

# Total Urinary Follicle Stimulating Hormone as a Biomarker for Detection of Early Pregnancy and Periimplantation Spontaneous Abortion

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Total concentrations of follicle stimulating hormone (FSH) were evaluated in daily urine samples from conceptive and nonconceptive menstrual cycles by measurement of the FSH beta subunit following treatment of the samples to dissociate the FSH heterodimer. Samples were self-collected by normal subjects during cycles in which daily blood samples also were obtained. Daily blood and urine specimens were collected prospectively from 10 subjects in conceptive cycles, which led to normal pregnancies, and from 10 subjects with bilateral tubal ligations to provide control samples from nonconceptive cycles. Mean serum and urinary FSH concentration profiles were parallel in both groups following ovulation and during the first 9 days of the luteal phase. Mean values for both serum and urinary FSH rose significantly above the postovulatory baseline by 10–12 days following the midcycle luteinizing hormone (LH) peak in nonconceptive cycles, but did not rise at any time following ovulation during conceptive cycles. Following regression analysis of the changing FSH concentrations between days 9–14 post-LH surge in conceptive cycles, a slope of  $\leq 0.02$  ng FSH/mg creatinine/day was selected as a cutoff point to identify conceptive cycles. There was a high concordance between the day of LH peak in serum and the day of FSH peak in urine. Therefore, in applying the algorithm, the day of FSH peak in urine was used to determine the days for which the FSH slope would be calculated, i.e., days 9–14 post-FSH peak in urine. The sensitivity and specificity of the change in urinary FSH concentrations to detect pregnancy in a different set of 55 cycles were found to be 88.9% and 89.3%, respectively. All six cases of early fetal loss in the sample set were correctly identified. These results suggest that urinary FSH can be used as an additional biomarker for the verification of early pregnancy in prospective epidemiologic studies in which early fetal loss is a suspected outcome. *Key words:* biomarker, early fetal loss, FSH, menstrual cycles, pregnancy, spontaneous abortion. *Environ Health Perspect* 105:862–866 (1997)

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Detection of spontaneous abortion in the periimplantation period is a challenging problem for reproductive epidemiologists. This pattern of reproductive failure, which has been termed early fetal loss (EFL), has been observed in 22% of pregnancies in normal unexposed women (1). There is a strong suspicion that this outcome may be more frequent when pregnant women are exposed to reproductive toxicants in the workplace or other environments (2). Recognition of EFL in population-based studies relies primarily on the measurement of human chorionic gonadotropin (hCG) in self-collected urine samples (3). The initial studies of normal women demonstrated the feasibility of this approach for detecting EFL (1). However, clinical studies of research subjects, in which circulating levels of hCG were measured on a daily basis, have suggested that in some cases of EFL there may not be adequate levels of hCG for detection of pregnancy (4). In population-based field studies, false positive detection of EFL was demonstrated to result from the presence of hCG in the urine of nonpregnant women (3).

The complexities involved in detection of EFL have necessitated the use of other

reproductive biomarkers to minimize the number of false positive and false negative results. The application of an assay for luteinizing hormone and its  $\alpha$  subunit (LH/LH  $\alpha$ ) has enabled us to identify the day of ovulation (5), which obviates concerns that false positive results may be caused by pituitary release of hCG during the midcycle gonadotropin surge (6,7). Measurements of urinary pregnanediol-3-glucuronide (PdG) in combination with the LH/LH  $\alpha$  subunit assay can be used to recognize the luteal phase of the cycle and to define an implantation window in which hCG excretion is likely to be a result of pregnancy (3). When hCG is detected outside of this window, it is unlikely to have been produced by a conceptus. Despite the use of multiple biomarkers to detect and interpret early pregnancy signals, it is still likely that many pregnancy events will be missed and that false positive pregnancies also may be misidentified.

