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Urinary steroids, FSH and CG measurements for monitoring the ovarian cycle and pregnancy in the chimpanzee

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Abstract: Non-invasive methods for monitoring reproductive status of chimpanzee based on the measurement of urinary steroids and gonadotropins were examined. A typical pre-ovulatory urinary estrone conjugate (E_1C) surge and post-ovulatory increase in pregnandiol glucuronide (PdG) were seen during the menstrual cycle. Urinary follicle stimulating hormone (FSH) showed two peaks over the infertile menstrual cycle. The earliest changes indicating pregnancy were a coincident rise in E_1C and chorionic gonadotropin (CG) levels and a concomitant fall in FSH levels. Urinary PdG levels showed a prolonged rise. Urinary E_1C in the pregnant chimpanzee was higher than during the menstrual cycle and increased with advancing gestation, with maximum levels occurring near term. In the case of stillbirth, E_1C and CG levels from mid- through late-pregnancy were low and the prepartum progressive increase in E_1C was not shown. The data presented here are of great practical value in captive breeding management of chimpanzees.

Key words: E_1C – PdG – gonadotropin – *Pan troglodytes* – ovulation – pregnancy monitoring – stillbirth

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Introduction

Chimpanzees (*Pan troglodytes*) are the best models for human reproduction because their hormonal profiles are very similar to those of humans [20]. Yet, the number of wild living has decreased considerably and they are listed as endangered. On the other hand, though some of them have never bred, the number of captive births has increased and management problems such as costs and limited genetic diversity still exist in most of the colonies [10]. To establish a successful breeding program in captivity for this species, assessment of reproductive status is indispensable.

Ovulation time is approximately determined in the chimpanzee by the observation of several parameters, such as sexual swelling detumescence [14, 20], cervical mucus ferning patterns [7], basal body temperature shifts, and gonadal hormone level changes [8, 20]. The most precise methods to ascertain the timing of ovulation are observation of ovarian morphology using laparoscopy or ultrasonography, but the most practical method for chimpanzees is to monitor the hormonal changes. Although serum hormone levels provide a more accurate representation of the temporal dynamics of endocrine events, urinary assessment offers the major advantage of allowing long-term monitoring of reproductive function from animals in which routine blood collection is impractical. The non-invasive characteristic of urinary hormone monitoring provides a stress-free approach to the accurate evaluation of great ape reproductive status [12]. For the past 30 years, various studies have shown that urinary steroids can be used to monitor menstrual cycle as well as early pregnancy in chimpanzees (e.g. [6, 11, 13, 20]). Also, chorionic gonadotropin (CG) has been used to detect

pregnancy in chimpanzees [17]. On the other hand, it has been reported that pituitary follicle stimulating hormone (FSH) is required for the normal development of ovarian follicle [19], however, changes in urinary FSH levels have not been reported in this species. Furthermore, endocrine characteristics throughout pregnancy and during early lactation have not been described in chimpanzees.

Knowledge of reproductive endocrinology would not only be important for enhancing reproduction, but would also provide the basis for controlling the fertility. The aim of the present study is therefore to describe hormonal changes during infertile menstrual cycles, throughout pregnancy, and during the early lactation period in captive chimpanzees by measurement of FSH, CG and steroid metabolites in urine.

Materials and methods

Subjects

Four female chimpanzees of reproductive age (17–24 years) and weighing 43–58 kg were studied. All were maintained at the Primate Research Institute (PRI), Kyoto University, Inuyama, Japan. They were nulliparous and had a history of regular menstrual cycles. Each female was checked daily for evidence of menstruation and changes in sex skin swelling as well as to assess general health.

Four pregnancies had been achieved in three chimpanzees between 1997–2000. Of these, three had been achieved by artificial insemination in two chimpanzees and one became pregnant by natural breeding. One pregnancy by artificial insemination did not yield a viable offspring, and was classed as a stillbirth, with a dead fetus delivered at or near term. The results of the necropsy showed that there were no deformities in the newborn and the cause of the stillbirth were not clear. Two years later, this animal became pregnant again by artificial insemination and then delivered a healthy offspring. The remaining three pregnancies were carried to term and resulted in the births of healthy singleton offspring. In addition to these four pregnancies in three chimpanzees, an infertile female was studied as a non-pregnant control.

