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The association of maternal factors with delayed implantation and the initial rise of urinary human chorionic gonadotrophin

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BACKGROUND: Late implantation and the pattern of early rise in hCG have been associated with early pregnancy loss. We explored factors that might be predictive of these markers of poor embryonic health in spontaneously conceived pregnancies.

METHODS: Participants in the North Carolina Early Pregnancy Study collected daily first-morning urine specimens while attempting to conceive. Samples were assayed for estrogen and progesterone metabolites (to identify day of ovulation) and hCG (to detect conception). Data were available for 190 pregnancies, 48 of which ended in early loss (within 6 weeks of the last menstrual period). We used logistic regression to identify characteristics associated with late implantation (≥10 days post-ovulation). For pregnancies surviving at least 6 weeks ($n = 142$), we used linear mixed models to identify factors associated with variations in hCG rise in the first 7 days from detection.

RESULTS: Later implantation was associated with current maternal smoking [odds ratio (OR): 5.7; 95% confidence interval (CI): 1.1–30] and with oocytes that were likely to have been fertilized late in their post-ovulatory lifespan (OR: 5.1; CI: 1.9–16). Older women had a faster rise in hCG ($P = 0.01$), as did women who had relatively late menarche (P for trend = 0.02). Women exposed *in utero* to diethylstilbestrol showed an unusual pattern of slow initial hCG rise followed by a fast increase, a pattern significantly different from that of unexposed women ($P = 0.002$).

CONCLUSIONS: Although limited by small numbers and infrequent exposures, our analyses suggest that a woman's exposures both early in life and at the time of pregnancy may influence early development of the conceptus.

Key words: smoking / DES / age at menarche / oocyte quality / early pregnancy loss

Introduction

hCG is secreted by trophoblast cells of the early conceptus and the developing placenta, and performs vital functions in early pregnancy. These include maintenance of the corpus luteum (Zeleznik and Pohl, 2006) and formation of the placental syncytium (Yang *et al.*, 2003). The appearance of hCG in maternal urine can be used as a marker of embryonic implantation (Wilcox *et al.*, 1999). Delayed implantation has been associated with early pregnancy loss (Wilcox *et al.*, 1999). Lower hCG levels and slower rates of hCG rise early in pregnancy are seen in ectopic pregnancies (Kadar *et al.*, 1981; Check *et al.*, 1992; Seeber *et al.*, 2006) and have been associated with pregnancy loss (Check *et al.*, 1992; Bjercke *et al.*, 1999; Urbancsek *et al.*, 2002; Baird *et al.*, 2003; Alahakoon *et al.*, 2004; Stone *et al.*, 2006; Shamonki *et al.*, 2009) (reviewed in Chung and Allen, 2008). Most of these studies have been based on clinical populations undergoing fertility treatment, which may not be representative of spontaneously conceived pregnancies. Given that timing of implantation and early pregnancy hCG rise may be markers of pregnancy health, we explored maternal and pregnancy characteristics that may influence these markers. We studied a cohort of women with spontaneously conceived pregnancies under observation from before the time of conception.

Materials and Methods

Study population and design

The North Carolina Early Pregnancy Study (NCEPS) (1982–1986) enrolled 221 women at the time they discontinued birth control in

order to conceive a pregnancy. The original purpose of the study was to determine the incidence of very early pregnancy loss (Wilcox *et al*., 1988). Women were eligible to participate if they had no known chronic health or fertility problems and were not using hormone medications. Participants collected daily first-morning urine specimens and kept daily diaries that included information on menstrual bleeding and sexual intercourse. All participants provided informed consent. The research was approved by the Institutional Review Board of the National Institute of Environmental Health Sciences.

Hormonal assays

To detect day of ovulation, first-morning urine specimens from a 17-day mid-cycle window were assayed for estrone 3-glucuronide (E_1G) and pregnanediol 3-glucuronide (PdG). E_1G and PdG were measured by direct radioimmunoassay (Wright *et al*., 1978; Samarajeewa *et al*., 1979). Specimens were analyzed in duplicate or triplicate, and the geometric means of the steroid hormone concentrations were divided by the corresponding creatinine concentration to adjust for variations in dilution. The day of ovulation for each cycle was defined based on the rapid drop in the estrogen-to-progesterone ratio (Baird *et al*., 1991), a measure validated by LH measurements (Baird *et al*., 1995) and by ultrasound (Ecochard *et al*., 2001).