The purpose of the present study was to investigate the utility of a urinary assay for follicle stimulating hormone (FSH) as an additional biomarker for detecting early pregnancy. The rationale for this approach is based on our understanding that the

increase in FSH production that occurs normally in the midluteal phase of the menstrual cycle is inhibited by the ovarian response to hCG immediately following implantation. The development and validation of a sensitive assay for total urinary FSH provided the opportunity to establish the value of this biomarker in confirming early pregnancies that are detected with hCG and to explore the possibility that identification of some early losses, which otherwise would be questioned, can be substantiated by the FSH profile.

## Materials and Methods

**Subjects.** Serum samples and first void early morning urine samples were collected daily during 65 menstrual cycles from 40 women aged 21–38 years, who were receiving artificial insemination with nonfrozen donor semen. All subjects in this study gave informed consent prior to participation. The women had normal reproductive function and received no medications during the study. They either had no sexual partner or an azoospermic partner. The procedures for recruitment and insemination of these subjects have been reported previously (4,8). These subjects are a subset of a larger study population and were selected for this data set because they all conceived a pregnancy in the absence of hormone therapy. Urine samples were collected by the subjects throughout the cycle and were frozen immediately without preservatives in their home refrigerator freezer ( $-10^{\circ}\text{C}$ ). They were delivered to the laboratory at the end of the cycle and were stored at  $-20^{\circ}\text{C}$ . Blood samples were collected at the clinic by venipuncture between 10:00 A.M. and 1:00 P.M., beginning on the expected day of ovulation and continuing until the first day of menses or until pregnancy was diagnosed by standard clinical procedures. The samples were allowed to clot, and the serum was stored

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frozen at  $-20^{\circ}\text{C}$  until analyzed. Ten of the 65 cycles that resulted in a normal pregnancy (positive control cycles) provided data which were used to compare the FSH profiles in serum and in urine. Ten healthy women between the ages of 33 and 47 years with bilateral tubal ligations (BTL) were recruited as the nonpregnant control group. These BTL subjects provided daily serum samples and early morning urine samples during one ovulatory menstrual cycle. The mean values of serum and urinary FSH concentrations in the BTL group and in the positive control cycles were compared by Student's *t*-test within each day following the LH peak in serum. All urine samples had been thawed at least twice prior to the present study in order to perform other analyses. Serum measurements of LH, FSH, estradiol ( $\text{E}_2$ ), progesterone (P), and hCG were evaluated to document that each cycle was ovulatory or conceptive.

**Assays.** Serum FSH, LH,  $\text{E}_2$ , and P concentrations were measured using commercial kits (Diagnostic Products Corp., Los Angeles, CA). Serum hCG concentrations were measured by immunoradiometric assay as described by Lasley et al. (3). The assay for total urinary FSH, which is based on heat dissociation of the FSH heterodimer and measurement of the FSH  $\beta$  subunit, was performed as follows. Briefly, microtiter plates (Nunc-Immuno Plate, Maxisorb, Applied Scientific, San Francisco, CA) were coated with 200  $\mu\text{l}$  of 10  $\mu\text{g}/\text{ml}$  monoclonal anti- $\beta$  hFSH antibody (FS2-4A10-G10, Scantibodies Lab, Santee, CA) in 0.2 M sodium bicarbonate buffer, pH 9.6, and incubated for 6 hr or overnight at room temperature (RT). Unbound sites in the wells were then blocked by overnight incubation with 250  $\mu\text{l}$  of 1% casein, 0.05% Tween 20, and 0.1% sodium azide in 10 mM PBS, pH 7.5. On the next day, 100  $\mu\text{l}$  of 0.5 M phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% sodium azide, pH 7.5, were added to every well. Urine samples or internal controls were transferred to polypropylene minitubes and placed into boiling water 2 min to dissociate the FSH subunits. Then, 100  $\mu\text{l}$  treated urine samples, internal controls or  $\beta$  FSH subunit standard (provided by the National Hormone and Pituitary Program) was added to each assigned well and incubated overnight at RT. Two hundred microliters polyclonal rabbit anti-hFSH- $\beta$  antisera (9) was diluted 1:5,000 in buffer B (0.1%  $\beta$  gamma globulin, 1% polyethylene glycol 8,000, 0.05% Tween 20, and 10 mM EDTA in 10 mM PBS) and added to each well before overnight incubation at RT. The next day, 200  $\mu\text{l}$  biotinylated goat anti-rabbit IgG (Bio-Rad 170-6401; Bio-Rad,

