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Laboratory Methods for Evaluating Early Pregnancy Loss in an Industry-Based Population

Bill L. Lasley, PhD, Peter Lohstroh, BS, Andrew Kuo, BS, Ellen B. Gold, PhD, Brenda Eskenazi, PhD, Steven J. Samuels PhD, Dennis R. Stewart, PhD, and James W. Overstreet, MD, PhD

Laboratory methods were adapted or developed to analyze approximately 70,000 daily urine samples collected during more than 2,500 menstrual cycles from 448 women working in the semiconductor industry. An immunoenzymometric assay (IEMA) for human chorionic gonadotropin (hCG) was employed for screening cycles in order to optimize laboratory resources and to reduce the number of samples requiring analysis by less efficient methods. The presence of hCG in urine was confirmed by the definitive immunoradiometric assay (IRMA). The screening assay eliminated 78% of cycles from further analysis because there was no evidence of conception. Thirty-eight of 448 cycles identified as having significant levels of hCG with the IEMA were confirmed as hCG positive with the IRMA. HCG-positive cycles were further evaluated by examination of daily diary data and by laboratory assays for ovarian and pituitary hormones. As a result of these evaluations, 17 of the 38 cycles identified by the IRMA as positive for hCG were found to be nonconceptive cycles. These results demonstrate the effectiveness of screening assays for hCG, as well as the importance of using multiple urinary biomarkers for the detection of early fetal loss with daily urine samples.

Key words: early pregnancy loss, urinary hormones, chorionic gonadotropin, semiconductor manufacturing, biomarkers

INTRODUCTION

The collection and analysis of daily urine samples for human chorionic gonadotropin (hCG) have been demonstrated to be practical methods for assessing early fetal loss (EFL) in population-based studies [Wilcox et al., 1988]. Direct measurements of hCG in urine samples with highly sensitive and specific immunoradiometric assays (IRMA) have been accepted as the gold standard for detection of EFL. How-

Institute of Toxicology and Environmental Health, and Division of Reproductive Biology and Medicine, Department of Obstetrics and Gynecology, School of Medicine, University of California, Davis (B.L.L., P.L., A.K., J.W.O.).
Division of Occupational/Environmental Medicine and Epidemiology, Department of Internal Medicine, School of Medicine, and Institute of Toxicology and Environmental Health, University of California, Davis (E.B.G., S.J.S.).
Maternal and Child Health and Epidemiology Programs, School of Public Health, University of California, Berkeley (B.E.).
Address reprint requests to Bill Lasley, PhD, Institute of Toxicology and Environmental Health, University of California, Davis, CA 95616-8615.
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ever, this methodology has significant limitations. Although IRMAs can be highly specific for the hCG molecule, they lack specificity for pregnancy events because authentic hCG is secreted by the pituitary and can be detected in nonpregnant individuals [Odell and Griffin, 1987, 1989]. The original laboratory methodology used by Wilcox et al. [1988] required the extraction of 4 mL of urine for each sample; being so labor intensive, this original IRMA was impractical for large-scale application. The direct IRMAs that are currently available [O'Connor et al., 1988; Taylor et al., 1992] require far less laboratory effort, but the use of radioactive materials makes these assays relatively expensive. Because large epidemiologic studies must consider laboratory costs as a factor in the design, it is beneficial to reduce the number of samples that must be analyzed with expensive methodology. Elimination of urine samples that are clearly negative for hCG is an approach that can be taken to reduce costs without decreasing the sensitivity or specificity of the definitive assays.

