Urinary hormone analysis as a diagnostic tool to evaluate the ovarian function of female gorillas (*Gorilla gorilla*)

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Summary. Daily urine samples were collected from 4 adult female gorillas over 7 menstrual cycles. Urinary oestrone conjugate and pregnanediol-3-glucuronide (PDG) were measured by radioimmunoassay; LH was measured by enzyme immunoassay and each hormone was indexed by creatinine. The quantity of urinary LH during the ovulatory surge was positively correlated with the quantity of PDG excreted during the luteal phase ($r = 0.87$, $P = 0.0013$). The observations indicate a relationship between the quality of the LH surge and the levels of PDG in the luteal phase and suggest that both the LH surge and subsequent luteal phase function may be predictable from the oestrogen excretion profile during the follicular phase.

Keywords: gorilla; oestrogen; progesterone; LH; luteal function

Introduction

Pregnancies and births of infants are relatively rare events among gorillas that are maintained in breeding situations in the United States. In an evaluation of ovulatory ovarian cycles, assessed by measurements of steroids in urine, Mitchell *et al.* (1982a) indicated that a post-ovulatory or luteal-phase defect may be responsible for some of the reduced fertility in this species.

To investigate this possibility further, a practical assay for urinary luteinizing hormone (LH) was developed and applied. The LH measurements together with simultaneous measurements of ovarian oestrogen and progesterone excretion were used to assess the relationship between ovarian and pituitary hormones in the gorilla menstrual cycle. The results of this study are intended to provide empirical evidence regarding the relationship between pituitary and ovarian hormone excretion as they are applied as indicators of normal ovarian function in gorillas and related species.

Materials and Methods

Animals and sample collection

Daily urine samples (2 ml) were collected from the cage floor from 4 mature female gorillas. Three of the gorillas are housed at the Toledo Zoo (P, 29 years old; H, 14 years old; and M, 12 years old) and Gorilla K (12 years old) is housed at the San Diego Zoo. The Toledo gorillas were all housed and copulated with an adult male gorilla known to have achieved at least one conception and with normal motile spermatozoa after electroejaculation. The last offspring of Gorilla P was in 1971; Gorilla H has not reproduced; Gorilla M has subsequently conceived with another male and delivered a normal infant; and Gorilla K has regularly copulated with a fertile male gorilla since her arrival (1 year) with no indication of conception or resulting offspring.

Menstruation was monitored by measuring blood in the urine using Hemastix (Miles Laboratories, Elkhart, IN, U.S.A.). Urine samples were frozen as soon as possible and kept frozen until assay. All hormone values were indexed by individual creatinine concentrations (Taussky, 1954) to correct for variation in urine concentration.

Oestrone conjugate assay

Urinary oestrone conjugates were analysed by radioimmunoassay as previously described (Shideler *et al.*, 1983). Briefly, 0.01 ml undiluted urine was combined with 0.29 ml Tris assay buffer pH 8.4 (0.1 M Tris, 0.9% NaCl, 0.1% NaN_3 , 0.1% gelatin). Antiserum which cross-reacts equally with oestrone sulphate and oestrone glucuronide (anti-oestrone glucuronide, 0.1 ml, 1:5000; D. Collins, Emory University) and tritiated oestrone sulphate (0.1 ml, 7000 c.p.m., sp. act. 55 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) were added to the urine and standard samples (30–500 pg oestrone sulphate). The assay was incubated at 15°C for 1 h. Charcoal–dextran (0.3 ml, 0.625% charcoal Norite A, 0.0625% in 0.1 M-phosphate buffer pH 7.0) was used to separate bound and free steroid during a 30-min incubation at 15°C. After centrifugation for 10 min the supernatants were mixed with Ready Solv HP (Beckman, Fullerton, CA, U.S.A.) and counted for 5 min each. The interassay coefficient of variation was 9.1% at 33% binding. The intra-assay coefficient of variation was 4% and 1% at 22% and 65% binding respectively. Sensitivity of the assay (zero minus $2 \times \text{s.d.}$) was 39 pg. Recovery of added oestrone sulphate (0.15–1.25 ng) to urine ranged from 105 to 112% (slope = 0.91).