Richmond, CA) diluted 1:64,000 in buffer B was added and incubated for 2 hr at RT, followed by incubation with 200  $\mu\text{l}$  of alkaline phosphatase streptavidin diluted 1:2,000 in APS buffer (1.0 M NaCl, 0.1% Tween 20 in 10 mM Tris-HCl, pH 7.5) for 1 hr at RT. Finally, 200  $\mu\text{l}$  of 1 mg/ml *p*-nitrophenyl phosphate (pNPP) in substrate buffer (1.0 mM  $\text{MgCl}_2$ , 1.0 M diethanolamine-HCl, pH 9.0) was added to each well. The plates were emptied and thoroughly washed with ELISA wash (0.15 M NaCl plus 0.05% Tween 20) between each step. Color development was expected within 1 hr, and each plate was read on a microtiter plate reader (Dynatec Microplate Reader model MR600; Dynatech Laboratory Inc., Alexandria, VA) using the dual wavelength mode, 405 nm minus reference 650 nm. The absorbances were automatically transferred to an enzyme immunoassay data reduction program for curve fitting and data analysis. Urinary concentrations of FSH were indexed by the creatinine concentration in the urine sample (3).

The detection limit (0.32 ng/ml) of the  $\beta$  subunit FSH assay was defined as the concentration corresponding to the mean plus three standard deviations of zero standard. For statistical evaluation, concentrations of FSH below the detection limit were given a value of 0.32 ng/ml. In the groups of nonconceptive and conceptive cycles 2.1% and 9.7% of the values were below the detection limit, respectively.

**Urinary FSH algorithm for detection of conceptive cycles.** The development of the algorithm was based on the change in FSH secretion that takes place in the luteal phase of conceptive cycles. In response to circulating levels of hCG, the ovarian secretion of  $\text{E}_2$  is increased and, through negative feedback at the level of the hypothalamus and pituitary, the secretion of FSH is inhibited. In the absence of hCG,  $\text{E}_2$  falls in the late luteal phase and FSH secretion increases as the hypothalamus and pituitary are released from negative feedback. A difference in the slope of change in FSH secretion in the late luteal phase of conceptive and nonconceptive cycles could therefore be expected. The slope should be steeper in nonconceptive cycles than in conceptive cycles. Urinary FSH concentrations were indexed by creatinine, and the resulting values were used to calculate a slope for days 9–14 post serum LH peak in each cycle using regression analysis. At least four FSH values from this 6-day interval were required for calculation of the FSH slope.

To develop the algorithm, the slopes of urinary FSH concentrations in the 10 positive control cycles were compared with the FSH slopes in the 10 cycles from the BTL group. A cutoff value for the FSH slope

was selected, which correctly classified 9 of 10 cycles in each group as conceptive or nonconceptive. The algorithm was evaluated for sensitivity and specificity by comparison to the gold standard of pregnancy detection by serum hCG. The algorithm was used to classify the remaining group of 55 artificial insemination cycles. Of these 55 cycles, 11 cycles resulted in a normal term pregnancy, 10 cycles resulted in a clinical spontaneous abortion (SAB, defined as a pregnancy that terminated after day 23 post LH peak in serum), and 6 cycles resulted in EFL (defined as a pregnancy that terminated on or before day 23 post LH peak in serum). The remaining 28 cycles were classified as nonconceptive because there was no evidence of serum hCG in the luteal phase of the cycle.

