Analysis of Implantation in Assisted Reproduction Through the Use of Serial Human Chorionic Gonadotropin Measurements

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Purpose: Our purpose was to examine implantation of singleton pregnancies achieved following various assisted reproductive technologies (ARTs) through the appearance and rising titers of serum human chorionic gonadotropin (hCG) levels.

Methods: A total of 114 singleton pregnancies resulting from in vitro fertilization and intrauterine insemination was analyzed. Patients were divided into five groups according to the type of ovarian stimulation protocol [gonadotropin stimulation with/without the use of gonadotropin-releasing hormone agonist (GnRHa), long protocol, or flare-up technique] and to the day of embryo transfer (day 2 or day 3 after oocyte retrieval). Serial serum hCG levels were measured between 10 and 25 days after fertilization and log-transformed. Linear regression analyses were performed and extrapolated to $\text{hCG} = 10 \text{ mIU/ml}$ (hCG_{10}), which was used as an estimate of detectable implantation. The slopes of the regression lines were used to estimate the rising speed of hCG.

Results: There were no significant differences in the days of hCG in maternal serum to reach 10 mIU/ml (implantation) or in the slopes of the regression lines for all five studied groups.

Conclusions: The appearance of hCG in maternal serum was used to assess the time of clinically detectable implantation. Furthermore, because hCG production is a marker of trophoblastic activity, its serum doubling time was used as an indicator of embryo quality. Results showed that in various ART protocols with and without GnRHa, there were no significant differences in implantation time or embryo quality. Embryo development in early pregnancy follows a

preprogrammed-timing schedule and depends mainly on the embryonic age of the healthy, successfully implanted conceptus.

KEY WORDS: assisted reproduction; embryo quality; human chorionic gonadotropin; implantation.

INTRODUCTION

The serial measurement of human chorionic gonadotropin (hCG) in maternal blood is an important tool for the prediction of pregnancy outcome in assisted reproductive technologies (ARTs) (1,2). In in vitro fertilization (IVF) therapy, the exact age of each embryo can be calculated following fertilization, and the subsequent appearance and rising of hCG concentration in the maternal plasma can be evaluated. Do the appearance and rising speed of hCG follow a similar pattern after various ART treatments [i.e., IVF with uterine transfer, tubal embryo transfer, gamete intrafallopian transfer, intrauterine insemination (IUI)] and after the use of different ovarian stimulation protocols?

According to some reports (3–6), some ART treatments may affect serum hCG patterns. The use of gonadotropin-releasing hormone agonists (GnRHa) in IVF cycles has been associated with delayed appearance of serum hCG (3–5). GnRHa are widely used in ovarian stimulation protocols combined with gonadotropins. The use of GnRHa has resulted in increased pregnancy rates, reduced cancellation rates, and enhancement of the number of mature oocytes retrieved (7–9). Although these are beneficial effects, some concerns have been brought up related to potential GnRHa effects on the ovary (10,11) and endometrium (3,12). It has been reported previously that the use of GnRHa combined with gonadotropins might delay hCG appearance by 1.3–2 days as a shift to the right of the rising curve in IVF (3–5). A different implantation time between IVF and IUI was also

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reported (6) in cycles where gonadotropins were used without GnRHa. Additionally, assisted hatching has been shown to facilitate earlier embryo implantation (13). Some of these studies, however, are confounded by the fact that various therapies, types of ovarian stimulation protocols, criteria used to establish the time between ovulation/fertilization and appearance of hCG, and analysis of singleton and multiple pregnancies have been considered (1).

The present study was designed to examine the appearance and increase of serum β -hCG titers following different ovulation stimulation protocols and ART procedures. We wanted to answer the following questions: (a) Does the use of GnRHa in IVF result in a delay in implantation as inferred from the analysis of serum hCG titers? To answer this question, we compared IVF patients who were stimulated with gonadotropins with/without GnRHa. (b) Is there a difference in implantation time when uterine transfer was performed 2 or 3 days following fertilization in IVF cycles using GnRHa? (c) Is there a difference in implantation time if GnRHa was used in long versus short protocols? For this purpose, we studied IVF cycles where gonadotropin stimulation was associated with GnRHa adjunctive therapy performed in a luteal suppression (long protocol) versus a follicular flare-up (short protocol) fashion. (d) Is there a difference in implantation time comparing IVF versus IUI cycles (in vitro versus in vivo fertilization) in cases where gonadotropin stimulation was performed without GnRHa?

