Monitoring Ovulation and Implantation in the Lion-Tailed Macaque (Macaca silenus) Through Urinary Estrone Conjugate Evaluations

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ABSTRACT

Urine samples were collected daily during ten nonfertile and four fertile ovarian cycles of four adult female lion-tailed macaques (Macaca silenus). Urine was analyzed for concentrations of total immunoreactive estrogen (E₂), estrone conjugates, and bioactive luteinizing hormones (LH). The estrone conjugates of selected samples were separated by high-performance liquid chromatography (HPLC) to evaluate the relative proportions of estrone glucuronide (E, G) to estrone sulfate (E, S) contributing to the sum total of the conjugate measured in the samples.

The estrone conjugate profile was found to accurately reflect the preovulatory estrogen peak in both nonfertile and fertile cycles as well as the early pregnancy increase which was found to be statistically significant on Day +14 postovulation (P=0.003). Estrone conjugate levels rose in the early follicular phase from 126.00 ± 24.07 (SEM) ng/mg creatinine to a preovulatory peak of 471.90 ± 62.95 ng/mg creatinine. Fertile cycles exhibited a postovulatory climb to a peak of 515.00 ± 38.00 ng/mg creatinine on Day +19, in contrast to the secondary rise observed in nonfertile cycles that peaked at 148.11 ± 13.80 ng/mg creatinine on Day +10. Bioactive LH evaluations confirmed ovulation and, in the fertile cycles, reflected the subsequent elevation of chorionic gonadotropin on Day +18. The estrone conjugate profile of fertile cycles and early pregnancy compared favorably to the E₂ profile: both showed the same time course and increases in estrogen excretion.

INTRODUCTION

Previous reports have demonstrated that evaluation of urinary gonadotropin levels provide a useful indicator of early pregnancy in most primate species (Tullner and Hertz, 1966; Marshall et al., 1968; Hodgen and Ross, 1974; Hodgen et al., 1974, 1978; Boorman et al., 1974; Lee and Ryan, 1975; Davis, 1977). Simplified to a practical kit (Nonhuman Primate Pregnancy Test) in which the presence or absence of chorionic gonadotropin is assessed, this approach to pregnancy diagnosis is a satisfactory method of determining pregnancy 18–20 days postovulation (Hodgen and Ross, 1974; Hodgen et al., 1978). In its more technical and time-consuming form, measurements of urinary chorionic gonadotropin have resulted in the development of sensitive radioimmunoassays that can detect pregnancy earlier, such as the assay developed for the rhesus macaque which can detect pregnancy at implantation, 8–12 days after fertilization (Hodgen et al., 1974). However, the hormone nonspecificity of some measurements, in addition to variations in follicular phase lengths and the short durations of the chorionic gonadotropin rise in some species, detract from the ultimate utility of approaching early pregnancy diagnosis through urinary gonadotropin measurements (Tullner and Hertz, 1966; Hodgen et al., 1974).

The present study was conducted to examine the potential utility of urinary estrone conjugate measurements by direct immunoassay for monitoring ovulation and subsequent implantation during the fertile menstrual cycle of the lion-tailed macaque (Macaca silenus). The close phylogenetic relationship of Macaca silenus to other, biomedically relevant macaque species which serve as models for human reproduction and cardiovascular research, in addition to an urgent need to develop an effective captive breeding program to insure their perpetuity, have both contributed to the lion-tailed macaque being the focus of such a study.
MATERIALS AND METHODS

Animals

Two multiparous and two nulliparous adult females were used in this study (age, 5-16 years; weight, 5.5-6.3 kg). Animals were caged alone and time-mated with an appropriate male during ovarian cycles, and lived in social groups for the duration of gestation. The study animals were housed in off-exhibit, open-air enclosures at the San Diego Zoo. They were fed a daily ration of commercial monkey chow and a measured mixture of fresh fruit and vegetables. Animals also had ad libitum access to water.