We employed a highly sensitive immunoradiometric assay to quantify hCG in urine samples (Wilcox *et al*., 1985). Variations in urinary creatinine were very small compared with the exponential rise in hCG (McChesney *et al*., 2005), making the adjustment of hCG for creatinine unnecessary.

Potential correlates

All potential correlates except two (season of implantation and oocyte-waiting time) were based on information reported by the participant at enrollment. 'Recent' oral contraceptive use was defined as use within 90 days of enrollment. Recent use of alcohol, caffeine, marijuana and vitamins was defined as any use in the 3 months prior to enrollment. Other characteristics of interest included age, reproductive history, menstrual cycle characteristics and BMI.

We considered season because in a prior analysis, risk of early pregnancy loss had been elevated in the last quarter of the year (Weinberg *et al*., 1994). We assessed whether late implantation was associated with season of implantation by assigning each day of the year to an angle in radians (subtracting 1 January from the date of implantation, dividing by 365 and then multiplying by 2π). By including the sine and cosine of these angles as exposures in our models using trigonometric regression, we allow a sinusoidal pattern for the risk of later implantation, with amplitude and day of greatest risk (phase) determined by the data.

Oocyte-waiting time is a measure of the time between ovulation and fertilization (presumably a matter of hours). We can measure oocyte-waiting time only indirectly, using the days of sexual intercourse (recorded in the woman's daily diary), relative to her measured day of ovulation (Wilcox *et al*., 1998). All conceptions in this study occurred with intercourse during a 6-day window ending on the day of ovulation. No pregnancies were observed with intercourse only on the day after ovulation (Wilcox *et al*., 1995). From this we can infer that the oocyte is short-lived and a long oocyte-waiting time would be most likely in pregnancies for which there was intercourse on the day of ovulation but not on the previous 5 days. That is, without capacitated sperm present when the oocyte arrives in the oviduct, the oocyte may need to wait many hours before capacitated sperm are present and fertilization can occur. The optimum analysis to address oocyte-waiting time would be limited to conceptions with intercourse only on the day of ovulation and not during the preceding 5 days. However, very few conceptions met that criterion. At the cost of some reduced specificity but with the benefit of added cycles, we defined a long oocyte-waiting time as intercourse on the day of ovulation but not during the 2 days before ovulation. [Intercourse before those 2 days being much less likely to result in conception (Wilcox *et al*., 1995).]

All of the potential correlates were examined with each of the reproductive end-points; however, we only present results from those variables that were statistically significant or suggestive of an association.

Reproductive end-points

A pregnancy was identified based on elevated hCG ($\geq$0.025 ng/ml) for three consecutive days (Wilcox *et al*., 1988). Once a pregnancy was identified, the day of implantation was assigned as the first day of a sustained rise in hCG in which each subsequent day exceeded 0.015 ng/ml. We dichotomized days to implantation as 9 or less and 10 or more. A pregnancy was categorized as an early loss if the initial rise in hCG was followed by a decline with menstrual-like bleeding within 6 weeks (42 days) of the start of the previous menstrual period. Pregnancies lasting longer than 42 days were considered clinical pregnancies.

Statistical analysis

We used logistic regression to identify factors correlated with late implantation (10 or more days after ovulation). To identify factors related to the pattern of hCG rise, we used a linear mixed model and generated *P*-values. The model was of the following general form for woman i on Day j:

$$\ln(\mathrm{hCG}_{ij}) = \beta_{0i} + \beta_{1i}(j) + \beta_0 + \beta_1(j) + \beta_2(j^2) + \beta_3(U) + \beta_4(Uj) + \beta_5(U(j^2)),$$

where β_{0i} and β_{1i} are zero-mean woman-specific random effects, j is the number of days starting with implantation and U (=1 or 0) indicates a dichotomous maternal or pregnancy factor that may be associated with the rise in hCG. This general model was expanded to accommodate multiple categories of a variable (U_1, U_2, U_3, etc.). To determine whether a variable was associated with the rate of hCG rise, the importance of β_4 and β_5 was assessed using a likelihood ratio test, based on a two-degree-of-freedom χ^2 distribution under the null hypothesis that U has no effect on the pattern of rise.