We retrospectively studied 114 ART cycles that were selected from our computerized data based on the establishment and delivery of a singleton pregnancy and which had a minimum of two determinations of serum hCG levels between 10 and 25 days after fertilization. The levels of β -hCG were measured from 10 to 25 days after fertilization and log-transformed; linear regression analyses of the log values of hCG versus the days after fertilization were performed and compared for the different groups.

MATERIALS AND METHODS

Patient Selection

A total of 114 patients who achieved a normal singleton pregnancy resulting in a live birth at The Jones Institute for Reproductive Medicine and who were treated from August 1988 through January 1997 (Norfolk series 32 to 66) were selected from the computerized database. To exclude known factors that might

affect pregnancy outcome and hCG curves, the following inclusion criteria were used: (a) pregnancies were singleton, with a single intrauterine gestational sac as detected by ultrasound at 6–8 weeks of gestation (4,5); (b) day 3 follicle-stimulating hormone (FSH) levels were within normal limits (14) [<15 mIU/ml; radioimmunoassay (RIA) kits (Leeco Diagnostics, Southfield, MI)]; (c) 10,000 IU hCG (Steris Labs, Phoenix, AZ) was administered for triggering ovulation in all cases; (d) all patients received 50 mg/day of progesterone in oil intramuscularly for luteal support and no extra hCG supplementation was given; (e) no micromanipulation of the oocytes or embryos was performed (13); (f) all patients had a normal uterine evaluation based on hysterosalpingogram and endometrial biopsy; and (g) in addition, all patients had a minimum of two determinations of serum hCG levels between day 10 and day 25 following fertilization (day of fertilization was estimated to be the day of oocyte retrieval and insemination in IVF and the day of insemination in IUI).

Five groups of patients who underwent gonadotropin stimulation were established according to the different ART protocols (Table I): group N (no GnRHa), IVF without GnRHa ($n = 18$ patients); group L2 (long GnRHa protocol, embryo transfer on day 2), IVF with GnRHa luteal suppression or long protocol ($n = 39$ patients); and group L3 (long GnRHa protocol, embryo transfer on day 3), IVF with GnRHa long protocol with embryo transfer on day 3 after oocyte retrieval ($n = 18$ patients). All other groups had embryo transfer on day 2 following oocyte retrieval: Group F (flare-up GnRHa regimen), IVF with GnRHa in a flare-up protocol ($n = 20$ patients); and group IUI, IUI without GnRHa ($n = 19$ patients). The ART protocols have obviously varied with the increasing knowledge and improvement of techniques used to alleviate human infertility in the Norfolk program during the study period. This made it difficult to control for all conditions to which patients were exposed to in the different protocols. For example, the non-GnRHa group was selected from August 1988 through March 1991; also, day-3 embryo transfers were selected from March 1993 through January 1997. These were unavoidable biases in sampling due to the retrospective nature of the study.

ART Protocols

Gonadotropin stimulation was accomplished using FSH (Metrodin; Serono Laboratories, Randolph, MA) alone or in combination with human menopausal gonadotropin (hMG; Pergonal; Serono Laboratories) starting on day 3 of the cycle (except in the flare-up

Table 1. Characteristics of the Cycles of the Five Groups of ART Patients Examined^a

Group	n	Age	D3F	Ovulation stimulation	GnRHa	Procedure	ET	hCG data
N	18	34.8 ± 3.3	7.2 ± 1.8	FSH/hMG	—	IVF	2	61
L2	39	33.4 ± 3.8	7.0 ± 2.3	FSH/hMG	+(long)	IVF	2	110
L3	18	33.2 ± 3.7	7.0 ± 1.6	FSH/hMG	+(long)	IVF	3	51
F	20	34.2 ± 3.5	8.0 ± 2.4	FSH/hMG	+(flare)	IVF	2	61
IUI	19	33.1 ± 3.6	7.7 ± 4.2	FSH/hMG	—	IUI	—	38

^a Age: mean ± SD. n: patient number in each group. D3F: day 3 FSH, mean ± SD. GnRHa: —, no-GnRHa used; +, GnRHa group. ET days: days of embryo transfer after oocyte retrieval. hCG data: number of data points between 10 and 25 days after fertilization.

protocol, when they were begun on day 5) and then adjusted in an individualized fashion and continued until the day of hCG administration.