Sample collection and preparations

Urine samples were collected during seven infertile menstrual cycles in four chimpanzees, and throughout pregnancy and during early lactation period in three pregnancies. In the chimpanzee impregnated by natural breeding, samples in early pregnancy could not be collected. Samples were obtained 2–5 days per week, with more frequent sampling during the periovulatory period of the menstrual cycles. For urine collection, non-invasive and non-stressful sampling method was used. Briefly, morning urine samples were aspirated by disposable syringe from the cage floor of their individual night dens after they were taken out to the playground. All samples were frozen immediately at −30 °C and stored until assayed.

All urine samples were analysed for E_1C, pregnandiol glucuronide (PdG), FSH and CG by enzyme immunoassays (EIAs). Frozen urine samples were thawed and centrifuged (3000 rpm [1870 × g], 10 minutes). The supernatant was diluted 1:50 in distilled water for E_1C and PdG assays, while it was diluted 1:4 for CG assay. For FSH assay, it was used without dilution. Those dilution factors were determined beforehand so that hormone concentrations of applied samples were in the ranges of the standards in the assays.

Hormone assays

Estrone conjugates (E_1C) and PdG in urine samples were determined by microtiter plate EIAs as previously described by us [5]. The intra- and inter-assay coefficients of variation were 7.3 and 8.0% for E_1C, 7.5 and 8.5% for PdG, respectively.

The assay for total urinary FSH, which is based on measurement of the beta subunit, has been described previously [19]. Before the analysis, urine samples were transferred to polypropylene minitubes and placed into boiling water for 2 minutes in order to dissociate the intact FSH dimer into alpha and beta subunits [19]. Dissociation of the subunits in this ELISA is necessary because of the fact that when in combination with the first antibody, the second antibody used will recognize only the free beta subunit and not the intact molecule. After dissociation, urine samples were analysed for the beta FSH subunit. The antibodies were monoclonal anti-beta hFSH (FS2-4A10-G10; Scantibodies Laboratory, Santee, CA, USA) and polyclonal rabbit anti-hFSH-beta antiserum (provided by Dr B.L. Lasley, University of California, Davis, CA, USA). The intra- and inter-assay coefficients of variation were 3.7 and 8.7%, respectively.

Concentrations of chorionic gonadotropin were determined by ELISA as previously described [15]. The primary capture antibody was anti-beta LH monoclonal antiserum, 518B7 (provided by

Dr Jan Roser, University of California, Davis, CA, USA), in conjunction with peroxidase-labelled second antibody against hCG, R76 (provided by Dr B.L. Lasley, University of California, Davis, CA, USA). The standard was hCG CR-127 (provided by Dr O'Connor, Columbia University, New York, USA). The intra- and inter-assay coefficients of variation were 9.8 and 12.0%, respectively.

In all assays, plates were washed thoroughly with wash solution by automatic plate washer (Sera Washer VII, Bio Tec, Co., Ltd, Tokyo, Japan) between each step. Each plate was read on a microtiter plate reader (Spectra I, Tecan, Grodig, Austria) using dual wavelength mode. The hormonal concentrations of each sample were calculated by fitting the absorbance to the standard curve using a curve-fitting program (DeltaSOFT 3, Biometallics Inc., Princeton, NY, USA). To compensate for variations in urine volume and concentration, the creatinine concentration of every urine sample was determined using the Taussky's method [21]. Urinary hormone concentrations are expressed as nanograms or micrograms per milligram of creatinine.

Validation of assays and statistical analysis

Serial dilutions of urine from samples of all reproductive stages gave displacement curves parallel to those obtained with the appropriate standards in all hormones. Accuracy was assessed by determining the recovery of known amounts of hormone added in quadruplicate to urine. Mean ± SD recovery values were 97.5 ± 10.5% for E_1C, 101.5 ± 9.2% for PdG, 91.7 ± 7.5% for FSH and 93.8 ± 8.9% for CG. Tests of assay validity indicated that these assays could be reliably applied to measure hormone concentrations in chimpanzee urine.

