A prospective study of early pregnancy loss in humans

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Objective: To test two hypotheses: In spontaneous conceptions, early pregnancy loss (EPL) is associated with [1] inadequate luteal (ovarian) P, and/or [2] elevated follicular (adrenal) P.

Design: A population-based prospective study.

Setting: Thirty rural Bolivian communities.

Patient(s): Women volunteers (n = 191), 19–40 years old, in stable sexual unions and not using contraception.

Intervention(s): Collection of serial saliva samples throughout sequential ovarian cycles and urine samples during late luteal phases. Collections continued throughout pregnancy for each detected conception.

Main Outcome Measure(s): Occurrence of spontaneous conceptions and subsequent outcomes. Salivary concentrations of P. Test for elevated urinary human chorionic gonadotropin (hCG).

Result(s): Luteal (through implantation) P levels were similar in pregnancies lost within 5 weeks after conception (EPL; n = 8) and those pregnancies that were maintained longer (sustained conceptions, SC; n = 32). Follicular P was significantly higher in EPL than in SC.

Conclusion(s): [1] Elevated follicular P was associated with EPL in natural conceptions in healthy women. [2] Early pregnancy loss exhibits absolute luteal P levels comparable to SC, but lower luteal/follicular P ratios. (Fertil Steril® 2006;86:373–9. ©2006 by American Society for Reproductive Medicine.)

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Early pregnancy loss (EPL) in humans is common [reviewed in Roberts and Lowe (1), Leslie et al. (2), Regan and Rai (3), Holman and Wood (4), and Macklon et al. (5)]. Several large prospective studies that relied on sensitive tests for detecting urinary human chorionic gonadotropin (hCG) in women attempting natural conception have reported similar EPL rates (about 25%–30%) despite different study populations and some differences in the frequency of urine sampling, the criteria for recognizing conception and loss, and the precise definition of EPL (6–10). Because preimplantation loss is not readily detectable, these estimates of EPL are necessarily minimums; the actual rate may be as high as 80% of all fertilized ova (1–5).

Despite its frequency, the causes of EPL are uncertain (3). Most of the evidence concerns risk factors for miscarriage in clinically recognized pregnancies instead of for EPL per se, and direct extrapolations may not be warranted.

Cytogenetic studies suggest that genetic abnormalities account for roughly half of the spontaneous miscarriages in clinically recognized pregnancies [reviewed in Goddijn et al. (11)]. Rising maternal age and some maternal behaviors (i.e., the use of cigarettes or other drugs) are known risk factors for miscarriage of clinically recognized pregnancies (3); high levels of caffeine or alcohol consumption may be additional behavioral risk factors (3, 12). However, none of these maternal behaviors was found to increase the risk of EPL in a study of US women (13), perhaps because of sample size limitations. A more recent Danish study (12) found some evidence that very high levels of alcohol consumption around the time of conception was associated with an increased risk of loss among all detected conceptions, but sample sizes precluded distinguishing any statistically significant increase in risk specific to EPL. Several endocrine, autoimmune, and thrombotic abnormalities are recognized risk factors for recurrent miscarriage (3), but their potential contributions to the risk of sporadic EPL in the general

population are conjectural. Although the advent of assisted reproductive technologies (ARTs) has prompted substantial attention to the possible hormonal correlates of EPL, the relevance of findings from ART for the outcomes of spontaneous conceptions is uncertain.

Progesterone is critical to uterine development, implantation, and pregnancy maintenance (14, 15). During the luteal phase (i.e., following ovulation), the corpus luteum, formed from the ruptured follicle, is the principal source of P until about 5 weeks after conception, at which time placental production begins to predominate (16, 17). During this initial period, either surgically removing the corpus luteum or biochemically blocking P with mefipristone leads to pregnancy failure (18, 19). Such observations underlie the clinical expectation that P therapy can prevent recurrent miscarriages and the epidemiological expectation that EPL will be associated with relatively lower P levels. Somewhat surprisingly, the evidence to date has not satisfactorily supported either prediction (20–23).

During most of the follicular phase (i.e., before ovulation), the adrenal cortex is the main source of P (24–26), typically produced at levels much lower than ovarian P and generally assumed to be of little significance for reproductive functioning. However, evidence that elevated adrenal P might affect reproduction is emerging from studies of the possible links between stress and disruption of the hypothalamic-pituitary-ovarian axis. Ovariectomized monkeys and postmenopausal women, when experimentally subjected to stress in the presence of replacement estradiol, exhibited a rise in glucocorticoids and adrenal P that triggered surges in LH (27, 28). In a longitudinal study of Guatemalan women who self-collected thrice-weekly urine samples, cortisol rises were accompanied by relatively higher follicular-phase P and LH (29, 30). Such excessive or poorly timed LH surges were hypothesized to impair follicular development (27, 28), but whether elevated follicular-phase P or LH is associated with pregnancy loss in humans has not been determined.