## Results

Individual serum profiles of  $\text{E}_2$ , P, LH, and FSH demonstrated normal follicular phase lengths, clear evidence of ovulation, and normal luteal function in both the positive control cycles and in the BTL cycles (data not shown). The lengths of the follicular phases (first day of menstruation until the LH peak) ranged from 12 to 16 days in the conceptive cycles and from 10 to 16 days in the nonconceptive (BTL) cycles and were not significantly different. Following ovulation, the rise of mean serum P  $\pm$  standard error (SE) for the initial 6 days was similar in the two groups ( $2.90 \pm 0.97$  ng/ml/day and  $2.50 \pm 0.98$  ng/ml/day in conceptive and nonconceptive cycles, respectively).

The mean profile for total urinary FSH in the nonconceptive cycles paralleled the mean serum FSH profile (Fig. 1 A–C). The FSH profile in serum and in urine was characterized by rising levels in the early follicular phase, followed by a decline prior to the midcycle gonadotropin surge. Following the midcycle surge, there was a gradual decline in FSH concentrations until approximately day 10 post LH surge, when luteal phase levels of FSH began to rise. This pattern was clearer in the urinary profile when the FSH values were indexed by creatinine (Fig. 1C).

The patterns for mean serum and urinary FSH profiles were similar in the conceptive cycles to those in the nonconceptive cycles until the midluteal phase, when the FSH values in conceptive cycles remained flat rather than rising (Fig. 1D–F). This difference reached statistical significance on day 10 post LH peak in the serum profile (Fig. 1D) and on day 10 or day 11 in the urine profile (Fig. 1E, F). This effect of conception was related temporally to the rise of serum hCG and  $\text{E}_2$  (Fig. 2) and coincided with the day when hCG was first detected in urine (data not shown).

Urinary FSH values, indexed by creatinine, were used to develop an algorithm for identifying conceptive cycles in the late luteal phase. The slope of urinary FSH concentrations between days 9 and 14 post LH peak ranged from -0.48 to 0.37 ng FSH/mg creatinine/day in positive control cycles and was <0.02 in 9 of 10 cycles. In the BTL cycles, the slope ranged from -0.02 to 1.55 ng FSH/mg creatinine/day and was >0.02 in 9 of 10 cycles. Based on these data, we selected a urinary FSH slope of >0.02 ng FSH/mg creatinine/day as the cutoff point for identifying nonconceptive cycles. Otherwise, the cycle was classified as conceptive. There was a high concordance between the day of LH peak in serum and the day of FSH peak in urine. In the 20 positive and negative control cycles, the day was the same in 14 cycles (70%), and the urinary FSH peak was 1 day later than the serum LH peak in 4 cycles (20%) and one day earlier in 2 cycles (10%).