Pearson product moment correlation was used to compare the serial dilutions of samples to the standard curve. Statistically significant differences were determined by using unpaired *t*-test.

Results

Endocrine changes during the infertile menstrual cycle

Profiles of mean (±SEM) urinary E_1C, PdG, and FSH values over seven infertile menstrual cycles from four chimpanzees are shown in Fig. 1. Data were centered around the midcycle FSH peak. The first day of menstrual flow was considered the beginning of the follicular phase. Menstrual cycle length varied from 31 to 35 days (mean 33.3 days). Most of this variation occurred in the follicular phase (16–22 days, mean 18.6 days), while the length of the luteal phase varied from 14–17 days (mean 15.2 days). All cycles were apparently ovulatory, as indicated by a cyclic pattern in which the follicular and luteal components of the cycle could be clearly distinguished.

Urinary E_1C concentrations exhibited conspicuous midcycle and luteal phase elevations during the chimpanzee menstrual cycle. Urinary E_1C levels were less than 40 ng/mg Cr during the early follicular phase and increased during the late follicular phase then reached a peak at or just before the midcycle FSH peak. Thereafter, E_1C levels declined to baseline values within 3 days after the peak. In all cycles, there was a secondary E_1C rise during the luteal phase. Urinary PdG levels were less than 1.0 μg/mg Cr during the follicular phase, then rose 1–2 days after the midcycle FSH peak to reach maximum values of 10 μ g/mg Cr on day 9–11 of the luteal phase in all cycles. Thereafter, PdG levels declined and returned to baseline values by the first or second day of menstruation. The urinary FSH rise during the early to mid follicular phase averaged 2 ng/mg Cr, and commenced 11–18 days before the day of the midcycle FSH peak. Then, FSH levels declined and returned to baseline values. Urinary FSH levels rose again sharply and reached maximum concentrations of 4.4 ng/mg Cr at midcycle and then decreased during the luteal phase to less than 1 ng/mg Cr.

Endocrine changes throughout pregnancy and early lactation period

All infants were delivered spontaneously, though one of the four pregnancies resulted in a stillbirth at 220 days of gestation, as described above. Mean gestation period of the three pregnancies resulting in livebirths was 233.3 days (231, 233 and 236 days). All three infants were nursed by their mothers following parturition for at least 1 month.

Changes in levels of urinary E_1C, PdG, FSH and CG throughout pregnancy and early lactation period in individual animals are shown in Fig. 2A–D. The earliest changes indicating pregnancy, occurring at about 10 days after the midcycle FSH peak, were a coincident rise in E_1C and CG levels and a concomitant fall in FSH levels to a nadir. Urinary PdG levels showed a prolonged rise followed by a modest fall at 15–18 days and then a secondary rise or a plateau.

Urinary E_1C increased during the late luteal phase of the fertile cycle and peaked at day 22.3 of pregnancy. We observed a subsequent decline of E_1C to a nadir at day 46.6, then a progressive

