleton		
Singleton	96	41.2
Spontaneously reduced twin	4	1.7
Twin pregnancy (n=44)		
Delivered Twin	44	18.9
Total number of pregnancies	233	100

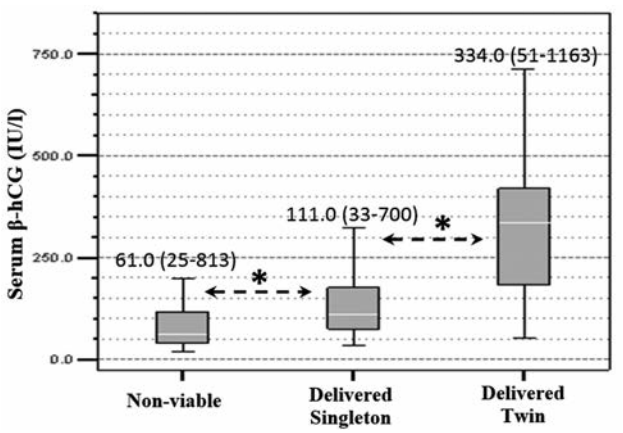

Figure 1. — The median (minimum-maximum) β-hCG concentrations for each pregnancy outcome are presented. *significant, $p < 0.001$.

Results

A total of 233 consecutive cycles (among 224 women) with DET were subject to analysis. The mean age at treatment was 33.8 (range 25–50) years. Viable pregnancies represented 61.8% and non-viable pregnancies 38.2% of all pregnancies, respectively. Table 1 shows the pregnancy outcomes of the studied patients.

A Kruskal–Wallis analysis was conducted to explore the impact of pregnancy outcome on serum β-hCG levels. There was a statistically significant difference in β-hCG

Table 2. — *Patient and IVF cycle characteristics between the pregnancy outcomes.*

		Non-viable pregnancies (n=89)	Singletons (n=100)	Twins (n=44)	*p*
Maternal age (years)		35.2 ± 5.9[a]	34.0 ± 5.7[a]	30.6 ± 5.2[b]	0.001
Duration of infertility (months)		81.9 ± 63.3	84.2 ± 70.2	75.9 ± 44.4	Ns
Previous unsuccessful attempts at IVF		25 (28.1%)	29 (29.0%)	13 (29.5%)	Ns
BMI (kg/m^2)		25.3 ± 3.7	25.3 ± 3.9	24.8 ± 3.7	Ns
Male factor		34 (38.2%)	49 (49.0%)	23 (52.3%)	Ns
Unexplained infertility		26 (29.2%)	29 (29.0%)	8 (18.2%)	Ns
Anovulation		14 (15.7%)	9 (9.0%)	10 (22.7%)	Ns
Tubal factor		11 (12.4%)	10 (10.0%)	2 (4.5%)	Ns
Poor ovarian reserve		14 (15.7%)	9 (9.0%)	10 (22.7%)	Ns
Duration of stimulation (days)		9.3 ± 1.9	9.7 ± 1.8	9.0 ± 1.2	Ns
Total gonadotropin dose (IU)		2974 ± 1425[a]	2716 ± 1215[ab]	2170 ± 720[b]	0.012
Number of retrieved oocytes		7.9 ± 5.5[a]	9.9 ± 6.5[ab]	13.0 ± 7.9[b]	0.001
Number of MII oocytes		6.6 ± 4.4[a]	8.3 ± 5.7[ab]	10.7 ± 6.5[b]	0.001
MII oocytes (%)		87.1 ± 12.7	84.5 ± 16.9	84.2 ± 14.4	Ns
Number of oocytes fertilized		5.4 ± 3.6[a]	6.4 ± 4.4[ab]	8.5 ± 5.8[b]	0.006
Oocytes fertilized (%)		82.6 ± 16.4	79.7 ± 18.6	78.3 ± 18.3	Ns
Number of embryos suitable for transfer		4.9 ± 3.5[a]	6.0 ± 4.3[ab]	8.0 ± 5.6[b]	0.002
Frozen-thawed cycles		22 (24.7)	14 (14.0)	6 (13.6)	Ns
Day of ET	Day 3	80 (89.9%)	84 (84.0%)	38 (86.4%)	Ns
	Day 5	9 (10.1%)	16 (16.0%)	6 (13.6%)	
Number of cells of the best embryo	Day 3	7.8 ± 1.8	8.1 ± 1.6	8.4 ± 1.3	Ns
Mean Morphology score per ET	Day 3	1.9 ± 0.3	1.9 ± 0.3	1.8 ± 0.3	Ns
	Day 5	2.5 ± 1.1	3.0 ± 0.6	2.7 ± 1.0	