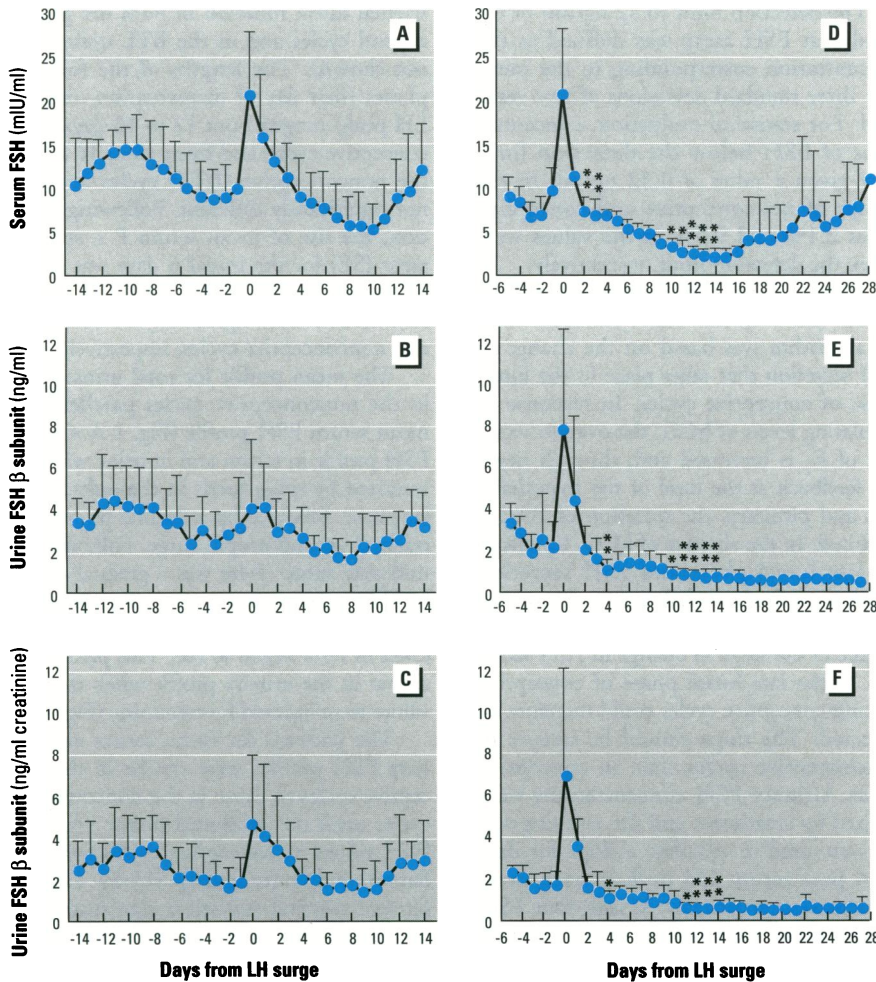
Therefore in applying the algorithm, the day of FSH peak in urine was used to determine the days for which the FSH slope would be calculated, i.e., days 9–14 post FSH peak in urine. In the combined groups of 75 cycles (controls and unknowns), the LH peak in serum and the FSH peak in urine was on the same day in 46 cycles (61.3%), and the urinary FSH peak was 1 day later in 23 cycles (30.7%) and 1 day earlier in 6 cycles (8.0%).

When the algorithm was used to classify the 55 unknown cycles, 9 of 11 normal conceptive cycles were identified, as well as 15 of 16 cases of spontaneous abortion, including the 6 cases of EFL (Table 1). Twenty-five of 28 nonconceptive cycles were also correctly classified. Therefore, in this sample set the slope of urinary FSH concentrations in luteal phase had a sensitivity of 88.9% to detect pregnancy and a specificity of 89.3%. The predictive value of a positive test was 88.9% and the predictive value of a negative test was

89.3%. The 55 cycles in the unknown sample set were provided by 35 subjects, four of whom provided cycles for the set of positive control samples. Of the remaining 31 subjects, 17 women provided a single cycle, 10 women provided two cycles, three women provided three cycles and one woman provided four cycles. To control for the lack of independence of multiple cycles provided by the same woman, a subset of single cycles was selected for testing with the algorithm. To form this set, all cycles from women who contributed to the positive control set were removed. All 17 subjects with single cycles were included in the set. When subjects provided multiple cycles and one of these was an EFL or SAB, the cycle with failing pregnancy was selected and the other cycle was removed. In the case of a subject with both an EFL and a SAB the cycle with EFL was selected. For all other subjects, the first cycle was used and the subsequent cycles were removed. In the resulting subset of 31 cycles, 18 of 18 conceptive cycles were correctly identified and 11 of 13 nonconceptive cycles were classified correctly. The sensitivity and specificity of the FSH algorithm to detect conceptive cycles in the subset of independent cycles were 100% and 84.6%, respectively (Table 2).