GnRHa Long Protocol (15). GnRHa [leuprolide acetate (LA); Lupron; TAP Pharmaceuticals, North Chicago, IL] was begun in the midluteal phase of the preceding cycle at the dose of 1 mg/day subcutaneously, dropped to 0.5 mg/day at the start of menses, and continued until the day of hCG administration.

GnRHa Flare-up Protocol (16). Patients were stimulated with 1 mg of LA subcutaneously on days 2–4, and on day 5 the dose of LA was decreased to 0.5 mg and continued through the day of hCG administration.

hCG Administration and Oocyte Retrieval (16). hCG (10,000 IU) was administered intramuscularly when two or more follicles were 16 mm or greater in diameter. Oocyte retrieval was performed transvaginally under ultrasound control 36 hr after administration of hCG in the IVF groups. Embryo transfer was carried out approximately 48 hr (in groups N, L2, and F) or 72 hr (in group L3) after oocyte retrieval. All embryos transferred derived from oocytes diagnosed as mature, preovulatory in metaphase II stage at the time of follicular aspiration. Luteal support was achieved by giving progesterone as described above.

IUI Protocol. IUI patients received FSH, hMG, or FSH+hMG for ovarian stimulation, and no GnRHa was used. Ten thousand IU of hCG was administered 36 hr before IUI (a single insemination performed with washed sperm). Luteal support was similar to all other groups.

hCG Assay

Quantitative measurement of β -hCG was performed with the IMx Total β -hCG assay [a microparticle enzyme immunoassay (MEIA); Abbott Laboratories, Abbott Park, IL] after November 1992. The interassay and intraassay coefficients of variation have remained 4.6 and 6.4%, respectively, throughout the study

period. The sensitivity of the IMx Total β -hCG assay was calculated to be 2 mIU/ml or better. The specificity of the IMx Total β -hCG assay was determined by studying the interference of bilirubin, hemoglobin, and triglyceride results, showing no effect on the determination of IMx Total β -hCG. There was no detectable cross-reactivity for IMx Total β -hCG with 1000 mIU/ml luteinizing hormone (LH), 1000 mIU/ml FSH, or 2000 mIU/ml thyroid-stimulating hormone (TSH). The Becton Dickinson hCG Mab Solid Phase Component System (BD RIA) was used before November 1992. The interassay and intraassay coefficients of variation were 4.9 and 6.1%, respectively. The sensitivity of the assay was approximately 0.5 mIU/ml. The specificity was tested by cross-reaction with 200 mIU/ml LH, 500 mIU/ml FSH, and 500 mIU/ml TSH, with BD RIA showing levels of 1.7, 1.4, and <1 mIU/ml, respectively. There were very few IVF patients treated without GnRHa after 1992, and also a clinical study using the flare-up technique was performed in our institution before that time (16). Therefore, patients from groups N and F were analyzed with the BD RIA assay. Our program performed an analysis of the correlation between the previously used BD RIA and the currently used MEIA in 51 randomly selected patients, showing an excellent correlation ($r^2 = 0.9935$; regression equation, MEIA β -hCG = $0.90 \times$ RIA -7.3). Data generated before 1992 were transformed using conversion charts so as to make results comparable.

Statistical Analysis

A simple linear regression model can provide a best estimate for log-transformed β -hCG levels at a particular gestational age (17). In this study, hCG values were measured between 10 and 25 days after oocyte retrieval or IUI (estimated to be the day of fertilization). Within this range, the log hCG–time relationship can be treated as log-linear (18). To exclude the effect of

exogenous hCG administration (17,19), only days ≥ 10 after fertilization were included. Two reference points were set to analyze the increasing level of hCG in very early pregnancy: (a) fertilization day (D_0 ; day = 0) was defined as the day of oocyte retrieval and insemination in IVF or the day of IUI; (b) hCG_{10} , the day of $hCG = 10$ mIU/ml postconception, was determined by the extrapolation of the regression line of $\log$ -hCG versus time. hCG_{10} was used to estimate the day of clinical detectable implantation (2,3,6). The slope (S) of the regression line is an estimate of the rising speed of hCG. The regression analysis was performed taking into account hCG data as repeated measures.