Each characteristic was assessed in a separate model; thus, all analyses are unadjusted for other factors except where specified in the text.

For graphical display, the patterns of rise in hCG over the 7 days beginning with implantation were quantified using an average relative increase in hCG for each day. This was calculated for Day j as

$$\exp[\text{average}(\ln \mathrm{hCG}_j - \ln \mathrm{hCG}_1)],$$

where '$\ln(\mathrm{hCG})_1$' indicates the natural log of the hCG concentration on Day 1, which is the day of implantation, '$\ln(\mathrm{hCG})_j$' indicates the natural log of the hCG concentration on Day j, which ranges from 2 to 7, and the 'average' is taken across pregnancies.

The absolute hCG curves from this population have been previously published (Nepomnaschy *et al*., 2008). In addition, that paper examined the association of hCG curves with twin pregnancies, pregnancy loss after 42 days gestation and infant sex. None of these factors were found to be important and thus all pregnancies are included here unless otherwise noted.

Study sample

There were 199 pregnancies detected in the Early Pregnancy Study. Of these, 151 were clinical pregnancies and 48 were early pregnancy losses. There were 10 clinical pregnancies which were missing a day of ovulation or a day of implantation, leaving 189 pregnancies in the

sample used to analyze late implantation. The patterns of hCG rise were assessed only among the clinical pregnancies. This is because early losses often do not last 7 days and do not necessarily have a clear pattern of rise (Baird et al., 2003). One additional clinical pregnancy could be used in this analysis because it had a measured day of implantation (day of ovulation was missing), thus providing 142 clinical pregnancies for the rate of rise analysis. Clinical pregnancies were mostly singleton live births ($n = 121$); other outcomes were 6 twin births, 13 miscarriages, 1 ectopic pregnancy and 1 molar pregnancy.

Some women contributed more than one pregnancy to the study (3 had more than one early loss, 21 had both an early loss and a clinical pregnancy and 1 had two early losses and a clinical pregnancy). We evaluated the effect of adjustment for this statistical dependency. By design, no woman had more than one clinical pregnancy in the study. Thus, the number of pregnancies is related to risk of loss: if a woman had more than one pregnancy, we know that at least one of those pregnancies was an early pregnancy loss. When (as in this case) cluster size is 'informative', the standard methods for adjusting for dependent observations (such as generalized estimating equations) are biased. Thus we used within-cluster resampling (Hoffman et al., 2001) to account for the multiple observations per woman. Other than a slight loss of precision, results were unchanged (data not shown).

Results

The median age was 29, and about two-thirds of the women had previously been pregnant (Table 1). About half of pregnancies in the study had implantation on Day 10 or later. Only 11 pregnancies (6%) were to women who were self-reported smokers. Seven pregnancies (5%) were to women who reported prenatal diethylstilbestrol (DES) exposure, and 47 (28%) pregnancies were to women who reported that their mother had smoked while pregnant with them.

Time from ovulation to implantation

Women who were current smokers had five times the odds of late implantation as never or former smokers [odds ratio (OR) (95% confidence interval, CI): 5.3 (1.3, 36), $P = 0.03$] (Fig. 1). There were not enough current smokers to evaluate a dose–response relationship. The association between husband's smoking and late implantation was also positive but weaker [OR (CI): 1.8 (0.64, 5.7)].

Implantation was also later among conceptions that resulted from a long oocyte-waiting time [OR (CI): 5.1 (1.9, 16), $P = 0.0008$] (Fig. 1). Late implantation was more common in exposed early losses, but not exclusive to them. When we restricted analysis to clinical pregnancies, the association between oocyte-waiting time and late implantation was still evident, but attenuated [OR (CI): 2.2 (0.63, 8.0)].