IVF: in vitro fertilization; BMI: body mass index; MII: metaphase 2; ET: embryo transfer; Ns: non-significant.
Values across a row with different superscripts (a–b) indicate significant differences between pregnancy outcome categories ($p < 0.05$), and values across an individual row with matching superscripts (a–b) indicate no significant differences between pregnancy outcome categories.
Data are presented as mean± standard deviation or n (%).

concentrations between the groups: non-viable (pregnancy loss), singleton, and twin pregnancies ($p < 0.001$). A Mann–Whitney U test with Bonferroni's correction was used to adjust for multiple comparisons between these groups, and the results indicated that the median score for each group was significantly different from the other ($p < 0.001$ for each). Lower serum β-hCG concentrations were observed in pregnancies that resulted in pregnancy loss than the singletons. In addition, twin pregnancies had higher β-hCG concentrations than the singletons did (Figure 1).

In terms of the impact of pregnancy outcome on the patient and IVF characteristics, there were no significant differences in body mass index (BMI), duration of infertility, number of previous unsuccessful attempts at IVF and type of infertility between the pregnancy outcomes ($p > 0.05$) (Table 2). Similarly, proportion of MII oocytes, proportion of oocytes fertilized, frozen-thaw cycles, day of ET, highest number of cells, and mean morphology score per ET were found to be comparable between the groups ($p > 0.05$). However, there were statistically significant differences in maternal age ($p = 0.001$), total gonadotropin dose ($p = 0.012$), number of retrieved oocytes ($p = 0.001$), MII oocytes ($p = 0.001$), oocytes fertilized ($p = 0.006$), and embryos suitable for transfer ($p = 0.002$). Subsequently, a

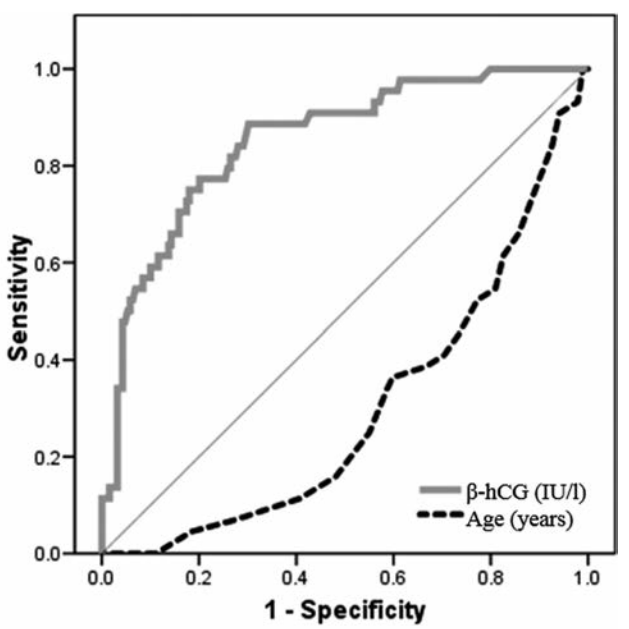

Figure 2. — ROC curves for serum β-hCG concentration and maternal age to predict twin pregnancy.

Table 3. — *Relationships between serum β-hCG and maternal age for occurrence of twin pregnancy analyzed by multivariate logistic regression.*

	OR	CI (95%)	*p*
Serum β-hCG ≥ 175 IU/l	13.33	5.92–30.02	< 0.001
Maternal age ≥ 35 years	0.28	0.12–0.64	0.003

OR: odds ratio; CI: confidence interval.

Mann–Whitney U test with Bonferroni's correction was used to adjust for multiple comparisons between groups (Table 2). It was found that the significance was maintained between non-viable and twin pregnancies ($p < 0.0017$ for each). The results also indicated that total gonadotropin dose, number of retrieved oocytes, MII oocytes, oocytes fertilized, and embryos suitable for transfer were comparable between singleton and twin groups ($p > 0.017$ for each). However, maternal age was found to be significantly lower in twin pregnancies than the singletons ($p = 0.001$).

ROC analysis showed a significant relationship between serum β-hCG (AUC = 0.85, CI 95% 0.79–0.91, $p < 0.001$), maternal age (AUC = 0.70, CI 95% 0.62–0.78, $p < 0.001$), and the occurrence of twin pregnancy (Figure 2). By using the ROC analysis, the optimal cutoff point for β-hCG was determined, and sensitivity, specificity, PPV, and NPV were calculated. For twins, when β-hCG ≥ 175 IU/l, sensitivity was 77.3%, specificity was 80.0%, PPV was 48.2%, and NPV was 93.8%.

A multivariate analysis by logistic regression was performed that included β-hCG and maternal age. As shown in Table 3, the results indicated that serum β-hCG ≥ 175 IU/l ($\beta = 13.33$, $p < 0.001$), and maternal age ≥ 35 ($\beta = 0.28$, $p = 0.003$) were still significant contributing factors to occurrence of viable twin pregnancy.