**Discussion**

Although much has been written concerning the methods for identification of EFL in populations exposed to environmental hazards, there has been limited experience in detecting this adverse outcome in large-scale epidemiologic studies. The largest study undertaken to date involved analysis of approximately 70,000 daily urine samples, collected in more than 2,500 menstrual cycles from 448 women employed in the semiconductor industry (3). Because of low fecundability in the study population, conclusive results were not obtained in this investigation (10). Nevertheless, valuable lessons were learned in the course of this study regarding the problems and pitfalls of interpreting urinary biomarkers of reproductive function in population-based studies. Based on the results of the initial studies of EFL in normal women (1), it was believed that a single assay for urinary hCG would be sufficient to detect EFL, providing that the assay was sufficiently sensitive and specific for the trophoblastic hormone. However, in the semiconductor study, it was necessary to use five separate assays for four different reproductive biomarkers in order to fully interpret the urinary hormone data (3). A highly sensitive but relatively nonspecific screening assay for hCG (11) was used to eliminate cycles with no evidence of hCG. Cycles positive for hCG in the screening



**Figure 1.** Mean values ± standard error for serum follicle stimulating hormone (FSH) and total urinary FSH during 10 nonconceptive cycles (A–C) and 10 conceptive cycles (D–F). FSH values for each day were compared between nonconceptive and conceptive cycles. \**p*<0.05; \*\**p*<0.01.

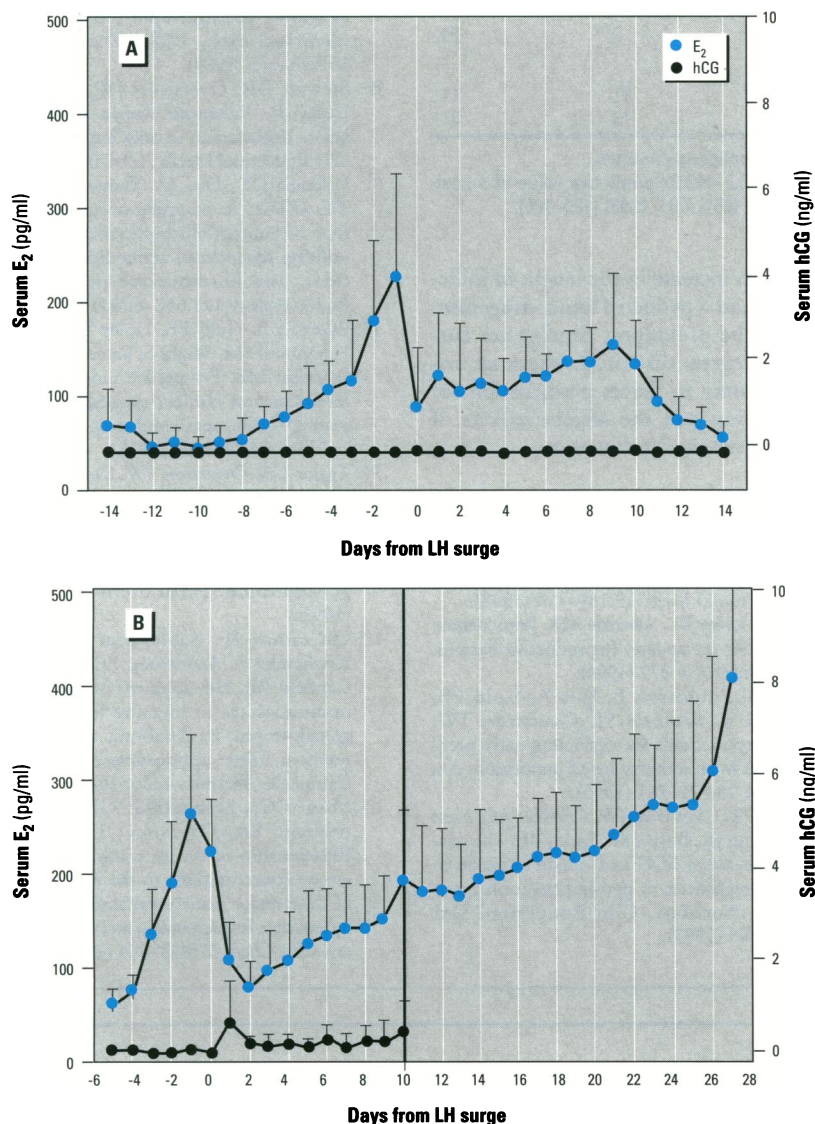
assay were reanalyzed with a specific assay for heterodimeric hCG (12). Only 21 of the 38 cycles confirmed to be positive for heterodimeric hCG were eventually classified as EFL (3). The detailed analyses of these cycles involved measurement of PdG (13) to