Given our previous observation that a long oocyte-waiting time led to early loss (Wilcox et al., 1998), we explored whether that association might be mediated by late implantation. We included both oocyte-waiting time and late implantation as predictors in a logistic regression of early loss. While the effect of oocyte-waiting time was attenuated when adjusted for late implantation (from an OR of 3.9 to 2.7), it was still significantly associated with early loss ($P = 0.04$).

Women who reported that their mother had smoked while pregnant with them had less late implantation [OR (CI): 0.48 (0.23, 0.96), $P = 0.04$]. The odds of late implantation also tended to be lower for women who had lived with one or more household smokers (mother, father or other) during their childhood ($P = 0.11$) (Fig. 1).

We explored the robustness of these associations with current smoking and prenatal exposure to maternal smoking by limiting the analysis to clinical pregnancies. The association between current smoking and late implantation was similar [5.5 (1.2, 39)] and the protective association of prenatal exposure to maternal smoking and late implantation was slightly stronger, 0.35 (0.14, 0.83). After further restricting to only live births (i.e. excluding the 15 clinical miscarriages), the associations again became slightly stronger [current smoking, 6.1 (1.3, 44) and prenatal maternal smoking exposure 0.31 (0.11, 0.77)].

Season of the year [previously associated with early loss in these data (Weinberg et al., 1994)] was not associated with the timing of implantation ($P = 0.97$, data not shown).

Rate of hCG rise

Three factors were associated with the steepness of the initial hCG rise: age, age of menarche and DES. Older women had a faster rise ($P = 0.01$) (Fig. 2). This could not be attributed to their increased gravidity which was not associated with rate of rise ($P = 0.25$). When pregnancy losses were removed, the *P*-value increased to 0.08. The *P*-value for age when age at menarche was included in the model was unchanged (0.01). Excluding the DES-exposed women led to a stronger *P*-value ($P = 0.006$). Age at menarche was weakly associated with hCG rise, with women who were younger at menarche having a slower hCG rise ($P = 0.09$) (Fig. 3). A linear trend test of the age at menarche categories was significant, $P = 0.02$. This *P*-value was not affected by adjustment for BMI ($P = 0.02$) or removing the pregnancy losses ($P = 0.03$); however removing the DES-exposed participants increased the *P*-value (0.06). Exposure to DES *in utero* was associated with a distinct pattern of hCG increase ($P = 0.002$) (Fig. 4). Exposed women had a slower hCG rise up to Day 4 and then a faster rise thereafter. Interpretation of this finding is limited by the fact that only four pregnancies were to DES-exposed mothers. Removing the pregnancy losses did not alter the *P*-value ($P = 0.002$).

Discussion

We found a number of maternal factors associated with time to implantation and with the pattern of initial hCG rise. Current smoking was associated with late implantation, while prenatal exposure to maternal smoking was associated with earlier implantation. Conceptions resulting from a long oocyte-waiting time were also more likely to implant later, but this pathway does not appear to explain their increased risk of early loss that we previously reported. Among those conceiving a clinical pregnancy, older women and women with a later age at menarche experienced a faster initial rise of hCG. Conceptions to the few women who were DES daughters showed a distinct pattern of rise, slower over the first 4 days and faster thereafter.

These data are unique in describing peri-conceptional events in a group of naturally conceived pregnancies. Nonetheless, the analysis has notable limitations. One is small numbers. For example, few women smoked or had been prenatally exposed to DES. While sample size limits the conclusiveness of our analysis, these

Table I Distribution of maternal and pregnancy characteristics within the two study samples, all conceptions ($n = 189$) and clinical pregnancies only ($n = 142$).