In designing an approach for surveillance of EFL in the Semiconductor Health Study, a laboratory strategy was employed that enabled efficient evaluation of tens of thousands of urine samples. Collaboration was established with an experienced data management/programming team. A highly sensitive, but nonspecific, screening assay (immunoenzymometric assay, IEMA) was employed to identify samples that contained no detectable hCG and thereby could be eliminated from subsequent analyses. The IEMA was a combination or "combo" assay that utilized capture antibodies capable of detecting not only heterodimeric hCG, but also the hCG free b subunit and the hCG b fragment. Such combo assays are considered highly sensitive for detection of pregnancy events [O'Connor et al., 1994]. The IEMA was based on colorometric rather than radiometric signals and was designed to save time and resources without sacrificing sensitivity [Taylor et al., 1992]. The screening analysis was restricted to samples collected during a physiologic "window" of time in which hCG would be expected to have a trophoblastic source. Thus, the only samples analyzed were those collected in the 10 days that preceded and the 5 days that followed the onset of menstrual bleeding. During screening, samples from the assay windows were also analyzed for pregnanediol-3-glucuronide (PdG), a metabolite of progesterone, to provide information on whether or not the cycle was ovulatory. Cycles that appeared to be ovulatory and that were positive for hCG in the IEMA (defined later) were retested for hCG with duplicate assessments by IRMA. The IRMA was configured to detect only heterodimeric hCG, in order to maximize the specificity of the definitive assay. Finally, additional biomarkers of reproductive function were measured in the IRMA-positive cycles to demonstrate that ovulation occurred (and therefore pregnancy was possible) and to identify other endocrine signals that would confirm an early pregnancy. These biomarkers included the combined measure of luteinizing hormone (LH) and its alpha subunit (LH/LH alpha) [Clough et al., 1992] as well as the estrone conjugates (E_1C) and PdG [Munro et al., 1991].

METHODS

Samples and Sample Handling

Urine samples were transported to the laboratory and logged using a bar code reader (model 162, Intermec, Lynnwood WA), which identified each sample and created the database fields for subsequent assay results. A series of 100 consecutively numbered bar-code labels (AC labels, Fremont, CA) was set aside for each subject,

and a unique human subject number (HSN) was assigned to each series. Vinyl labels rather than paper labels were used because they were tear resistant and remained glued to vials after repeated freezing and thawing. Appropriate bar-code labels were affixed to 5 mL collection vials. Label data included the sample sequence number in bar-coded and uncoded form and the collection date in uncoded form. Vials were placed by the subject in monthly collection boxes with a calendar in the bottom, to ensure that the vials were placed correctly in the box. When a box was returned, the bar-code label was read into a relational database written in the Paradox® Programming Language (Borland International, Scotts Valley, CA). Missing or empty vials were flagged at entry. "Cycle day 1" of each menstrual cycle (first day of menstrual bleeding) was determined from the daily diary completed by each study subject [Gold et al., 1995]. The cycle days that corresponded to the collection dates were manually entered into the database, and a red tag was placed on the cap of the sample, which was collected on cycle day 1 as it was transferred to the "megabox," which contained all of the samples from an individual subject. Samples from known conceptive cycles (normal pregnancy or clinical spontaneous abortion) were not analyzed. All samples were stored frozen in an 8 × 8 × 16 ft. walk-in freezer at −20°C until they were analyzed.

Megaboxes were retrieved from the freezer and the samples representing the 16-day screening window (i.e., 10 days before and 5 days following the day of the onset of each menstrual period) were logged into the data base to create a text file listing each sample and its location in the assay plate format. Bar codes of each sample were re-scanned to create assay log sheets and appropriate labels for each microtiter plate. The samples for the screening window were placed in a refrigerator (4°C) and left overnight to thaw, while megaboxes containing the remainder of the frozen samples were returned to the freezer. The following morning the thawed samples were inverted once to mix the contents and were placed on a robotic pipetting machine (ICN Micromedic Accuflex Plus: ICN Biomedicals, Inc., Costa Mesa, CA) for sample transfer and dilution.