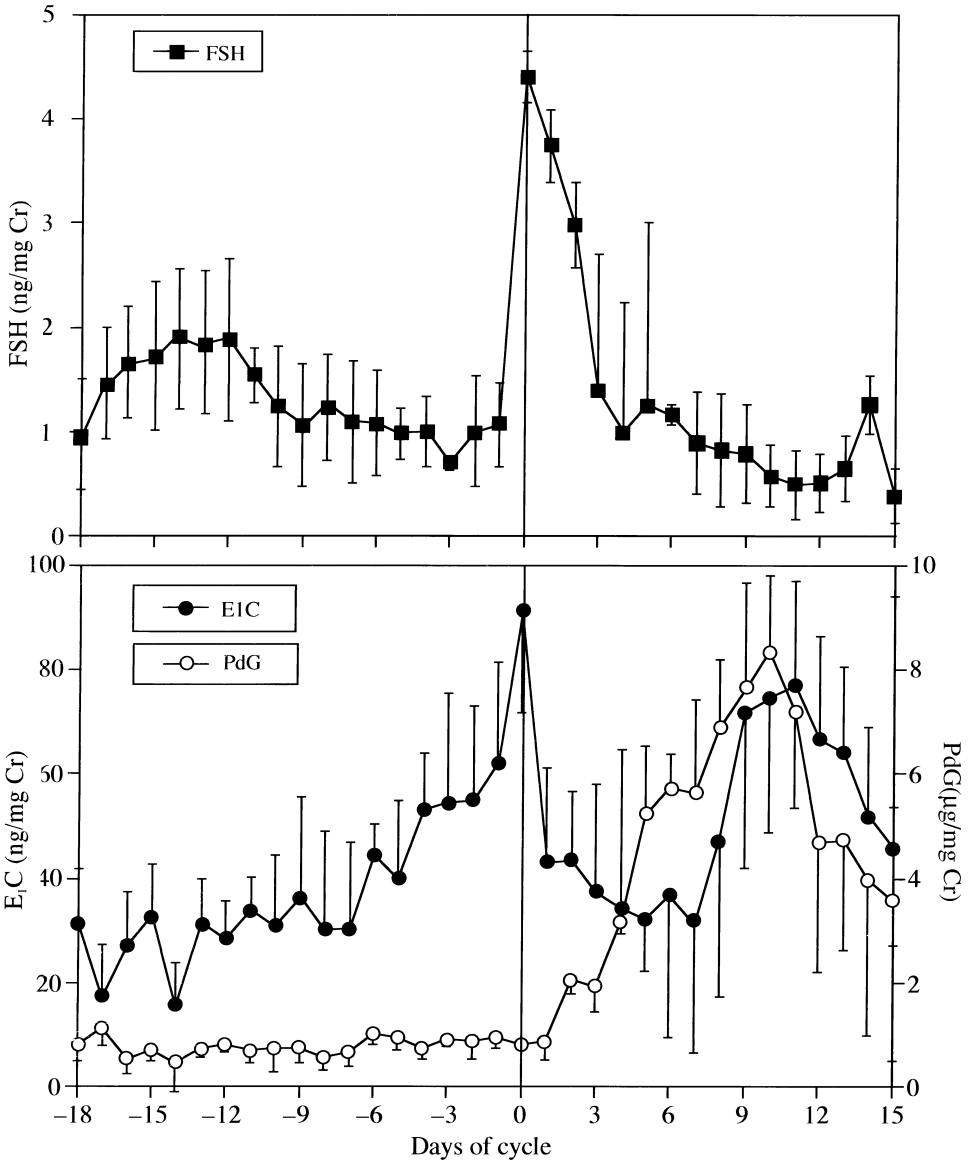

Fig. 1. Hormonal profiles of urinary E_1C (●), PdG (○), and FSH (■) during seven ovulatory menstrual cycles in four female chimpanzees. All data were indexed by creatinine (Cr) content and normalized to the day of urinary FSH peak (day 0). Results are expressed as mean ± SD for four animals. The vertical line reflects the day of FSH peak. The first day of menstrual flow was considered the beginning of the follicular phase.

elevation toward term, with maximum levels occurring just before parturition. After parturition, E_1C abruptly decreased to non-pregnant levels. Urinary PdG increased during the luteal phase until day 15.0 of pregnancy, when the first gestational peak was observed. Thereafter, PdG decreased to a nadir at day 23.6 of pregnancy. The second gestational peak in PdG occurred at day 36.3 of pregnancy, again followed by a subsequent decline. Urinary PdG remained modestly elevated from day 70–150 followed by a progressive rise or plateau toward term. After parturition, PdG abruptly decreased to non-pregnant levels.

Urinary CG levels in four pregnancies are also shown in Fig. 2. A significant rise in CG of more than 2 ng/mg Cr was found after day 13.6 of pregnancy, and levels remained high for about 100–150 days, with a peak at day 20.6 of pregnancy. The CG peak was coincident with the PdG peak. Thereafter, CG levels fell gradually and remained consistently low during the last 50–100 days of pregnancy up to parturition. After parturition, CG decreased to non-pregnant levels.