Sample Collection

Early morning urine samples were collected between 0600 and 0800 h. Females, while caged alone, entered specially designed sections of their nestboxes where they remained until samples were obtained from clean collecting trays beneath the nestboxes. While living in social groups, females entered metabolism cages where they remained until samples were obtained. Collected samples were then aliquoted, labeled and frozen without preservatives at -20°C until evaluated.

Assays

Estrone conjugate assay. Unprocessed urine (0.02 ml) was combined with 0.280 ml buffer (0.1 M Tris, 0.9% NaCl, 0.1 Na₂, 0.1% gelatin, pH 8.4) for a total volume of 0.3 ml. Antiserum which cross-reacts equally with E₁ and E₂ (antiestrone glucuronide, 1:5,000 dilution, 0.1 ml in Tris buffer; D. Collins, Emory University) and titrated estrone sulfate (7000 cpm, specific activity 55 Ci/mM, 0.1 ml in Tris buffer; New England Nuclear, Boston, MA) were added to unknowns and to prepared standards. Standards of estrone sulfate (Sigma Chemicals Co., St. Louis, MO) ranged from 31 to 5000 pg/tube and were assayed in duplicate. After incubation at 15°C for 1 h, antibody-bound hormone was separated from unbound steroid by the addition of 0.3 ml of charcoal dextran (0.065 charcoal, 0.00065% dextran in 0.1 M phosphate buffer), incubated at 15°C for 30 min and centrifuged at 2500 rpm for 10 min. Supernatants were decanted into scintillation vials with 6.0 ml of a single-phase scintillation cocktail (Ready-Solve HP Beckman, Fullerton, CA) and counted for 5 min.

High-pressure liquid chromatography (HPLC). The contributions of the sulfate and glucuronide conjugates of estrone were assessed following HPLC separation. A chromatographic system consisting of two single piston pumps (Altex Scientific, Berkeley, CA), solvent mixer (Altex Scientific), and in-line injector (Rhodyne Model 7020, Berkeley, CA) was fitted to a Lichrosorb reverse-phase C18 column (4.6 mm X 25 cm, 10 µm particle size) and controlled by a microprocessor (Altex Scientific). Eluates were collected in 12 X 75 mm culture tubes with a fraction collector (FC-100, Gilson Electronics, Middleton, WI). Chromatography grade methanol (Mallinckrodt, Paris, KY) and filtered distilled deionized water were degassed prior to use. A linear gradient system of 20-100% methanol in water within 80 min at a flow rate of 1 ml/min was used to separate the components. The column was characterized by placing pure samples of crystalline estrone sulfate and estrone-3-glucuronide dissolved in alcohol on the column and assaying fractions for conjugated estrone.

Urine samples were prepared for chromatography as follows: 0.5 ml of urine was combined with an equal volume of methanol:ethanol (1:1 v/v) which contained 1000 cpm radiolabeled estrone sulfate. The samples were then centrifuged for 10 min at 2500 rpm to take down the precipitated inorganic matter. The supernatant was dried down to 0.4 ml under nitrogen stream and an aliquot (0.05 ml) was injected onto the column. Twenty-five 1-ml fractions were subsequently collected and an aliquot of these eluates were directly taken for recovery of tracer and another was dried down under N₂, reconstituted in Tris buffer and assayed for immunoactivity.

LH bioassay, total estrone radioimmunoassay, creatinine assay and hematuna. Details for the assay for biologically active LH are described by Hodges et al. (1979). The total urinary estrogen radioimmunoassay used in this study is described by Hodges et al. (1979) and Czekala et al. (1981) and was applied without modification. Hormone concentrations in urine were normalized by creatinine indexing. The creatinine content of 0.1 ml urine/sample was estimated by the method described by Taussky (1954). To detect menstruation or placental sign, blood in the urine was evaluated by the use of Hemastix® (Miles Labs.).

Statistical Analyses

Standard descriptive statistics (mean, standard deviation, standard error of the mean, and covariance) were used to obtain the value given for the nonfertile cycle length and the data points of the composite graphs. Statistically significant differences between data points within and between nonfertile and fertile cycles were determined by using the Student's t test.