An aliquot of each urine sample (100 μL) was pipetted directly into microtiterplate wells for the IEMA. A second aliquot (14 μL) was delivered with 700 μL of distilled water into a 1 mL plastic minitube (DBM Scientific, San Fernando, CA) and stored for up to 1 week prior to analysis for creatinine and PdG. Megaboxes from which the samples had been taken were retrieved from the freezer, and the thawed samples were returned to their appropriate megaboxes until needed for a subsequent assay. All samples from windows that were positive for PdG (i.e., had evidence of ovulation) and positive for hCG by IEMA, and a subset of samples from windows that were positive for PdG but negative for hCG by IEMA, were retrieved at a later date, rethawed, and repipetted in an identical fashion for the IRMA analysis, which was performed in duplicate. When windows were positive for hCG by IRMA, all samples in the cycle were thawed and diluted for PdG, E_1C, LH/LH alpha, and creatinine analyses.

Immunoenzymometric Assay (IEMA) and Immunoradiometric Assay (IRMA) for hCG

The IEMA for hCG was adapted from Taylor et al. [1992] and O'Connor et al. [1988]. The IRMA was adapted from O'Connor et al. [1988]. Briefly, standards for both assays were prepared from intact hCG (CR127, Columbia University, New

York, NY). Standard solutions of 1.6, 0.8, 0.4, 0.2, and 0.1 ng hCG/mL were prepared in "coarse filtered" 1:1 pre-pubescent male urine (PPMU) and 50 mM phosphate buffered saline (PBS, pH 7.0). The zero standard was 1:1 PPMU:PBS with no added hCG. Controls with hCG concentrations of 0.2 and 0.8 ng hCG/mL were prepared similarly. Because of the differences in the matrix effect of PPMU in the zero standard and the matrix effect of urine samples from individual women, negative values for the hCG concentrations (less than 0% binding compared with PPMU) were obtained in some baseline samples from study subjects. The microwells were filled to a 200 μL total volume at all steps in both assays. The samples were thawed at room temperature (RT), inverted several times to ensure thorough mixing, and allowed to settle overnight at 4°C. Aliquots of 100 μL were added to each of the sample wells with the robotic pipetting station. There were 48 sample wells per plate for each IEMA or IRMA. Standards and controls were placed in minitubes and pipetted, by hand, with 12 channel pipettors. Both the standards and the controls were added to each plate in quadruplicate. All of the assay plates were washed between steps with 0.15 M NaCl, 0.05% Tween 20 with an Elcawash (Elcatech, Winston-Salem, NC) 96 tip plate washer. For the IRMA, radioactive solutions were aspirated and plates were washed with a 12 channel Nunc Immunowash plate washer (Nunc, Naperville, IL).

For the IEMA, Nunc Maxisorb flat-bottomed microplates were coated with a solution of monoclonal antibodies B109 (2.5 μg/mL) and B204 (5.0 μg/mL) in 0.2 M $NaHCO_3$ (pH 9.6). These capture antibodies, which detect heterodimeric hCG (B109) as well as the hCG free b subunit and hCG b fragment, had been frozen at −20°C overnight and were thawed at RT. The coating incubation time was 48 hr. The remaining binding sites on the microwell surfaces were blocked with 1% bovine serum albumin (BSA), 10 mM PBS (pH 7.5) for 1–4 hr at RT or overnight at 4°C. Immediately prior to the addition of samples, standards, or controls, 100 μL of 0.5 M PBS (pH 7.0) was added to each well. This equilibration step was carried out overnight at RT. The detection antibody was B108, an anti-hCG b subunit monoclonal antibody that was biotinylated (b-B108) using a method adapted from Bayer et al. [1986]. A 25 ng/mL solution of b-B108 in 0.1% bovine gamma globulin (BGG), 10 mM PBS (pH 6.0), and 10 mM EDTA was added to each IEMA plate and incubated overnight at 4°C. On the final day of the assay, alkaline phosphatase-streptavidin (APS, Zymed Laboratories, South San Francisco, CA), was diluted, first 1:1 with glycerol, and then 1:1000 in 1.0 M NaCl, 0.1% Tween 20, and 10 mM Tris-HCl (pH 7.5) before incubation for 1 hr at RT. A substrate solution of 1.0 mg/mL p-nitrophenylphosphate (PNPP) in 1.0 M diethanolamine-HCl (DEA-HCl, pH 9.0), and 1.0 mM $MgCl_2$ was then added and allowed to incubate until the darkest standard well reached an optical density (OD) of 1.2. The ODs of the IEMA plates were measured using a Dynatech MR600 Microplate reader (Dynatech Laboratories, Alexandria, VA) at 410 nm (test) and 570 nm (reference). The OD information on the plate was used to construct a standard curve from the standards and to calculate concentrations for quality control samples and unknowns with the EIA© software program written by Dr. Dennis Stewart.