Pregnanediol-3-glucuronide assay

Urinary pregnanediol-3-glucuronide (PDG) was analysed as described by Mitchell *et al.* (1982b). Undiluted urine (0.0001 ml) was combined with 0.1999 ml 0.1 M-phosphate assay buffer–0.1% gelatin pH 7.0. The PDG antiserum (No. 02/zoo, 1:18 000; Courtauld Institute, London, U.K.) cross-reacts 100% with pregnanediol-3-glucuronide and 19.5% with pregnanediol. This was combined with tritiated PDG (Courtauld Institute; sp. act. 42 Ci/mmol, 7000 c.p.m./0.1 ml) and the urine or standard samples (30–5000 pg) and incubated overnight at 15°C. Bound and free steroid were separated by addition of 0.5 ml charcoal–dextran and a 45-min incubation at 15°C. After centrifugation the supernatant was counted in Ready Solv-HP. Interassay coefficient of variation was 12% at 48% binding and 20% at 78% binding. Intra-assay coefficient of variation was 5% and 2% at 47% and 79% binding respectively. Assay sensitivity was 78 pg. PDG (0.15–1.25 ng) added to urine was recovered between 86% to 98% (slope = 0.98).

LH assay

Monoclonal antibodies. Monoclonal antibodies with affinities for human luteinizing hormone (hLH) were produced by stable clones of cell hybrids isolated from the fusion of BALB/c mouse spleen cell with NS_1 mouse myeloma cells (Milstein & Kohler, 1975). Mice were immunized with hLH (Parlow) or human chorionic gonadotrophin (hCG; Organon, East Orange, NJ) over a period of several weeks. Specific mice were selected for fusion when serum titres were at least 1:1000 at 50% maximum binding in specific radioimmunoassays (RIA) for hLH and hCG. After fusion and screening of cell hybrids for specific antibodies, positive hybrids were cloned and injected into pristine primed mice for ascites antibody production (Leak *et al.*, 1985). The two antibodies, anti-hLH (102G₁₁) and anti-hCG (716G₁₁), were purified by passage through a protein A–sepharose affinity column (Ey *et al.*, 1978). Clone 102G₁₁ was found to be a monoclonal IgG₁ by immunoelectrophoresis and by double diffusion in agarose gels with subclass-specific antisera. Clone 716G₁₁ appeared to be IgG₁ based on elution patterns generated by the protein A–sepharose column (Ey *et al.*, 1978).

Characterization of antibodies by RIA. The purified antibodies were evaluated in a competitive overnight RIA system previously described (Wada *et al.*, 1982). Briefly, unlabelled hormone in serum was added to tracer and antibody, incubated overnight at room temperature and precipitated with polyethylene glycol 6000 (200 g/l) in place of the second antibody. To characterize Clone 102G₁₁ hLH (Parlow 9/80) was used as the tracer and standard. To characterize Clone 716G₁₁, hCG (Calbiochem, Cat no. 869031) and hLH (Parlow 9/30) were used both as the tracer and standard.

The association constants (K_a) were calculated by Scatchard analysis (Scatchard, 1949) of the data from RIA displacement experiments and found to be approximately 2×10^{11} l/M for Clone 102G₁₁ and 3×10^9 l/M for Clone 716G₁₁.

Cross-reactions of the related hormones in the RIA systems were determined by displacement of labelled hormone by cross-reacting hormone. Percentage cross-reactivity was calculated from amount of hormone causing half displacement (ED_{50}) of tracer-labelled hormone on a weight–ratio basis: i.e. $[\text{ED}_{50} \text{ LH (ng)}/\text{ED}_{50} \text{ FSH (ng)}] \times 100 = \%$ cross-reactivity of FSH in LH assay system. Cross-reactivities of Clone 102G₁₁ with hFSH and hTSH were 0.9% and 18.4% respectively. Cross-reactivities of Clone 716G₁₁ with hFSH and hTSH were 91% and 141% respectively (hLH as trace). Minimal detectable dose at 10% displacement was 0.2 ng hLH/ml for Clone 102G₁₁ and 18 ng hLH/ml for Clone 716G₁₁.