confirm that ovulation occurred in the cycle, as well as assays for LH/LH  $\alpha$  (5) and/or estrone conjugates (14) to determine the approximate day of ovulation. Only when hCG was shown to be present in an ovulatory cycle and during the time period when

implantation could take place was the cycle classified as having EFL (3).

The assay for total urinary FSH, which we have used in this study, provides an additional biomarker for early pregnancy. Like the changes in the ovarian steroid hormones E<sub>2</sub> and P and in the peptide hormone relaxin, the change in FSH secretion that takes place in the luteal phase of conceptive cycles results from the presence of circulating hCG and is a biological response to the pregnancy signal. The results of the present study suggest that this biomarker has good sensitivity for detecting early pregnancies, including those that terminate in EFL. Declining concentrations of FSH between 9 and 14 days post FSH peak are not sufficient evidence alone that conception has occurred because other factors could be responsible for this outcome. For example, acute physical or emotional stress could prevent or delay the expected rise of FSH and give the appearance of FSH suppression due to pregnancy. However, when immunoreactive hCG has been detected in a cycle, the failure of FSH to rise following the midcycle gonadotropin surge indicates that there has also been an ovarian response to a gonadotropic signal. This evidence of bioactivity is important for evaluation of transient low concentrations of hCG immunoactivity in urine samples.

Because FSH is released together with LH during the preovulatory gonadotropin surge, the FSH profile also provides information on the approximate day of ovulation and can be used to locate the implantation window. In our sample set, the peak of FSH in urine was on the same day as the LH peak in serum in more than 60% of cycles, and the two events were never more than 1 day apart. Our results suggest that the urinary FSH biomarker, in conjunction with measurements of urinary hCG, may be sufficient to demonstrate most EFL events. Cycles that demonstrate a clear gonadotropin surge and a rise of hCG in the implantation window, but no suppression of FSH, would require evaluation with other biomarkers. Either ovarian dysfunction or an abnormality of hypothalamic-pituitary function could result in such an outcome. However, measurements of ovarian steroid metabolites, such as estrone conjugates and PdG, would distinguish between these alternative explanations. The combination of a normal nonconceptive steroid hormone profile and a normal nonconceptive FSH profile would lead to the conclusion that the hCG was not of trophoblastic origin and that the cycle must be classified as nonconceptive. Our experience to date indicates that the presence of hCG in nonconceptive cycles should be



**Figure 2.** Mean values  $\pm$  standard error for serum estradiol (E<sub>2</sub>) and serum human chorionic gonadotropin (hCG) in 10 nonconceptive cycles (A) and 10 conceptive cycles (B).