The procedure for the IRMA differed from the IEMA procedure in the following ways. First, 12 Dynatech Removawell strips (96 wells total) in a Removawell holder were used instead of the standard one piece microplates used in the IEMA. These Removawell plates were coated with 2.5 mg/mL B109 in 0.2 M $NaHCO_3$ (pH 9.6). The IRMA procedure was the same as the IEMA procedure through the sample/

standard incubation step. The signal antibody (B108) was iodinated with $Na^{125}I$ in presence of choramine-T as described in Greenwood et al. [1963], at a working concentration of about 200,000 cpm/mL in 0.1% BGG, 10 mM PBS (pH 7.5), and 10 mM EDTA, and was incubated overnight at 4°C. On the final day of the IRMA, the Removawell strips were aspirated, washed, and broken into individual wells. The individual wells were then counted with a Beckman 5500 gamma counter (Beckman Instruments, Palo Alto, CA) using a Beckman Data Transporter to capture the counts. The raw counts stored in the Data Transporter were edited into discrete, microplate-sized blocks, with 96 counts corresponding to a complete plate, including standards, controls, and samples. The 96 count blocks were then converted to the plate data file format and stored within the database.

The configuration of each microtiterplate was formatted to contain quadruplicate zeros, quadruplicate standard curves, two quadruplicate internal controls, and 48 unknown samples. Each plate was treated as an individual assay in terms of quality control, and assays not meeting the predetermined quality control conditions were repeated. For the screening assay (IEMA), samples were analyzed once, in singleton, whereas samples in the IRMA were analyzed twice, that is, in singleton but in two separate assays. The criteria for quality control were based on the calculated sensitivity as well as the measured values for the internal controls in each assay according to Taylor et al, [1992].

The calculated sensitivity for the hCG assays was defined as the minimum mass detected with 99% confidence and was calculated for each assay by the following formula: sensitivity = 3 × standard deviation of the four zeros divided by the slope of the first two standards. Individual zero values were considered outliers if an individual value fell outside of three standard deviations of the mean of the other three. The mean sensitivity of the IEMA was 0.02 ng hCG/mL (n = 649), with coefficients of variation of 20.6% and 10.4% at 0.2 and 0.8 ng hCG/mL, respectively. The mean sensitivity of the IRMA was 0.035 ng hCG/mL (n = 747) with coefficients of variation of 13.9% and 15.8% at 0.2 and 0.8 ng hCG/mL, respectively. For both the IEMA and the IRMA, each pair of internal controls (CR127 at two doses) were compared with the previous average. Assays for which both of the internal controls fell outside the mean plus or minus the 20% of the mean of previous assays were rejected and repeated regardless of the sensitivity. Fifty of the IEMA and 78 of IRMA assays did not meet quality control standards and were repeated in duplicate.

Treatment of the IRMA and IEMA raw data was nearly identical once the raw optimal densities or count values were converted to plate data files. The EIA® program transformed the data (logit-log transformation) and used linear regression analysis to convert the raw counts or ODs in the plate data files into print files that contained hCG concentration data. In generating the print files for the assays, the operator also linked the concentration data to the actual sample identities contained in the text files.