Discussion

The maternal characteristics and IVF cycle parameters were found to be comparable, apart from the maternal age, between the singleton and twin pregnancies. The authors found that the occurrence of twin pregnancy decreases by 3.6-fold in women ≥ 35 years. Thus, the woman's age affects occurrence of twin pregnancy, even when DET is specified. Similar to the present findings, it was shown that the female age was negatively correlated with the occurrence of multiple pregnancy [12-16]. Few studies have also reported that developmental stage and morphology score might be predictors of multiple pregnancy [14, 17]. Although number of retrieved oocytes / MII oocytes / fertilized oocytes/ embryos suitable for transfer seems to be higher and developmental stage / morphological scores superior in twin pregnancies when compared with singletons, the differences did not reach statistical significance. In fact, the strict embryo selection criteria that the present authors used, might lead to transfer of only good quality embryos with appropriate stage of development. A larger sample size, however, might allow increasing the significance level of these findings, since the confidence of these results is likely to increase.

The present results indicated that there was an increasing β-hCG trend from non-viable toward singleton, and twin pregnancies. Similarly, in the series of Poikkeus *et al.* (included single to three ET cycles), intact hCG concentrations were about four-fold higher in viable pregnancies than in non-viable ones [8]. In another study, Bjercke *et al.* evaluated 417 IVF pregnancies and concluded that when intact hCG value was > 55 IU/l, the chance of having a vital pregnancy on day 12 after IVF-ET was 90% [1]. In the present study, however, the AUC values of β-hCG obtained from ROC curve analyses (data not shown) were found to be much lower for discriminating viable than twin pregnancies (0.77 *vs*. 0.85). It was likely due to a more prominent overlap in β-hCG ranges between non-viable and singleton pregnancies. Therefore, β-hCG might be a more efficient marker for discriminating twin than viable pregnancies after IVF-DET treatment cycles. In addition, Kathiresan *et al*. performed a study in order to assess the degree to which maternal characteristics and cycle parameters were predictive of higher β-hCG levels. They measured serum β-hCG concentrations on day-15 after oocyte fertilization and proposed that β-hCG concentration > 250 IU/l in cycles involving day-3 ET might be suggestive of multiple pregnancy [9]. However, the probability of predicting multiple pregnancy with this β-hCG value was found to be low (18%). On the other hand, it was observed in the present study that when β-hCG ≥ 175, sensitivity was 77.3, specificity was 80.0%, PPV was 48.2%, and NPV was 93.8% for multiple pregnancies. Thus, the present authors found a higher PPV than they had reported. In cases with β-hCG < 175 IU/l, the probability of twin pregnancy was found to be mostly excluded. On the other hand, one of two women has twin pregnancy with β-hCG ≥ 175 IU/l. Similarly, Urbancsek *et al.* evaluated 120 IVF pregnancies in which a maximum of four embryos per cycle were replaced two or three days after oocyte aspiration, and when they sought a cutoff value for multiple gestation, they found that a β-hCG value of 135 IU/l on day 11 after ET had a sensitivity of 80%, specificity of 88%, PPV of 80%, and NPV of 88% [7]. Nevertheless, the previous studies included single to four ET cycles in their analyses when interpreting the multiple pregnancy. The optimal β-hCG cutoff points that were reported in these studies might not be accurate for the present authors' current practice with IVF-DET, as the prevalence of twinning was different in this population. Recently, Shapiro *et al.* conducted a study including 767 IVF cycles each with transfer of two blastocysts [18]. They measured serum β-hCG concentration five days after the blastocyst transfer, which was earlier than in other studies, and they found that multiple pregnancy was predicted by day-5 β-

hCG ≥ 5 IU/l with a sensitivity of 97%, specificity of 36%, PPV of 39%, and NPV of 97%. Finally, it is evident that the current literature lacks homogeneity when dealing with the markers measured (intact hCG *vs*. β-hCG), time of measurement, or characteristics of the populations studied.

The limitations of this study are mainly due to its retrospective nature, which prevented the authors from controlling for potential bias. Thus, further prospective studies investigating β-hCG concentrations in a subgroup of patients with only DET are still warranted. Moreover, each institution should analyze their own data to determine β-hCG cutoffs based on their own experience, as β-hCG concentrations might vary depending on the type of assay used.

Conclusion

β-hCG might be a useful marker for discriminating twin pregnancies following IVF-DET treatment cycles. The present data also demonstrates that younger the women's age is associated with higher the probability of achieving a twin pregnancy. As the individualized transfer policy is now being seriously considered, the present data might provide clues for dizygotic twinning when IVF-DET is still indicated.

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