A power analysis was performed based on the total number of subjects evaluated ($n = 114$) and the number of hCG measurements obtained between day 10 and day 25 ($n = 321$). The study had an overall 88% power ($\alpha = 0.05$, $\beta = 0.12$) to detect significant differences between the compared groups with respect to the day at which the $\log$ of hCG equaled 1 and the slopes of the regression lines.

RESULTS

There was a total of 114 subjects with hCG values obtained from as early as day 10 to as late as day 25. Table II presents the estimated day the $\log$ of hCG equaled 1 (hCG_{10}) and the slopes and intercepts of the regression lines. The day of the $\log$ of hCG equaled 1 and the slopes of the regression lines for all five groups compared were not significantly different. The day the $\log$ of hCG equaled 1 was virtually identical for all groups except for group N. Figures 1 and 2 were made for these two sets of comparisons that had the largest differences, i.e., groups N and L2 and groups N and F. For N and L2 (shown in Fig. 1) and N and F (shown in Fig. 2), the 95% confidence intervals

of regressions for the groups overlapped at the day the $\log$ of hCG equaled 1 (hCG_{10}).

To answer our first question, we compared groups N and L2 or groups N and F (IVF groups, non-GnRHa and GnRHa treated, embryo transfer on day 2). The implantation time, which was estimated by the day the $\log$ of hCG equaled 1, was not significantly different in the GnRHa groups with long protocol (group L2) or flare-up protocol (group F) compared with the non-GnRHa group (group N). The largest differences in these comparisons occurred in the estimated slopes of group N (slope = 0.1826) and groups F (slope = 0.2008) and L2 (slope = 0.1999). The slopes of F and L2 were approximately 1.1 times greater than the slope of N. As mentioned above, the study had about 88% power to detect a difference in slopes about 0.03 unit or a slope that was about 1.2 times smaller (or larger) than the slopes of F and L2. Thus the conclusion that there was no difference between the groups with respect to hCG_{10} , which was based on the regression equations, is not unreasonable given the small difference in slopes that the study had the power to detect.

To answer the second and third questions, we compared groups L2 and L3 (IVF groups, GnRHa treated, embryo transfer day 2 or 3) and groups L2 and F (IVF groups, GnRHa treated, luteal suppression or follicular flare-up fashion, embryo transfer day 2). The regression lines for both hCG_{10} and slopes were very similar for the analysis of groups F, L2, and L3 (and also the IUI group). The implantation day, estimated by the hCG regression lines, was the same in the long protocol, the flare-up protocol, and day 2 or day 3 embryo transfer groups (and also in the IUI group). Furthermore, the rising of hCG levels, which was estimated by the slope of the regression lines, was statistically the same in all groups.

The same result was observed when answering the fourth question. The comparison of groups N and IUI (IVF and IUI group, where gonadotropin stimulation was performed without GnRHa) showed an overlap of the 95% confidence intervals in hCG_{10} and that the slopes of the two regression lines were not significantly different.

DISCUSSION

The concentration of hCG level in the maternal circulation during a normal pregnancy has two phases in the very early stages of conception: first, hCG increases rapidly within 48 hr, and second, it increases exponentially (20). The rate of the rise of hCG is

Table II. Days from Fertilization to Detectable Implantation (hCG_{10}) and Regression Coefficients for hCG Measurements in the Various Groups of Patients Assessed

Group	hCG_{10}	Regression coefficient	
		Slope	Intercept
F	9.60	0.2008	-0.9272
IUI	9.62	0.1951	-0.8775
L2	9.52	0.1999	-0.9028
L3	9.31	0.1849	-0.7215
N	8.54	0.1826	-0.5590

Fig. 1. Regression lines ($\pm 95\%$ confidence intervals) for serum hCG data comparing groups N (no GnRHa) versus L2 (GnRHa, long protocol), both groups having received embryo transfer on day 2.