Enzymeimmunoassays for Pregnanediol-3-Glucuronide and Estrone Conjugates (PdG and E_1C EIAs)

The assays for PdG and E_1C were performed as previously described by Munro et al. [1991]. Briefly, previously diluted samples were pipetted by hand directly into 96-well microtiter plates that had been coated with capture antibodies for the respec-

tive steroid conjugates. The unknowns were placed in the most central rows (rows C, D, E, and F); therefore, a maximum of 48 unknowns was analyzed on a single plate. In both assay systems, the upper- and bottom-most rows of wells were not used due to uncontrollable plate drift in these outside wells. Standards and two internal controls, all in duplicate, were placed in rows immediately above (row B) and below (row G) the unknowns. The standards were placed in an ascending pattern in row B and a descending pattern in row G in order to control for plate drift. The functional sensitivities of the assays were $\leq$10 ng/mL for E_1C and $\leq$0.2 μg/mL for PdG using 40 μL and 20μL of the 1:50 dilution of urine, respectively. The interassay coefficients of variation were 20.2% and 18.2% for PdG and 13.1% and 16.8% for E_1C using the high and low urine pools, respectively.

LH/LH Alpha Assay

The LH/LH alpha assay was performed as described by Clough et al. [1992], utilizing a total alpha gonadotropin configuration that detects both intact LH and the free alpha subunit of all three pituitary glycoproteins. The sensitivity of this assay was <2 fM with inter-assay and intra-assay coefficients of variation of 18.2% and 6.1% for the high and low urine pools, respectively.

Definitions

Cycles were defined as having a positive window during screening when there was evidence of ovulation as indicated by a PdG concentration $\geq$3 μg PdG/mg Cr in four or more samples, and a rise of hCG was detected in the IEMA that was >0.15 ng hCG/mg Cr on 2 of 3 consecutive days. The criteria for identification of EFL among the IEMA-positive cycles included: 1) confirmation of the rise in hCG with the IRMA; 2) evidence that the rise of hCG occurred in an ovulatory cycle, as confirmed by the presence of an LH/LH alpha peak and/or an E_1C peak followed by a sustained rise of PdG; and 3) evidence that the rise of hCG occurred during a time in the cycle when implantation could occur.

A computer algorithm was used to identify EFL using both steroid and IRMA data. Any sample with a creatinine concentration <0.15 mg Cr/mL was not included for consideration, and all measurements were rounded to two significant digits. A cycle was determined to be potentially positive for EFL if at least 11 samples in the 16-day window were available for analysis (i.e., no more than 5 missing samples) and 2 of 3 consecutive samples had >0.15 ng hCG/mg Cr as measured in either individual IRMA or as determined by the arithmetic mean hCG value for the duplicate IRMAs. The two samples that were positive in the IRMA did not have to be the same two samples that were positive by IEMA because the two assays do not detect the same molecules. In order to remain classified as potentially positive for EFL, the cycle must also have had evidence of a midcycle gonadotropin surge (one or more samples in which the E_1C concentration was >30 ng E_1C /mg Cr or the LH/LH alpha concentration was >250 fM LH/mg Cr) and evidence of a luteal phase (the PdG concentration was $\geq$3 μg PdG/mg Cr in four or more samples).

Cycles that were classified as potentially positive for EFL by the computer algorithm were then evaluated individually by a single observer (BLL), who was blinded with respect to the subjects' identities, work sites, work groups, and exposures. First, the cycle was assessed for evidence of ovulation. When multiple peaks of E_1C or LH/LH alpha were observed, the approximate day of ovulation was iden-

tified as the day when LH/LH alpha and E_1C peaks coincided, and were followed by a rise of PdG that was ≥3 μg PdG/mg Cr for 4 or more days. If either of the E_1C or LH/LH alpha peaks were missing, then the peak of LH/LH alpha or E_1C that immediately preceded a sustained PdG rise, as defined earlier, was identified as the approximate day of ovulation. The length of the luteal phase, as measured from the day of the LH/LH alpha peak and/or E_1C peak to the first day of menses, must have been at least 8 days for the cycle to be classified as ovulatory. The final criterion for EFL was met if the rise of hCG occurred more than 8 days after the LH/LH alpha peak and/or E_1C peak. This time period for implantation was chosen because it corresponds to the time of the cycle when the joining of maternal vessels to the trophoblast is known to occur, that is, 7–8 days following ovulation [O'Rahilly and Muller, 1987]. All cycles not classified as EFL by the observer were classified as nonconceptive cycles.