Urinary FSH decreased during the luteal phase of the fertile cycle, fell to a nadir at day 22.0 of pregnancy, and then remained at low or

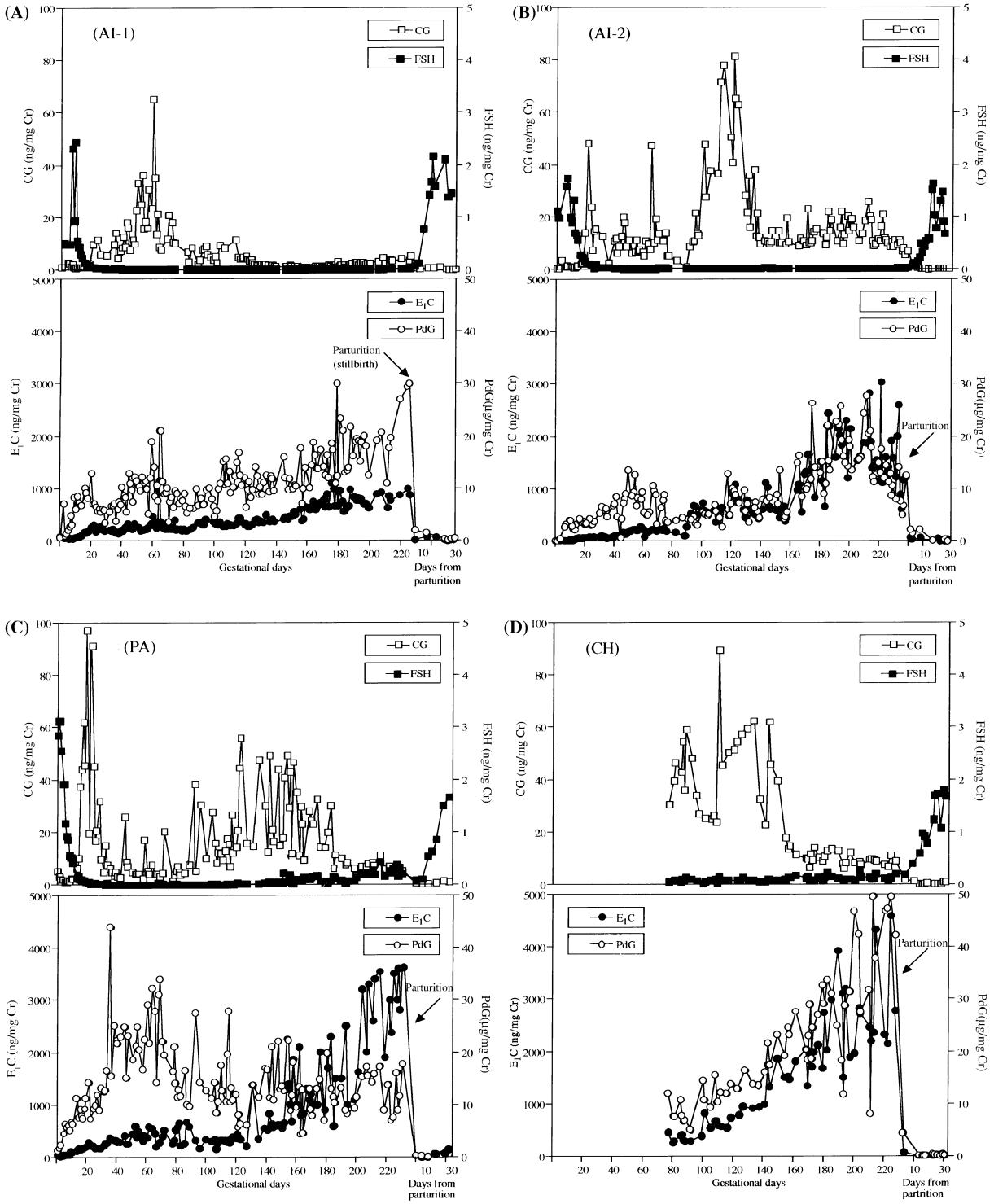

Fig. 2. Hormonal profiles of urinary E_1C (●), PdG (○), FSH (■) and CG (□) during pregnancy and early lactation period in an individual chimpanzee. (**A**) Chimpanzee AI-1, underwent spontaneous stillbirth on 220 days of gestation, (**B**) Chimpanzee AI-2, (**C**) Chimpanzee PA, (**D**) Chimpanzee CH, resulted in livebirth. Urine samples in early pregnancy period in Chimpanzee CH could not be collected. All data were indexed by creatinine (Cr) content and normalized to the day of FSH peak (day 0) in the fertile cycle and to the day of parturition (day 0). Arrows indicate the day of parturition.