This report presents results from a study of conception and pregnancy outcome in a "natural fertility population" (i.e., women were neither actively seeking to prevent nor to become pregnant). Specifically, we tested two hypotheses regarding P levels in those conception cycles in which pregnancy loss occurred before 5 weeks after conception, about the time at which the corpus luteum ceases to be the primary source of P. First, we evaluated whether EPL is the result of inadequate luteal P, which may be indicative of a luteal phase defect. Second, we ascertained whether EPL is associated with elevated follicular P, which can be produced by the adrenals in response to stress (27, 28, 31).

MATERIALS AND METHODS
Population and Participants
Data collection was conducted within the framework of Project REPA (Reproduction and Ecology in Província

Aroma), a multidisciplinary longitudinal study of reproductive functioning and health among rural Aymara families indigenous to the Bolivian altiplano (32). All study protocols were approved by the Institutional Review Board, University of California, Riverside. Study participants, who were recruited during 12 months beginning in November 1995, represented >80% of the eligible women (19–40 years old, currently in stable sexual unions, and not using contraception) in 30 communities scattered over 200 km² situated about midway between the cities of La Paz and Oruro, Bolivia. Of 316 adult female participants, 125 were pregnant and/or lactating and noncycling at recruitment and throughout the study, 98 were lactating at the time of the first observed menstrual segment, and 93 were menstruating and not breastfeeding at recruitment.

Hormonal Measurements
The data collection protocol has been previously described (32). In brief, throughout participation, menstruating women (n = 191) were visited every other day to record menstrual status and collect a 5-mL saliva sample. Beginning at 24–25 days following each first day of menstrual bleeding, a urine sample was tested for hCG, evidence of conception, and implantation, using a commercially available pregnancy test (QuPID; StanBio, Boerne, TX; sensitive at 25 IU/L hCG; accuracy and specificity are both >99%). Due to the isolated conditions and lack of electricity, urine samples could not be frozen for more sensitive laboratory testing at another time. Urine collection continued every other day until the next menses or, in the case of a positive test, until pregnancy loss or the 6th month of gestation.

Of 853 menstrual cycle initiations (1–8 cycles per woman, median = 4 cycles per woman), 65 were associated with at least one positive hCG test. Of these putative conceptions, 23 were observed to term, 27 were lost before the third trimester, 1 was medically aborted, and 14 women withdrew from the study while still pregnant, principally due to waning participant interest. The total number of conception cycles available for statistical analyses was reduced to 45 because of laboratory handling or storage failure (12 cycles), incomplete sample collection (5 cycles), and inability to estimate probable day of ovulation or conception (3 cycles). We have previously compared progesterone levels in the subsample of conceptions observed through full term births with those in a sample of conceptions from Chicago women (32); neither the data from the other conceptions in these Bolivian women nor any of the analyses presented here have been previously published.

Saliva samples for all cycles were assayed for P at Northwestern University, following previously published methods (33, 34); the correlation for P in concurrently collected saliva and serum samples was 0.75 ($P<.001$) in a sample of 48 US women (33). Other than samples collected during late pregnancy, all samples from the same woman were assayed together.

Analytical Samples

The timing of pregnancy loss was defined as the first day of vaginal bleeding following a positive hCG test (if subsequently confirmed by sequential negative hCG tests). Conceptions ending within 5 weeks of ovulation were classified as EPLs (n = 8). Sustained conceptions (SCs, n = 32) were those persisting beyond this period. Pregnant women withdrawing before 5 weeks after conception (n = 5) were not included in either sample.

A putative day of ovulation for each cycle was estimated using a previously described algorithm that takes advantage of the well-established fact that P, having been relatively low and flat during the follicular phase, rises sharply after ovulation (32): The graphed profile of the serial P values for each cycle was inspected for a marked change in slope, and this point was designated as the day of ovulation. The three conception cycles in which this method was unable to identify a day of ovulation were not included in the present analyses. Follicular and luteal P levels were analyzed separately.