RESULTS

A total of 70,119 urine samples were obtained from 448 women during 2,552 menstrual cycles, and of these cycles 2,036 (80%) were evaluated by the IEMA. Screening could not be performed because of "missing" samples in 516 cycles. Altogether, 6,834 samples were recorded as missing. All urine samples were missing for 21 cycles, and an additional 450 cycles could not be analyzed because they were incomplete cycles collected at the beginning or end of the study. At least one sample was missing in 1,349 (52.9%) of the menstrual cycles, but only 45 of the urine sets that were collected in complete cycles could not be screened for hCG because there were fewer than 11 samples in the 16-day assay window.

Of the 2,036 cycles screened for hCG with the IEMA, 448 cycles (22%) met the criteria for a positive screening window (PdG ≥3 μg/mg Cr in 4 or more samples and hCG > 0.15 ng/mg Cr in 2 of 3 consecutive samples) and were evaluated subsequently with the IRMA (Fig. 1). From the set of 1,588 cycles that were negative for hCG in the IEMA, the first 481 cycles with positive PdG also were tested with the IRMA. All of these cycles were confirmed to be negative for hCG.

Only 38 of the 448 IEMA positive cycles (8%) were classified as positive for hCG following analysis with duplicate IRMAs. After additional evaluation of the PdG, E_1C, LH/LH alpha, and diary data, 17 of these cycles were determined not to be EFL (Table I). In two of these cycles (263–5, 327–2), exogenous hCG was given for treatment of infertility. One subject (subject 62) contributed three cycles in which baseline levels of hCG were consistently elevated. All three cycles had evidence of ovulation and vaginal bleeding following progesterone withdrawal, which is indicative of consecutive nonconceptive cycles. The source of hCG was not determined, and follow-up by the subject's personal physician was recommended. Other hCG-positive cycles were excluded because the source of hCG was a pregnancy that terminated in clinical spontaneous abortion during a *previous* cycle (21–2, 145–2, 1002–3) or the pregnancy occurred in the presence of an intrauterine contraceptive device (1011–2). Other reasons for exclusion included the appearance of hCG in an anovulatory cycle (94–2, 1050–6) or in cycles earlier than luteal day 8 (76–6, 230–3).

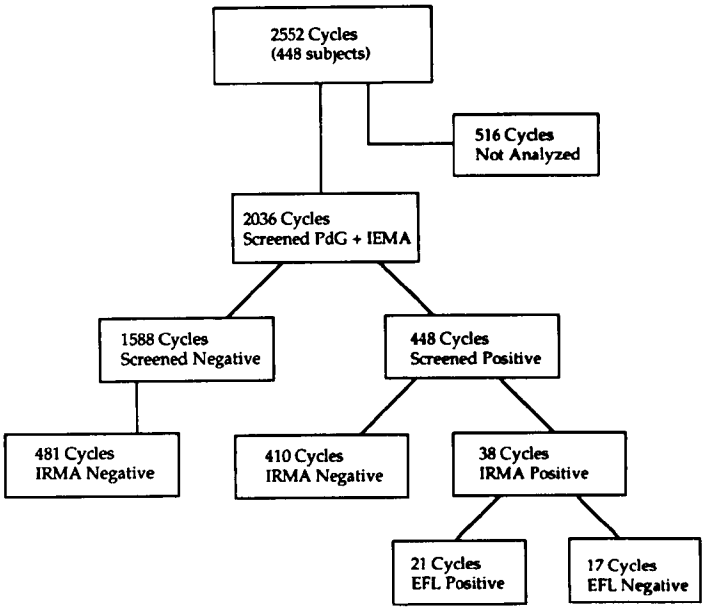

Fig. 1. Flow chart for evaluation and classification of urine samples in the prospective study of early fetal loss.