Fig. 2. Regression lines ($\pm 95\%$ confidence intervals) for serum hCG data comparing groups N and F (GnRHa, flare-up), both groups having received embryo transfer on day 2.

not constant but decreases gradually with advancing pregnancy (17). To analyze the hCG levels in the maternal circulation, the gestational age should be considered. After detectable levels are reached (5–10 IU/ml), hCG levels increase exponentially during the next 6 weeks of gestation and reach a plateau at about 9 weeks' gestation (17). The log hCG–time relationship is linear between 10 and 30 days (18). Therefore, the regression analysis of hCG offers a good approach for a global analysis of embryonic age between 10 and 25 days postconception (17,18).

In early pregnancy, there is a correlation between trophoblastic cell number and β -hCG levels in maternal plasma (21). Furthermore, the doubling time of embryonic hCG can be used as an indicator of embryo quality (22). By the formula of doubling time (DT) $DT = \log 2 / \text{slope}$ (17), the slope of the regression line can be used as an estimate of embryo quality (22). The correlation between the levels of hCG in the maternal circulation and embryo quality should be accepted during the linear phase of the exponential increase (10–30 days postconception) (18). After this period, hCG levels in maternal circulation show reduced increasing rates and reach a peak between 8 and 10 weeks (23). hCG is secreted by the syncytiotrophoblast in the blastocyst stage (23). In vitro, hCG secretion might increase 50- to 150-fold within 48 hr after hatching and attachment (24). For this reason, the appearance of hCG in maternal circulation can be used as an indicator of implantation in the clinical setting.

Our studies demonstrated that there were not significant differences in the clinically detectable implantation day for all groups examined. These results differ from those of previous studies (3–5). Our study included only singleton pregnancies which were segregated based on defined and different ovarian stimulation protocols. Importantly, we assessed serum hCG levels during days 10–25 following fertilization, the period where the log hCG–time relationship can be treated as log linear (18). These facts may explain the differences observed with other reports. As for the embryo quality, which was estimated by the slope of the regression lines in log hCG, results were also almost the same in all groups. This may come as no surprise since, by design, all embryos implanted successfully and developed into a term pregnancy. Comparison of the regression analysis of log hCG in GnRHa with long or flare-up protocols, without GnRHa, IVF with day 2 and day 3 embryo transfer, and IUI treatment showed that all singleton pregnancies had statistically similar hCG curves. Based on analysis of our data, we propose that the clinically

detectable implantation day (hCG = 10 mIU/ml) ranged between 9.3 and 0.9 days postconception, with a 95% confident interval. The major difference in the detectable implantation day between GnRHa-treated (group F; L2, L3, and hCG₁₀ = 9.6, 9.5 and 9.3, respectively) and non-GnRHa-treated (group N; hCG₁₀ = 8.5) IVF groups was not found to be significantly different in this study. The discrepancy between our studies and those published elsewhere (4,5) should be discussed carefully.

Within the five groups studied, IUI, L2, L3, and F had almost-identical regression lines. The biggest difference in log hCG curves was found in the non-GnRHa IVF group (group N). There was a 0.8- to 1.1-day delay in hCG₁₀ and the slopes of the regression lines were also slightly higher in the GnRHa-treated IVF groups. This “delayed” appearance of hCG in maternal serum was found to be accompanied by an increased hCG secretion rate (higher slope of regression line) in our studies and in previous communications (4–6). However, as demonstrated above, these results were not significantly different from those of the other groups according to our data.

Questions that arise are (a) Can hCG levels in maternal serum be used as a biomarker of embryo development during the preimplantation to periimplantation periods? (b) Is there any reason, if actually present, for a GnRHa-dependent delayed appearance of maternal serum hCG? and (c) Which are the major factors that control embryo growth?