Statistical Approach

Trends in P levels were evaluated by fitting population-averaged linear models with correlated errors [also known as "marginal models" (35)] using the MIXED command in SPSS version 13.0 (SPSS Inc., Chicago, IL). This approach to the analysis of longitudinal data, such as repeated sampling during the course of a menstrual cycle, has several advantages over the use of summary indices (e.g., area under a curve). Most important, these models account for a variety of potential correlation structures in the data, which can arise from longitudinal measures but avoid the reduction in statistical power that accompanies summary indices (35). In addition, different numbers of observations for each subject are permitted; thus, the approach makes the maximum use of all available data.

Progesterone observations were transformed (either natural log or square root) to satisfy assumptions of normality for the random errors in the linear models. In addition to evaluating absolute P levels, each P observation in a given cycle was standardized as a percentage of that same cycle's average midfollicular P level. Specifically, setting ovulation to day 0, midfollicular was defined as days −3 through −10, and the average P across this span of days was calculated. Each P observation was divided by this average midfollicular value and multiplied by 100.

In each linear model analysis, covariates of interest included EPLvSC (an indicator variable: conception loss or maintenance), AGE (in completed years), and LACTATING (an indicator variable: yes or no). Peak midluteal P in nonconception ovulatory cycles is known to vary with age and lactation status, being lower in menstruating women who are still breastfeeding. Fixed effects associated with these covariates were included in the models, with the objective being to estimate the population-averaged fixed effects and take the correlation structure of the residuals into account when calculating the standard errors of the fixed effects.

To capture the time-dependent changes in P over the ovarian cycle, and to reflect the different principal sources of P before and after ovulation, time was coded relative to the estimated day of ovulation (RELDAY). In separate analyses, RELDAY ranged from either −14 through 0 (before ovulation) or 0 through 12 [in humans, natural implantation initiated after day 12 is rarely if ever successful (6)]. Based on initial descriptions of the data, RELDAY-squared was included in the analyses to model possible quadratic trends in P levels. For 6 of the 8 women who had an EPL, a nonconception ovulatory cycle had also been observed. In the analyses of P from these cycles, EPLvOVUL (an indicator variable: conception loss or nonconception ovulatory cycle) was substituted for EPLvSC.

Alternative correlation structures for random errors associated with observations on the same subjects were considered when fitting the models, and likelihood ratio tests were used to determine the simplest correlation structures with the best fits to the observed data.

RESULTS

Those women with sustained conceptions averaged 6 years younger than those who experienced EPL (27.1 ± 4.63 vs. 33.0 ± 4.57 years, $P=.003$); in each linear model analysis, AGE was included as a covariate to account for age variation in P. Of those women with SC, 66% (21 of 32) were lactating compared with 38% (3 of 8) of those experiencing EPL, but because the sample sizes were small, the difference was not statistically significant. Of the eight EPLs, if duration is defined as the first day of last menstrual bleeding to the day of vaginal bleeding associated with EPL, then three conceptions ended on day 26, and one ended on each of days 30, 31, 33, 43, and 46 (median = 30.5 days). If duration is defined as the estimated day of ovulation to the day of vaginal bleeding associated with EPL, then one ended on each of days 11, 13, 14, 23, 30, and 32, and 2 ended on day 16 (median = 16 days). Of the 32 SCs, 19 persisted beyond 22 weeks gestation, while 7 ended and 6 were lost to follow-up before this time.

Table 1, Table 2, and Table 3 present the estimated models generated by the linear model analyses. The estimated coefficient for each variable (referred to as a "fixed effect") indicates the expected change in predicted P level associated with a one-unit change in that variable.

As presented in Figure 1A, following ovulation (day 0), the SC and EPL samples had similar P levels through the luteal phase and during implantation (i.e., through day 12). This similarity was borne out by statistical analyses (not shown). None of the many models fitted to the data from days 0 through 12, or from any subset of days during this period, demonstrated a significant difference in P levels between SC and EPL.

TABLE 1

Estimated linear model 1.[a,b]

Fixed effect	Coefficient	Standard error	P value
Intercept	9.8724	1.2206	<.000
RELDAY	−0.4196	0.0859	<.000
RELDAY2	−0.0211	0.0040	<.000
AGE	−0.1269	0.0402	.010
LACTATION (Yes)	0.7397	0.4122	.081
EPLvSC	1.9764	0.4559	<.000

[a] Regression model 1 estimates follicular P (square root transformed): sqrt (P_F) = 9.8724 − (0.4196 RELDAY) − (0.0211 RELDAY2) − (0.1269 AGE) + 0.7397 [if lactating] + 1.9764 [if EPL].
[b] Covariance structure is heterogenous autoregressive; estimation procedure is restricted maximum likelihood.