DISCUSSION

The results of this study show clearly that daily urine samples can be collected from a large population of working women and can be utilized to measure biomarkers of reproductive function. The compliance of workers with the urine collection protocol was excellent; less than 10% of samples were found to be missing. In only 1 of 2,552 cycles collected was there evidence that one urine sample was distributed in several collection vials. Nevertheless, more than 20% of cycles were incomplete and could not be analyzed. The procedures developed in this study for identification and confirmation of EFL require entire cycles for analysis, including the periovulatory period, the menstrual period, and the early follicular phase of the subsequent cycle. The incomplete cycles obtained in this study were collected primarily at the beginning or end of the investigation when one or more of these components was missing. This situation arose because workers were instructed to begin urine collection on the day following the baseline interview and to discontinue collection at the end of a calendar month. If in future studies the instructions for urine collection were changed so that women entering the study began and ended collection on the fourth day after beginning a menstrual period (cycle day 5), virtually all cycles would be eligible for analysis.

The computerized database management system facilitated three critical tasks: accurate logging and tracking of samples (e.g., assigning unique study identifiers, tracking sample storage locations); reliable integration of information from different study components (e.g., field questionnaire results and laboratory assay results); and producing written and graphic reports for monitoring study progress and carrying out data analyses. The key components of the system (bar-code labels, bar-code reader,

TABLE I. Characteristics of hCG-Positive Cycles that Were Determined not to be EFL in Semiconductor Health Study

Cycle no.	Reason for exclusion
263-5	Subject treated with human menopausal gonadotropins and hCG for infertility
327-2	Subject treated with human menopausal gonadotropins and hCG for infertility
332-2	Anovulatory cycle
62-3	E_1C, PdG, and LH/LH alpha consistent with repetitive nonconceptive cycles; hCG from undetermined nontrophoblastic source
62-6	
62-9	
21-2	hCG from clinical spontaneous abortion in antecedent cycle
145-2	hCG from clinical spontaneous abortion in antecedent cycle
1002-3	hCG from clinical spontaneous abortion in antecedent cycle
1011-2	hCG in presence of intrauterine contraceptive device
94-2	Anovulatory cycle
1050-6	Anovulatory cycle
76-6	hCG detected before luteal day 8
230-3	hCG detected before luteal day 8
256-5	Endocrine profiles uninterpretable; subject treated with prednisone
1052-3	Missing samples prevent interpretation of hCG

high-speed microcomputer and database management software) are widely available and relatively inexpensive. Furthermore, continued technological development promises to make such hardware and software even cheaper, faster, and more flexible in the future.

The IEMA proved to be a useful screening assay for assessing large numbers of cycles. As predicted during the development of the assay [Taylor et al., 1992], the majority of cycles (78%) were identified as nonconceptive during screening and were eliminated from further evaluation. Nevertheless, more than 90% of the IEMA positive cycles were negative for hCG when evaluated subsequently by IRMA. The large number of false-positive cycles was due in part to the deliberately low cutoff point (>0.15 ng hCG/mg Cr in 2 of 3 consecutive days) for designation of a positive window and in part because the capture antibodies for the IEMA recognize the hCG free b subunit and hCG b fragment as well as heterodimeric hCG, which is the only molecule measured in the IRMA.

There is controversy concerning the most appropriate hCG assay for detection of EFL. In this study a conservative approach was taken in which detection of heterodimeric hCG was required for definitive classification of a cycle as having EFL. The original IRMA utilized by Wilcox et al. [1988] also had primary specificity for the heterodimeric hCG molecule. However, it has been suggested that a combo assay, such as the IEMA utilized in the present study, may be more appropriate for detection of EFL because of its superior sensitivity in detecting the onset of normal pregnancy [O'Connor et al., 1994].