	Number of conceptions[a] (%)	Number of clinical pregnancies[b] (%)
Time from ovulation to implantation		
≥10 days	87 (46)	52 (37)
≤9 days	102 (54)	89 (63)
Age		
≥29	97 (51)	72 (51)
<29	92 (49)	70 (49)
Prior pregnancy		
Yes	128 (68)	94 (66)
No	60 (32)	48 (34)
History of miscarriage		
Yes	27 (14)	18 (13)
No	101 (54)	76 (53)
Never pregnant	60 (32)	48 (34)
Age at menarche		
<12	24 (13)	20 (14)
12, 13	114 (60)	83 (58)
>13	51 (27)	39 (28)
Cycle length		
<29	86 (48)	66 (49)
≥29	95 (52)	69 (51)
Regular cycles		
Yes	162 (86)	120 (84)
No	27 (14)	22 (16)
Recent oral contraceptive use		
Yes	17 (9)	12 (9)
No	172 (91)	130 (91)
Oocyte-waiting time		
Long	23 (12)	11 (8)
Short or average	166 (88)	130 (92)
BMI		
<18	18 (10)	14 (10)
18 to <25	155 (82)	115 (82)
≥25	16 (8)	13 (9)
Prenatal DES exposure		
Yes	7 (5)	4 (4)
No	135 (95)	100 (96)
Prenatal exposure to maternal smoking		
Yes	47 (28)	36 (29)
No	118 (72)	88 (71)
Number of smokers in the home when participant was <10 years old		
0	64 (35)	44 (31)
1	64 (35)	49 (36)
≥2	56 (30)	46 (33)
Husband's current smoking		
Yes	15 (8)	12 (9)
No	174 (92)	130 (91)
Smoking status at enrollment		
Current	11 (6)	8 (6)
Former	52 (27)	40 (28)
Never	126 (67)	94 (66)
Alcohol intake		
Any	150 (79)	114 (80)
None	39 (21)	28 (20)
Caffeine intake		
Any	179 (95)	133 (94)
None	10 (5)	9 (6)
Marijuana use		
Any	26 (14)	20 (14)
None	163 (86)	122 (86)
Vitamin use		
Yes	99 (53)	79 (55)
No	89 (47)	63 (45)

[a]This sample includes early pregnancy losses and clinical pregnancies, women could have experienced either or both leading to more than one observation per woman.
[b]This sample includes pregnancies that survived to clinical recognition. Women only contributed one clinical pregnancy leading to only one observation per woman.

preliminary data may serve to generate hypotheses for future research. Given that we explored a total of ~20 variables and 2 outcomes, a few statistically significant results would be expected by chance. We have therefore focused our interpretations on biological plausibility and coherence rather than statistical significance. Our data set represents a relatively healthy population of embryos; in order to be observed, embryos had to be able to implant and secrete hCG. Embryos that failed sooner or were unable to produce enough hCG to meet our cutoffs are unobservable; as in any pregnancy study, their unobservability may have influenced the observed associations. Another possible limitation is that our measure of hCG was in urine. Some of the observed differences could be due to exposure effects on maternal disposition of hCG rather than to the actual amount of hCG secreted by the conceptus, although urinary and serum concentrations are in general highly correlated (Wehmann and Nisula, 1981; Norman *et al*., 1987; McChesney *et al*., 2005).

Time to implantation

Experimental data suggest that when oocytes wait many hours before fertilization they produce less viable embryos (Lanman, 1968; Butcher, 1976; Longo and So, 1982; Juetten and Bavister, 1983; Perreault,