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TABLE 3

Estimated linear model 3.[a,b]

Fixed effect	Coefficient	Standard error	P value
Intercept	−1.0924	0.4174	0.012
RELDAY	0.3581	0.0480	<0.000
RELDAY2	−0.0232	0.0044	<0.000
AGE	0.0350	0.0144	0.019
LACTATION (Yes)	−0.4216	0.1407	0.005
EPLvSC	−0.6686	0.1848	0.001

[a] Regression model 3 estimates standardized luteal P (ln transformed): ln (P_{SL}) = −1.0924 + (0.3581 RELDAY) − (0.0232 RELDAY2) + (0.035 AGE) − 0.4216 [if lactating] − 0.6686 [if EPL].
[b] Covariance structure is heterogenous autoregressive; estimation procedure is restricted maximum likelihood.

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In contrast, follicular (days 0 through −14) P levels were significantly greater (*P*<.001) in EPL than in SC (Table 1: The coefficient for EPLvSC is the additional increase in predicted P in EPL cycles relative to SC cycles). Progesterone decreased with rising maternal age (*P*=.01) but was higher if breastfeeding (*P*=.081).

Figure 1B compares P levels in EPL cycles to nonconceiving ovulatory cycles from the same women. Although luteal P levels did not differ (analyses not shown), follicular P was higher (*P*=.061) in those conception cycles that led to EPL (Table 2). Although the significance level was marginal,

TABLE 2

Estimated linear model 2.[a,b]

Fixed effect	Coefficient	Standard error	P value
Intercept	9.0186	3.6597	.011
RELDAY	−0.6632	0.2062	.002
RELDAY2	−0.0494	0.0155	.003
AGE	−0.0217	0.1024	.835
LACTATION (Yes)	−2.9740	1.0843	.015
EPLvOVUL	1.5355	0.7815	.061

[a] Regression model 2 estimates follicular P (square root transfomed): sqrt (P_F) = 9.0186 − (0.6632 RELDAY) − (0.0494 RELDAY2) − (0.0217 AGE) − 2.9740 [if lactating] + 1.5355 [if EPL].
[b] Covariance structure is first order auto regressive; estimation procedure is maximum likelihood.

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owing to the small sample size, the effect size (1.5355) was roughly comparable to that in Model 1 for EPL (1.9764).

When expressed as a percentage of the midfollicular level in the same cycle (Fig. 1C), standardized luteal P levels were significantly lower (*P*=.001) in EPL than in SC cycles (Table 3). Standardized luteal P levels increased with age but were relatively lower in lactating women.

DISCUSSION

The demands of measuring hormones in spontaneous conception cycles ending in EPL are evidenced by the paucity of data. We know of three investigations of nonclinical populations followed prospectively for this purpose: The North Carolina Early Pregnancy Study (NCEPS) (6), a study of Guatemalan women (29), and the present study (Project REPA) (32). The findings of these three investigations have similarities and differences.

The times to loss in the samples of EPL from the three studies are reasonably comparable. For the eight EPL conceptions analyzed here, five (62.5%) ended before 21 days after ovulation; the median duration from ovulation to loss was 16 days; and the median duration for cycles in which early losses occurred was 30.5 days. In the Guatemala study (29), of 22 conceptions observed in 16 women, 9 went to term, and 13 were lost; the median duration from ovulation to loss was 14 days (range = 13–47 days). In the NCEPS (36), the median duration for the 43 cycles in which early losses occurred was 32 days.

In the present study, absolute levels of P during the first half of the luteal phase were comparable in EPL cycles, in prior ovulatory cycles from the same women who experi-