To maximize the possibility that all conceptive cycles would be recognized in the present study, the cutoff point for positive hCG in the IRMA was set lower that those of other recent investigations. For example, O'Connor et al. [1988] defined conceptive cycles as those with urinary concentrations of hCG exceeding 0.25 ng

hCG/mL for 3 consecutive days, and Taylor et al. [1992] required that hCG levels reach 0.25 ng hCG/mg Cr for 3 of 4 successive days. The more liberal definition used in the present study contributed to the 45% false-positive rate of EFL as ascertained by hCG measurements alone (17 of 38 cycles; see Fig. 1). However, the additional requirements for evidence of ovulation and an appropriate time of appearance of hCG effectively identified nonconceptive cycles. There is an obvious tradeoff between variables such as the sensitivity of the assay and the detection level at which the hCG discriminator is set and the number of false-positive cycles that must be evaluated using other biomarkers. There are insufficient data to make firm recommendations on the optimum strategy for detecting EFL [O'Connor et al., 1994]. Nevertheless, the results of this population-based study clearly demonstrate that as discriminator levels are lowered, nontrophoblastic hCG will be detected in nonconceptive cycles and must be recognized in order to avoid misclassification of cycles as having EFL.

In the present study, 21 of 57 (36.8%) conceptions were EFL [Eskenazi et al., 1995], as compared with 22% of all conceptions identified as EFL in the only previous large population-based study [Wilcox et al., 1988]. This difference may be related to physiological and sociodemographic differences between the populations of women (e.g., age of the cohort), but laboratory methodology also could be involved. Although the cutoff point for positive hCG was higher in the present study (0.15 ng hCG/mg Cr) than in the study of Wilcox et al. (0.025 ng hCG/mL), in the present study only 2 days of positive hCG were required to classify a cycle as positive for hCG, whereas 3 days of positive hCG were required in the previous study. The possibility that the criterion used in the present study could lead to misclassification of nonconceptive endocrine events as EFL is suggested by the high percentage of IRMA-positive cycles that were subsequently classified as nonconceptive when other reproductive biomarkers were considered. We cannot rule out the possibility that nontrophoblastic hCG, if present during the implantation window, would have been misinterpreted as a sign of early pregnancy in the present study. Such misclassification could also contribute to discrepancies between the results of the present study and the previous study of Wilcox et al. [1988].

The fecundability of the population that was studied during this investigation [Eskenazi et al., 1995] was approximately one third that of the women studied by Wilcox et al. [1988]. The reasons for lower fecundability in this population are unknown, but abnormalities of ovarian function may be involved. It is clear that many of the cycles that were false positive for EFL were initially misclassified because of inappropriate secretion of hCG from a nontrophoblastic source. Some of these false-positive EFLs occurred in ovulatory cycles, and others were associated with anovulation. These abnormal cycles would not have been recognized without the use of additional biomarkers of ovarian and pituitary function. The appearance of such cycles may be expected in women exposed to reproductive hazards in the workplace or in the environment, and also may be found in heterogeneous groups of unexposed women. Future studies should be planned to include laboratory protocols and procedures for data analysis that are capable of evaluating altered ovarian function or other endocrine abnormalities.

The complexity of the hormone patterns in the present study required subjective assessment of cycles with suspected EFL. Because the evaluator was blinded to the subjects' identities, work groups, and exposures, this process did not bias the outcomes reported in the study. However, subjective assessments cannot be standard-

ized, and their application may lead to difficulties in comparisons between studies. Objective algorithms are needed to identify anovulatory cycles and the day of ovulation based on urinary hormone measurements. Although not used in this study, such algorithms have been published [Baird et al., 1991], and their application in the future will lead to more standardized definitions of EFL that are based on biomarker data.

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