RESULTS

The creatinine concentration of all urines analyzed ranged from 0.100-2.50 mg/ml over nonfertile and fertile cycles, and early pregnancy (X=0.50; SD=0.4; SEM=0.003; CV=0.71; N= 200). Samples having creatinine values less than 0.100 were not included in the study. Estrone conjugate values ranged from 6.0-1237.0 ng/mg creatinine. The interassay coefficient of variation for the estrone sulfate assay was 9% at 32% binding (N=20) and 13% at 27% binding (N=22).

Figure 1 is a composite graph representing ten Macaca silenus nonfertile ovarian cycles in which estrone conjugate values have been aligned on the day of their midcycle peak (Day 0). One nulliparous female contributed eight cycles and two were contributed by one multiparous female. On the basis of these data, a 31 ± 0.63-day (SEM) nonfertile cycle length is estimated for this species and is characterized
by a preovulatory increase in estrone conjugates beginning on Day −5 at 126.00 ± 24.07 ng/mg creatinine that peak on Day 0 at 471.90 ± 62.95 ng/mg creatinine. This preovulatory increase in estrone conjugates is statistically significant on Day −5 (P=0.04). The peak reached on Day 0 is followed by a rapid decline, with estrone conjugate levels falling to 62.22 ± 4.85 ng/mg creatinine on Day +4, a secondary rise reaching 148.11 ± 13.80 ng/mg creatinine on Day +10, and a return to early preovulatory levels of less than 90.00 ng/mg creatinine from Days +15 to +18 at which time the onset of menstruation is observed (Day +17.9 ± 0.38). The midcycle LH surge was confirmed in all cycles by evaluation of bioactivity in daily urines.

Four fertile cycles and early pregnancies of the lion-tailed macaque are represented in Fig. 2. All four pregnancies produced normal live offspring. Unlike the nonfertile cycle of Fig. 1, postovulatory estrone conjugate levels do not return to early preovulatory levels on Day +14. Instead, the fertile cycle mean level of Day +14 shows a statistically significant increase over the nonfertile cycle mean value for the same day (P=0.003). Fertile cycle estrone conjugate levels continued their postovulatory climb until Day +19, reaching a level of 515.00 ± 38.00 ng/mg creatinine. From Day +20 on, levels decline, falling to 121.00 ± 36.00 ng/mg creatinine on Day +28.

After the midcycle LH peak of the fertile cycle (Day 0), bioactive LH does not show a large increase in early pregnancy until Day +18 and peaks on Day +20. Placental sign, as indicated by asterisks in Fig. 2 for two pregnancies, occurred on Day +24 — +28 in one, and on Day +25 in the other. It was not observed until Day +34 — +39 in a third pregnancy and was not seen at all in the fourth.

Three of the four previously described fertile cycles and early pregnancies are represented in Fig. 3 which is a composite of total estrogen measurements (Et) over the same time frame as Fig. 2. The profiles from both figures are quite similar except that the midcycle total estrogen peak lacks the sharp resolution of the conjugate peak, as does the early pregnancy increase. Both may reflect a slower rate of

FIG. 1. Estrone conjugate levels indexed by creatinine (Cr) in ten menstrual cycles of Macaca silenus (N=10). The midcycle LH peak (not shown) was concomitant to the estrone conjugate peak in six cycles and ranged from Day −1 to Day +3 in the remaining four cycles. The onset of menstruation (dark bar) on Day +17.9 ± 0.4 further indicates that cycles were ovulatory and that alignment of cycles by the estrone conjugate peak is appropriate.
FIG. 2. Estrone conjugate and bioactive LH levels indexed by creatinine (Cr) in urine samples from four lion-tailed macaques that became pregnant (N=4). The estrone conjugate levels (closed circles) reflect the preovulatory estrogen peak (Day 0) as well as an early pregnancy rise which is statistically higher (P=0.003) than non-pregnant levels by Day +14. Bioactive LH levels reflect the appearance of elevated chorionic gonadotropin levels on Day +18 which is 4 days after the first indication of pregnancy by the estrone conjugate assay. Placental sign is indicated by asterisks.