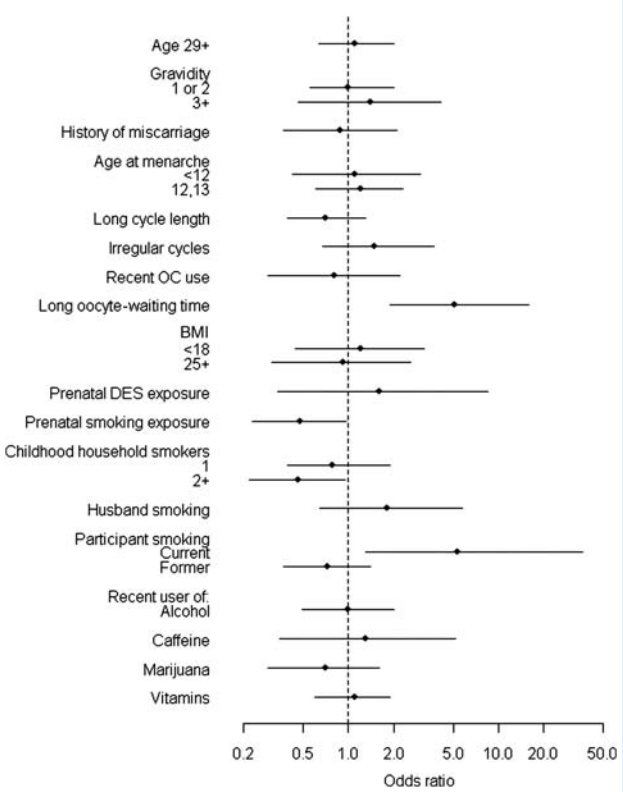

Figure 1 ORs and 95% CIs for late implantation (≥10 days post-ovulation).

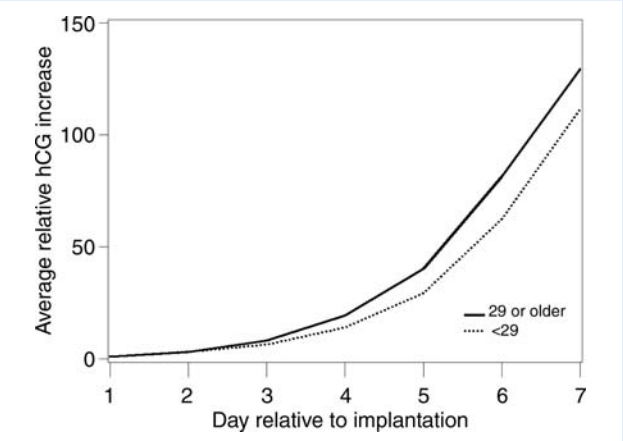

Figure 2 Average relative increase in hCG over the first 7 days of pregnancy beginning with the day of implantation (Day 1), stratified by participant age at intake, $P = 0.01$. Solid line represents women 29 or older; the dotted line represents women under 29.

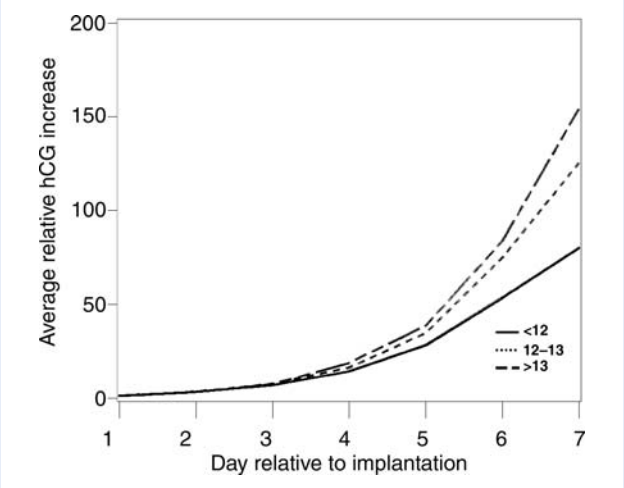

Figure 3 Average relative increase in hCG over the first 7 days of pregnancy beginning with the day of implantation (Day 1), stratified by age at menarche, $P = 0.01$. The solid line represents women <12 at menarche, the small dashed line represents women aged 12–13 at menarche and the long dashes represent women >13 at menarche.

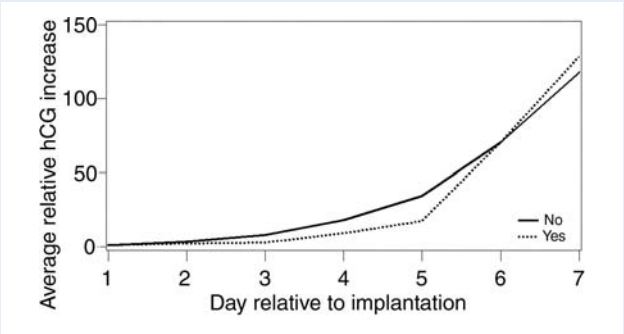

Figure 4 Average relative increase in hCG over the first 7 days of pregnancy beginning with the day of implantation (Day 1), stratified by *in utero* exposure to DES, $P = 0.002$. The solid line represents women not exposed to DES *in utero*; the dotted line represents women who did report exposure to DES *in utero*.