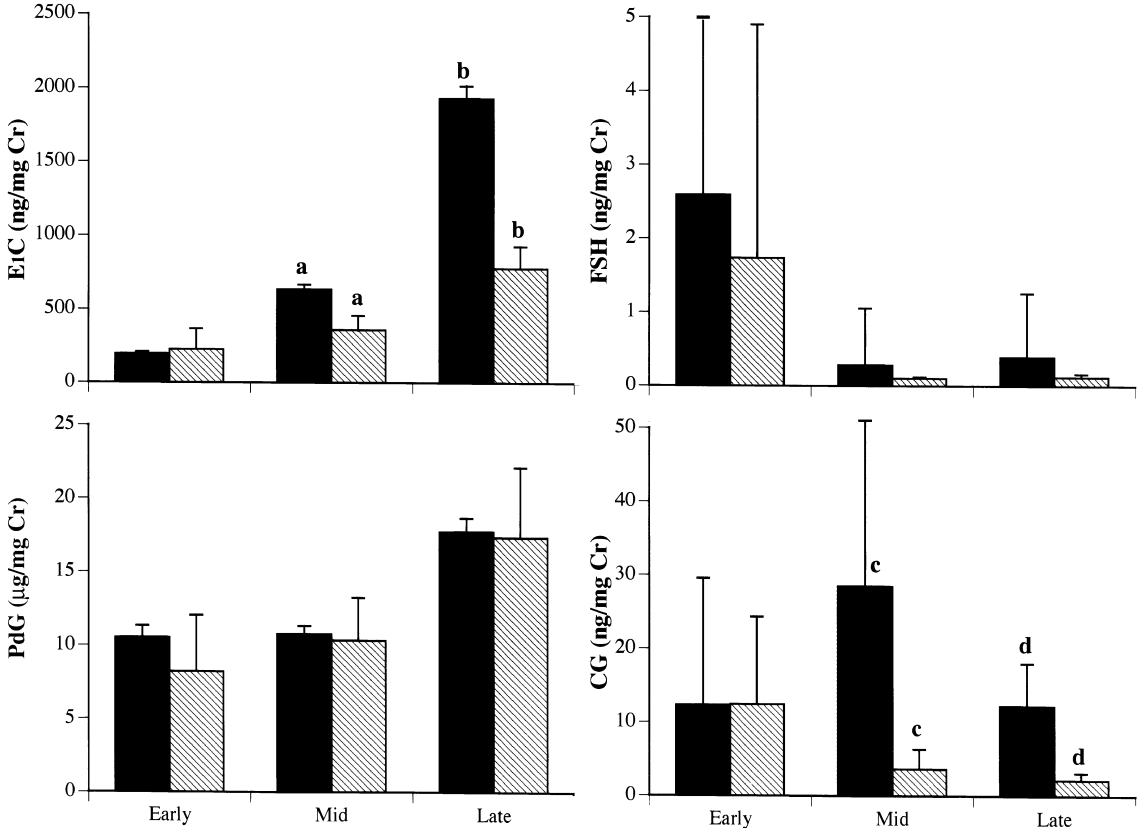

Fig. 3. Changes in urinary levels of E_1C, PdG, FSH and CG during early, mid- and late-pregnancy in the livebirth (■) and stillbirth (▨). Each of the three stages of pregnancy comprised one-third of respective gestation periods for individual females. Each value represents the mean ± SD. Different letters in each bar indicate a significant difference ($P > 0.001$) between each group.

undetectable levels throughout pregnancy. Immediately after parturition, FSH increased.