FIG. 3. Total urinary estrogen (E₄) and bioactive LH levels indexed by creatinine (Cr) in three of the four fertile cycles shown in Fig. 2 (N=3). Although this measurement of estrogen is more laborious in requiring hydrolysis and extraction of each sample, the profile lacks the resolution of the preovulatory estrogen peak and early implantation seen in Fig. 2. Total estrogen and estrone conjugate profiles are, however, similar in reflecting the preovulatory estrogen rise and an increase at Day +14 just prior to the detection of chorionic gonadotropin.
increase in $E_t$ as compared to estrone conjugates. While no $E_t$ data on nonfertile cycles were available to compare with fertile cycles, total estrogen measurements of early pregnancy showed a statistically significant increase on Day +14, +15 and +16 over Day +11, +12 and +13 ($P=0.05$), which compares favorably to the estrone conjugate data.

The estrone conjugates of selected urine samples taken from different stages of one lion-tailed macaque fertile cycle were separated by HPLC and measured, and compared to the total of the conjugates measured without chromatography. The results are presented in Table 1. Estrone sulfate accounted for 64−74% of the sum of the two conjugates after chromatographic separation in all samples.

**DISCUSSION**

Previous reports have confirmed the reliability of hormone measurements obtained from small, early morning urine samples, indexed by creatinine concentrations in the same samples (Hodges et al., 1979, 1981; Lasley et al., 1980; Shideler and Lasley, 1982). Briefly, when changes in urine volume, creatinine concentration, estrogen concentration, and estrogen indexed by creatinine in *Macaca silenus* are compared, the quality (in terms of hormone evaluations) of individual samples was found to vary throughout a 24-h period. However, measurements obtained from urine samples collected in the early morning (0600−0800 h) when urine production resumes after a nighttime reduction are found to be consistently close to measurements obtained from 24-h urine collections (Shideler and Lasley, 1982). In addition, results from the application of this procedure, when compared to results obtained from serum analyses of estrogens in well-described species, indicate that relative changes in estrogen secretion are reflected accurately by urinary estrogen measurements (Hodges et al., 1979, 1981; Lasley et al., 1980).

Chromatographic separation studies have demonstrated that, while urinary estriol is the dominant estrogenic component of pregnancy in humans, chimpanzees, gorillas and orangutans (Czekala et al., 1983), estrone is also a major estrogenic component, which, unlike estriol, appears to be shared in common by primates across the entire order. Estrone is not only the second largest component of hominoid pregnancy but it is the major classical estrogen in most cercopithecoid and ceboid pregnancies (Liskowski et al., 1970; Hodgen et al., 1972; Challis et al., 1977; Setchell et al., 1980; Hodges et al., 1981). It is also the major component of at least one lower primate, *Lemur variegatus* (Shideler et al., 1983). Since estrone has only two major conjugates (estrone glucuronide and estrone sulfate), it is logical to predict that an assay which cross-reacts with both conjugates would be informative and applicable across the order.

In an initial report by Branch et al. (1980), estrone glucuronide concentrations measured directly in early morning urine of human subjects were shown to reflect ovulation (n=6), and ovulation and implantation (n=5) accurately. These data were the first to describe a simple and practical method of obtaining longitudinal profiles which reflect ovarian and trophoblastic activity without venipuncture.