FIGURE 1

Progesterone levels before and after ovulation (day 0) in naturally occurring conception and nonconception cycles. Panels **A** and **B** plot the daily geometric mean of observed P values. Preovulatory P was significantly higher in the sample of conceptions lost early (EPL) compared with those pregnancies persisting for at least 5 weeks after conception (**Panel A**) and compared to nonconception ovulatory cycles (**Panel B**). Panel **C** plots the daily geometric mean of P expressed as a percentage of the average midfollicular P level in the same cycle. Postovulatory standardized P is significantly lower in EPL than in sustained conceptions.
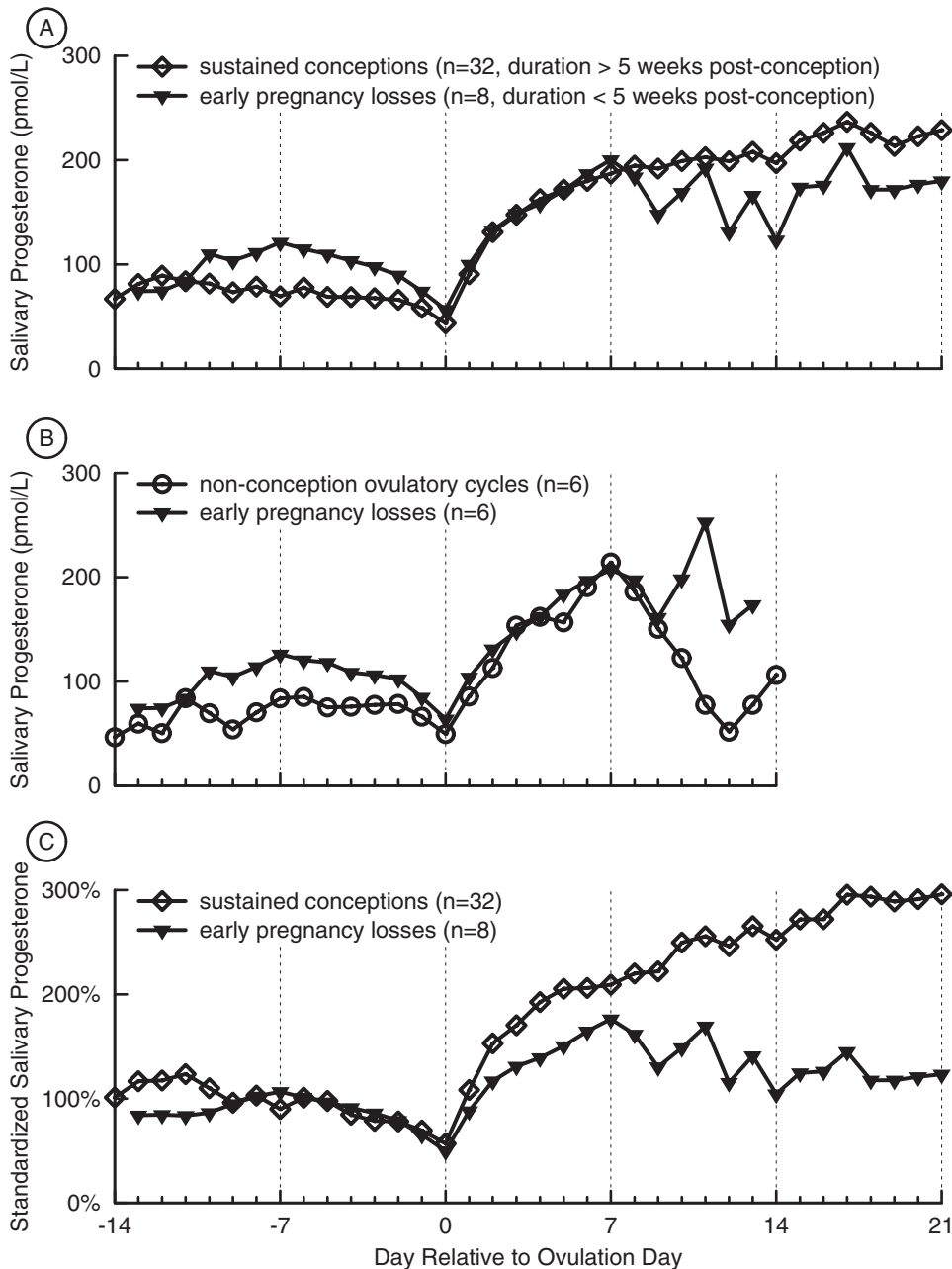
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enced EPL, and in SC cycles (Fig. 1, panels A and B). Subsequent to implantation, P continued to climb in SC but began to decline in EPL. Similarly, in a subsample of 20 women from the NCEPS, absolute levels of P did not differ in EPL and subsequently successful pregnancies until after implantation, at which time P in EPL began to fall (23). Our findings are consistent with the suggestion by Baird et al. (23) that "most early losses in reproductively normal women

do not result directly from deficiencies in ovarian steroid production."