Within 1 month of early lactation period, FSH increased rapidly, while other urinary hormone levels were significantly low.

Stillbirth

In the case of the stillbirth (Fig. 2A), E_1C levels from mid- through late-pregnancy were significantly low ($P < 0.001$) compared with those in livebirths, and the prepartum progressive increase was not shown (Fig. 3). Urinary CG levels were also significantly low ($P < 0.001$) in the latter half of pregnancy for stillbirth (Fig. 3). On the other hand, no differences were found in PdG and FSH levels in comparison with pregnancies that resulted in livebirths (Fig. 3).

Discussion

The present study demonstrated the endocrine changes that accompany the events of ovulation, pregnancy, parturition and early lactation in chimpanzees. The profile of urinary FSH was firstly described in this species. The results showed that measurement of urinary hormones are reliable methods for assessing reproductive function in this species.

In infertile cycle, the approach of ovulation was signaled by an E_1C peak, concomitant with or followed by a rise in FSH. The subsequent rise in PdG provided indirect evidence that ovulation had occurred. During early and mid-follicular phases of infertile cycles, rises in FSH levels were observed. Similar findings have been reported in humans [22], and the increase in FSH production during the follicular phase is thought to be involved in follicle selection in the subsequent cycle [2]. When this rise of FSH is absent or delayed, the recruitment of follicles for the next cycle will likewise be absent or delayed [13, 22]. Therefore, alterations in FSH during this time may determine the normalcy of the subsequent cycle. Our study also described that during the luteal phase, increases in E_1C levels were observed, and

these elevations coincided with increases in PdG. These findings were previously described in chimpanzees [3] and in humans [1].

Estrone conjugates (E_1C) and PdG are abundant steroid metabolites excreted into urine in great apes [9] and known to accurately reflect changes in corresponding circulating hormones in chimpanzees and other species of great apes, macaques and in humans [16, 20]. Therefore, measurements of these hormones in urine can reliably assess endocrine changes during the ovarian cycle. Detailed analysis of cycles requires measurement of PdG in order to confirm that ovulation occurred, as well as assays for FSH and/or E_1C to determine the approximate day of ovulation. At present, prediction of ovulation by measurement of these hormones alone is not possible, but the method is clearly of practical value for monitoring ovarian cyclicity and retrospective determination of ovulation.

The present study demonstrated that pregnancy could be detected as early as 12 days after fertilization, but after 15 days pregnancy may be determined very reliably. Pregnancy could be detected by a sustained increase in E_1C and PdG beyond the length of the normal luteal phase. Moreover, the simultaneous increase in CG and fall in FSH levels in urine by approximately 12 days following the midcycle gonadotropin surge are indicative of pregnancy. Like the changes in E_1 C and PdG, changes in FSH levels that take place in the luteal phase and result from the presence of CG are a biological response to the pregnancy signal. Thus, the information provided by the measurement of urinary FSH, in conjunction with measurements of urinary CG and steroids allows for early and accurate detection of pregnancy, and gives additional definition to the endocrine changes associated with implantation and to placental function during pregnancy.

Spontaneous abortion and stillbirth are common problems in primate breeding colony [10]. In this study, one of four pregnancies resulted in stillbirth. The cause of the stillbirth could not be clear, however, the female's E_1C and CG levels in mid-through late-pregnancy were significantly low compared with other normal pregnancies. In accordance with our results, previous studies reported that serum estrogen was low in high-risk pregnancies and in growth-retarded babies in humans [4]. In the gorilla, abortion was preceded by a fall in estrogens and the pregnancy associated with placental infracts and abruption was also attended by chronically low estrogen values [1]. Furthermore, it had been reported that urinary CG was characterized by a precipitous fall in urinary CG before spontaneous abortion in the chimpanzee [18]. Thus, although the data of the present study are limited, the pregnancy can be accurately monitored using the urinary hormone profile and these hormones aid in the evaluation of the progression and ultimate end of gestation in chimpanzees.

The availability of reliable non-invasive methods for monitoring reproductive function in female chimpanzees offers new opportunities for improving the captive breeding management.

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