The lion-tailed macaque data confirm what

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**TABLE 1. Urinary estrone conjugate measurements with and without chromatography in the lion-tailed macaque, pre- and postovulation in a fertile cycle.**

<table>
<thead>
<tr>
<th>Day from ovulation</th>
<th>Urinary estrone conjugates (ng/mg creatinine)</th>
<th>Total estrone conjugates (without chromatography)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_1$ G</td>
<td>$E_1$ S</td>
</tr>
<tr>
<td>−1</td>
<td>15</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>(26)*</td>
<td>(74)</td>
</tr>
<tr>
<td>+11</td>
<td>18.2</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>(35)</td>
<td>(65)</td>
</tr>
<tr>
<td>+18</td>
<td>108</td>
<td>292</td>
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<td></td>
<td>(27)</td>
<td>(73)</td>
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</table>

*The number in parentheses indicates the percentage of the sum of immunoreactivity of the two components.
Branch et al. (1980) demonstrated in their human study, namely, that estrone conjugate measurements accurately reflect a preovulatory and early implantational increase in estrogen excretion. However, unlike the human, in which estrone glucuronide is the single estrone conjugate component, both estrone glucuronide and estrone sulfate are present in the lion-tailed macaque. Although Branch et al. (1980) did not address the question of whether or not the quantitative increase observed at implantation coincided with a qualitative change in conjugate components, it does not appear to be the case in the lion-tailed macaque as shown in Table 1.

The early pregnancy increase in urinary estrone conjugates that is apparent on Day +12 in Macaca silenus, but which is not statistically significant until Day +14, appears to be the direct result of the action of chorionic gonadotropin acting at the level of the ovary. This is consistent with its appearance in the plasma of rhesus monkeys 8–12 days postconception (Hodgen et al., 1974). The urinary estrone conjugate data shown here suggest the estrone conjugate assay can detect pregnancy earlier and over a broader range of time than the LH bioassay, but not sooner than more specific and laborious measurements of urinary gonadotropins.

Whether or not these Macaca silenus data are comparable to data on circulating estrogens in other macaques remains an open question that we hope to resolve, in part, by future studies on a more common macaque species. To the best of our knowledge, serum measurements of estradiol on Macaca radiata (Lasley et al., 1974), Macaca nemestrina (Eaton and Resko, 1974) and Macaca mulatta (Hotchkiss et al., 1974; Bielert et al., 1976) yield cycle profiles of the same configuration as seen in Fig. 1, and serum measurements of estradiol in the early pregnancy of Macaca mulatta, as reported by Atkinson et al. (1975), show an early increase similar to that seen in the estrone conjugate and total estrogen profiles of Figs. 2 and 3.

Data presented here further suggest that a direct measurement of estrone conjugates may have important application in terms of physiologic studies. The higher levels (a 4-fold increase) of the combined measurement of estrone sulfate and estrone glucuronide, when compared to total urinary estrogen levels, indicate that enzymatic hydrolysis of urinary estrogen is less efficient than previously thought (Baker et al., 1979). The greater mass of the conjugate molecules accounts for only a moderate (60%) increase of the observed estrone concentrations on a mass basis. Estrone conjugate levels, in terms of molar concentrations, are much greater than what can be accounted for by the total amount of estrogen measured following enzymatic hydrolysis (Baker et al., 1979; Wright et al., 1979). The sharper resolution of the estrone conjugate preovulatory and preimplantational profiles, reflecting urinary estrogen increases, suggests measurements of hydrolyzed urinary estrogen underestimate changes in production and excretion, especially when estrogen production is increasing. If this is true, then urinary estrone conjugate evaluations may be a more meaningful approach to studying changes in ovarian estrogen production than either total urinary estrogen or plasma unconjugated levels.

The major advantage of the estrone conjugate assay stems from the fact that both ovulation and early pregnancy can be monitored through the same simple technique. This fact suggests that the technique may have practical applicability in timed matings. Parkin and Hendrickx (1975) have shown that the fertile period of female macaques is limited to a 24-h period immediately following the estrogen peak and, therefore, can be predicted on the basis of the timing of the preovulatory estrogen rise. The short turnover time required to obtain results from the estrone conjugate assay, combined with the nonstressful aspect of sample collection, offer other advantages over more traditional methods of steroid measurements. Additionally, preliminary data from this laboratory on the gorilla, orangutan, owl monkey, tamarin and lemur indicate that the estrone conjugate assay is not only applicable across the Order Primates, but has particular utility in longitudinal studies.

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REFERENCES