1992). Our earlier finding of oocyte-waiting time being associated with early pregnancy loss is consistent with those experimental data, and suggests that aging of the oocyte prior to its fertilization (even by a few hours) may be detrimental to embryonic development (Wilcox *et al.*, 1998). Such effects on development may contribute to delays in implantation. In the present study, we find that long oocyte-waiting time is associated with late implantations both in conceptions destined to be early losses and (albeit more weakly) in surviving conceptions.

After restricting to clinical pregnancies only, a long oocyte-waiting time still tended to be followed by later implantation. This could reflect a less competent conceptus. It is also possible that the association is a natural consequence of later fertilization. Oocytes are thought to remain viable for ~24 h. Oocytes that are fertilized quickly will produce embryos that are as much as a day ahead of embryos that result from late fertilization. Thus, the late fertilizations may be up to 1 day behind in their implantation time. Consistent with this hypothesis, the implantation delay we observed in the clinical pregnancies was primarily a shift from post-ovulation Day 9 to Day 10. Such a delay may or may not have negative consequences for the embryo.

The delay in implantation among smokers, if not a chance finding, could be due to disruption of ovum retrieval by the oviduct or of tubal transport (Neri and Marcus, 1972; Mitchell and Hammer, 1985; Knoll *et al*., 1995; Knoll and Talbot, 1998) (reviewed in Lyons *et al*., 2006). The IVF literature suggests that maternal smoking can also have adverse effects on a range of other reproductive functions. Smoking has been associated with decreased ova retrieval (Van Voorhis *et al*., 1996; Joesbury *et al*., 1998; Fuentes *et al*., 2010), lower fertilization rates (Elenbogen *et al*., 1991; Rosevear *et al*., 1992), lower implantation rates (Van Voorhis *et al*., 1996) and increased miscarriage risk (Maximovich and Beyler, 1995; Van Voorhis *et al*., 1996). A meta-analysis reported lower IVF pregnancy rates for smokers compared with non-smokers (Augood *et al*., 1998).

We previously reported that women with prenatal exposure to maternal smoking had reduced fertility (Weinberg *et al*., 1989). It is possible that among these women only the most robust of the embryos succeed in implanting. This selection could explain both a fertility effect and the observed association between prenatal exposure to maternal smoking and earlier implantation if the slower-developing embryos often fail to implant.

hCG rise

The slower initial hCG rise among women with earlier age at menarche was unexpected. Early menarche has been associated with endometrial pathology [endometrial cancer (Fujita *et al*., 2008; Dossus *et al*., 2010), adenomyosis (Templeman *et al*., 2008) and endometriosis (Treloar *et al*., 2010)]. Early menarche may also be associated with endometrial factors that retard early development of an implanted conceptus (and thus slow the production or release of hCG). Polymorphisms in chemokine receptor 3 (CCR3) have been associated with age at menarche and CCR3 is associated with endometrial inflammation (Yang *et al*., 2007). Since embryonic implantation is an inflammatory process, it is possible that this gene indirectly influences the early rise of hCG.

DES was associated with a distinct pattern of hCG rise in these data. Women who were DES daughters have been found to have numerous uterine and cervical abnormalities (Kaufman *et al*., 1984) that may contribute to their higher risk for infertility (Herbst *et al*., 1980; Kaufman *et al*., 1984) and pre-eclampsia (Troisi *et al*., 2007). DES exposure *in utero* may also influence immune function (Ford *et al*., 1983; Ways *et al*., 1987). Structural abnormalities of the uterus and immune impairments may influence the ability of an embryo to invade the endometrium, which might have resulted in the unusual pattern of hCG rise.

Conclusion

In summary, these data from a group of naturally conceiving women who participated in the NCEPS provide an opportunity to examine factors affecting the timing of implantation and the patterns of early pregnancy hCG. These data suggest that early life factors, as well as current exposures, may affect early pregnancy.

Authors' roles

A.M.Z.J. performed all analyses and wrote the manuscript. C.R.W. provided statistical expertise. C.R.W., D.D.B. and A.J.W. designed and implemented the original study, provided input on these analyses and edited the manuscript.

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