**Table 1.** Sensitivity and specificity of total urinary follicle stimulating hormone (FSH) to detect conceptive cycles

| Slope of FSH concentrations days 9–14 post FSH peak *    | Pregnancy outcome |     |     |               | Total |
|----------------------------------------------------------|-------------------|-----|-----|---------------|-------|
|                                                          | Conceptive        |     |     | Nonconceptive |       |
|                                                          | Normal pregnancy  | EFL | SAB |               |       |
| Conceptive cycle ( $\leq 0.02$ ng FSH/mg creatinine/day) | 9                 | 6   | 9   | 3             | 27    |
| Nonconceptive cycle ( $> 0.02$ ng FSH/mg creatinine/day) | 2                 | 0   | 1   | 25            | 28    |
|                                                          | 11                | 6   | 10  | 28            | 55    |

Abbreviations: EFL, early fetal loss; SAB, spontaneous abortion; CI, confidence interval. Sensitivity (95% CI) = 88.9% (77.8–101.0); specificity (95% CI) = 89.3% (83.5–95.1); predictive value of a positive test (95% CI) = 88.9% (77.8–101.0); predictive value of a negative test (95% CI) = 89.3% (83.5–95.1).

**Table 2.** Sensitivity and specificity of total urinary follicle stimulating hormone (FSH) to detect conceptive cycles in a subset of cycles in which each subject contributed a single cycle

| Slope of FSH concentrations<br>days 9–14 post FSH peak  | Pregnancy outcome |     |     |               | Total |
|---------------------------------------------------------|-------------------|-----|-----|---------------|-------|
|                                                         | Conceptive        |     |     | Nonconceptive |       |
|                                                         | Normal pregnancy  | EFL | SAB |               |       |
| Conceptive cycle<br>( $\leq 0.02$ ng/mg creatinine/day) | 6                 | 5   | 7   | 2             | 20    |
| Nonconceptive cycle<br>( $> 0.02$ ng/mg creatinine/day) | 0                 | 0   | 0   | 11            | 11    |
|                                                         | 6                 | 5   | 7   | 13            | 31    |

Abbreviations: EFL, early fetal loss; SAB, spontaneous abortion; CI, confidence interval.

Sensitivity (95% CI) = 100% (100–100); specificity (95% CI) = 84.6% (65.3–103.9); predictive value of a positive test (95% CI) = 90% (83.3–96.7); predictive value of a negative test (95% CI) = 100% (100–100).

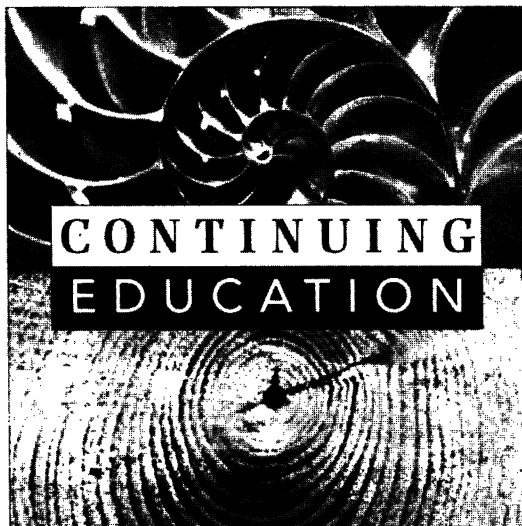
anticipated in any large-scale epidemiologic study of pregnancy wastage (3). However, in all such cases identified so far, the hCG was not found within an implantation window (3). Thus, there may be little necessity of employing additional biomarkers in hCG-positive cycles as long as the FSH peak can be identified and the FSH profile can be clearly interpreted as either normal or abnormal.

While the focus of this report is on pregnancy detection, the assay for total urinary FSH also can be used to evaluate ovarian function in nonconceptive cycles. The initiation of the next ovarian cycle is dependent on the release of pituitary suppression from the negative feedback of ovarian steroids in the luteal phase of the cycle. The resultant rise of FSH in the midluteal phase of the antecedent ovarian cycle is the initiator of the follicular phase of the following cycle. When this rise of FSH is absent or delayed, the recruitment of follicles for the next cycle will likewise be delayed. This outcome would be associ-

ated with an increase in the length of follicular phase and a period of hypoestrogenism that could be prolonged. Studies are currently in progress that utilize the total urinary FSH assay to detect perturbations of FSH secretion and the adverse effects of such events on ovarian function.

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