In answer to question a, recent studies showed that the onset of hCG production appears to be time dependent and that the embryonic age is the predominant factor controlling the growth of the conceptus (25). In vitro, the secretion of hCG was found to be similar in hatched blastocysts and intrazonal blastocysts (25,26). Although there was a dramatic increase in hCG secretion after implantation, the relationship between hatching and hCG secretion was still questionable (25,27). Furthermore, an embryo that appears to develop normally to the expanded blastocyst stage in culture does not necessarily acquire the competence to secrete significant amounts of hCG (25). In vivo, maternal serum hCG is not an effective biological marker in the pre- and periimplantation periods (28), because the hCG levels in the maternal blood cannot be detected before embryo–maternal circulation is established. Therefore, serum hCG levels should not be used as an indicator of embryo development in the preimplantation period (28).

In answer to question b, although the day of the appearance of detectable hCG levels (hCG = 10 mIU/

ml) in the maternal serum was not significantly different in this study, since other studies reviewed showed a delayed appearance of maternal serum hCG in GnRHa-treated cycles (3–6). The impact of GnRHa in implantation has been a major concern in some studies (3–5) and is discussed below. In humans, receptors for GnRH have been described in placental tissue (29). The presence of GnRH receptor mRNA in the human ovary has been reported (11). GnRH may function as an autocrine/paracrine factor in the ovary (10,11,30). GnRH is also produced in the placenta (31), where it is thought to exert a paracrine/autocrine action through specific receptors located on the syncytial cell membrane (32). GnRH appeared to exert a receptor-dependent stimulatory effect on pulsatile hCG secretion in a superfusion model in the first-trimester placenta (33). Also, GnRH (antagonists) reduced spontaneous hCG pulsatility by blocking endogenous GnRH action. All these studies strongly suggest that GnRH may have an autocrine/paracrine impact on hCG secretion. Thus, a delayed appearance of hCG in GnRHa-treated IVF cycles is theoretically possible. Since maternal serum hCG levels should not be used as a biomarker of embryo development during the pre- to periimplantation periods, the delayed appearance of hCG, if actually present, could not be explained by a delay of embryo growth during the pre- to periimplantation period. Nevertheless, in our studies, the regression of log hCG analysis was not significantly different in patients receiving GnRHa therapy or not.

In answer to question c, embryo development between the preimplantation periods to early postimplantation pregnancy is poorly understood. In vivo, the human embryo can be surveyed only by maternal blood markers and ultrasound examination. Implantation is dependent on embryonic age and independent of endometrial maturation during the implantation window (34). There were no significant differences in implantation as judged by early transvaginal ultrasonographic studies between IVF and IUI (35). In vitro, the human embryo culture system is restricted by survival days (up to 14 days after fertilization in recent studies) (27). Blastocysts form at a definite time and irrespective of cell numbers in the embryo (23). The cleavage rate and morphological grade do not appear to be good indicators of embryo quality to predict subsequent development (27). Preimplantation differentiation is closely timed by an internal embryonic clock, independent of the number of cleavage divisions (23). Because embryos develop according to their preprogrammed time schedule before implantation in vitro, it is reasonable that the development of the normal embryo might

follow the same schedule after implantation. Our studies show that for the same preprogrammed time-dependent schedule, all healthy and successfully implanted concepti will exhibit the same development pattern. An interesting finding (albeit not statistically significant) was the delayed appearance of hCG in the maternal serum usually accompanied by a higher slope, i.e., increased detectable hCG. This delayed appearance and compensatory increase in hCG in the maternal serum, if proved, would provide evidence of embryo growth dependent on embryonic age and independent of other external conditions. The use of GnRHa in ovulation induction or artificial reproductive procedures may disturb the hCG secretion or the trophoblastic invasion in the embryo temporarily. At some given point in time, hCG levels in the maternal serum may increase in a compensatory fashion to reflect embryo age.

We suggest that embryo development in early pregnancy follows a programmed-timing schedule and depends mainly on the embryonic age of the healthy, successfully implanted conceptus. A delayed appearance of maternal serum hCG might not mean delayed implantation of an embryo. Our data demonstrate that implantation timing is similar in various ART protocols involving different ovarian stimulation regimens and embryo transfer timing. It appears that neither the use of GnRHa nor variations in the timing of embryo transfer affect embryonic implantation if the embryo is normal. This embryonic age-dependent preprogrammed schedule is the main control mechanism that governs embryo development from the preimplantation to the postimplantation period.

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