To our knowledge, the analyses presented are the first evidence of an association between elevated follicular P and EPL in natural conceptions in healthy women (neither the NCEPS nor the Guatemala study specifically evaluated this link). This finding is consistent with other observations that elevated P during the follicular phase can affect reproductive outcome. In the NCEPS (37), conception was less likely in cycles with higher follicular P even when controlled for body mass and smoking (two factors associated with variation in follicular P). Elevated P during the late follicular phase increases the probability of failure in ART (38), and persistently elevated P is associated with amenorrhea and infertility in some adrenal disorders (39, 40).

Although the adrenals are the principal source of P throughout most of the follicular phase (24–26), P elevations above typical follicular levels could, in theory, be of ovarian and/or adrenal origin. For example, perhaps follicular P elevation is an indication of ovarian dysfunction, poor oocyte quality, or some alteration in follicular function, possibly related to age. Increases in adrenal P can occur because of adrenal pathology (39, 40) or stress stimuli (27–31). The Guatemala study (30) found increases in follicular P to be associated with increases in cortisol (produced by the adrenals in response to stress). Ovariectomized monkeys and postmenopausal women exhibited rises in glucocorticoids and adrenal P when experimentally subjected to stress in the presence of replacement estradiol (27, 28).

It is not possible to ascertain the origin(s) of elevated follicular P observed in EPL in the present study. The linear models included AGE as a covariate to control for the older average age of the women who experienced EPL compared with those with SC; therefore, elevated follicular P in these EPLs is not readily attributable to this age difference. The absence of elevated follicular P in the ovulatory nonconception cycles of the women who experienced these EPLs suggests that some temporary factor (e.g., poor oocyte quality specific to that cycle or a transient stress) instead of a constant condition (e.g., adrenal or ovarian pathology) underlay the elevated follicular P associated with EPL in this study.

Stress has been linked to reproductive functioning and outcomes in several studies of both animals and humans (reviewed in 29, 41). For example, those conceptions in the Guatemala study characterized by temporarily higher cortisol levels during the first 3 weeks after conception were significantly more likely to end in loss (29). That study has yet to evaluate, however, whether the observed EPLs were also associated with elevated follicular P, as we have described here, although they have reported that temporarily higher cortisol during the follicular phase is associated with an increase in P (30).

Our observation that standardized, but not absolute, luteal P levels are lower in EPL may help to explain the contradictory findings regarding the predictive value of P levels for endometrial maturation or risk of EPL (21, 42, 43). Experimental data demonstrating a critical role for ovarian P in early pregnancy maintenance (18, 19) lend credence to the hypothesis that insufficient ovarian P (often referred to as luteal phase defect, LPD) may increase the risk of EPL. However, a meta-analysis of 15 randomized control trials concluded that exogenous P supplementation in early pregnancy could not be justified (21). The debate regarding the efficacy of hormonal interventions for LPD or EPL continues, in part because of inconsistencies in the diagnostic criteria for LPD (3). The findings of the present study suggest it may also be helpful to examine relative as well as absolute luteal P levels; perhaps some women in these studies experienced unrecognized elevations of follicular P that confounded interventions and/or analyses. Likewise, a study of ART pregnancies reported successful gestation and birth at exceptionally low P levels (44). Perhaps the induction of P-receptors is more important than absolute P levels, or perhaps the relative change in P from before to after ovulation is a more salient signal for endometrial development and implantation.

Clearly, these hypotheses require further investigation, as does our suggestion that elevated follicular P in EPL may reflect adrenal response to stress. In addition to the small sample size, other limitations of the present study include [1] an inability to ascertain whether the elevated follicular P in EPL is of adrenal or ovarian origin, and [2] the use of hCG tests that were less sensitive than the tests used in either the NCEPS or Guatemalan study. Our study protocol recognized only those pregnancies in which hCG reached at least 25 IU/L, a sensitivity typical of over-the-counter pregnancy tests. Unquestionably, some conceptions and losses went undetected.

Conceptions that fail to achieve ≥25 IU/L hCG and/or are lost >5 weeks after ovulation may have a different etiology than the EPLs in this study. We restricted EPL to those conceptions lost within 5 weeks, the period during which placental P is not yet dominant. This definition arises from the evolutionary argument that stressors prompting maternal adrenal response are likely to have a greater impact on maternal reproductive functioning than on placental functioning (29). If this argument is correct, pregnancy losses occurring later than those as defined in our study are less likely to be associated with elevated follicular P levels.

Our results argue that more attention should be directed to follicular P levels in the study of EPL and to the factors that may stimulate adrenal production of P. A better understanding of the role of adrenal P may help to reduce the risk of EPL in both ART and spontaneous conceptions.

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