Coupling of antibodies to alkaline phosphatase. The purified monoclonal antibody, Clone 716G₁₁, was coupled to alkaline phosphatase (Sigma Chemical Co., St Louis, MO, U.S.A.) by the modified two-step method described by O'Sullivan & Marks (1981). Briefly, 20 000 units of alkaline phosphatase were dialysed against 6 litres of a phosphate-buffered saline (PBS) pH 7.0 overnight at 4°C. The alkaline phosphatase solution was adjusted with PBS to 10 mg/ml

Fig. 1. Measurements of daily urine from one gorilla (M) during the mid-cycle. The LH surge is compared using the LH enzymeimmunoassay techniques and the rat Leydig cell bioassay for bioactive LH. Both methods indicate that peak LH concentration is on 29 January and follow a similar pattern.

in a total volume of 3 ml. Exactly 0.2 ml of a 4% solution of glutaraldehyde in PBS was added slowly with gentle mixing. The glutaraldehyde-alkaline phosphatase solution was incubated for exactly 50 min at room temperature. The mixture was loaded onto a Sephadex G-25 column (0.7 cm × 27 cm) at 4°C to remove the glutaraldehyde. About 1 mg antibody, IgG, was dialysed overnight in 0.7 M-carbonate buffer at 4°C. During the coupling procedure, 0.2 ml of dialysed IgG (1 mg) was slowly added to a solution of 4 mg purified alkaline phosphatase in 0.6 ml PBS. The reaction was allowed to proceed at room temperature for 8 h. The solution was loaded onto an ultrogel ACA-34 column (0.7 cm × 43 cm; LKB, Paris, France) equilibrated at 4°C with a 0.05 M-Tris buffer pH 7.2, containing 0.9% (w/v) NaCl and 0.001 M-MgCl₂. Flow rate was adjusted to 0.6 ml/5 min. Fractions were tested for IgG-alkaline phosphatase activity in an enzyme-linked immunosorbent assay (ELISA) (see below). Positive fractions were pooled with the addition of BSA (1% w/v; Fraction V, Sigma) and parabens (10% methyl-propyl parabens in H₂O; 0.01% v/v; Sigma). The solution was stored at -20°C until needed.

The validity of the enzyme-linked immunosorbent assay to monitor LH activity in gorilla urine was assessed by the rat Leydig cell bioassay for LH as described by Ramachandran & Sairam (1975). Similar LH patterns were obtained by both assay systems (Fig. 1).

ELISA for LH. Capture antibody (Clone 102G₁₁; Monoclonal Antibodies Inc., Mt View, CA) was diluted to 2 µg/ml with phosphate-buffered saline (0.01 M-PBS pH 7.0). Then 96-well microtitre plates (Immulon I, Dynatek) were coated, 0.2 ml/well, with the diluted capture antibody and incubated overnight at room temperature. After incubation the plates were emptied and washed twice with wash buffer (17.5 g NaCl, 0.8 ml Tween 20/2 litres deionized water). Plates were inverted and desiccated at room temperature. To each well in triplicate was added 0.2 ml standard (Parlow 9/80) in a dose from 0.25 to 16 ng/ml. Samples (0.1 ml urine) were assayed in duplicate, and the plate was incubated for 2 h at room temperature. The plate was washed 3 times with wash buffer and 0.150 ml Clone 716G₁₁-alkaline phosphatase (1:1000 in assay buffer) was added to each well and incubated for 1 h at room temperature. After incubation the plate was washed 3 times with wash buffer and 0.2 ml substrate solution, 5-bromo-4-chloro-3-indolylphosphate (BCIP, Sigma) was added to each well. Incubation was carried out at room temperature for 25–50 min and plates were read on a Dynatek plate reader at an optical density of 630 nm. The interassay coefficient of variation for 13 ng was 11% and for 4 ng was 9%. Intra-assay coefficient of variation at 1 ng was 6% and at 8 ng was 2%. hLH (4 ng) added to baseline urine was recovered at 100%. Assay sensitivity was 0.5 ng/ml.

Statistics

The area under the curve was analysed by triangulation. LH area was calculated from baseline to baseline ($n = 4-6$ days) during the ovulatory peak. Oestrone conjugate area was calculated as Day -4 to Day 0 (oestrone conjugate peak = Day 0). Day -4 was the first detectable elevation of oestrone conjugate above baseline levels. PDG area was calculated from Day +1 to the day before menstruation ($n = 14-17$).

Hormone values were compared using linear correlation and Student's *t* test. All mean values are expressed as $\pm$ s.e.m.

Fig. 2. Mean $\pm$ s.e.m. daily values of urinary oestrone conjugates, PDG and LH of 4 mature female gorillas (7 cycles). Peak values of oestrone conjugates correspond with the peak level of LH.

Results

The composite urinary hormone profiles of oestrone conjugate, PDG and LH for 7 menstrual cycles of 4 adult female gorillas are shown in Fig. 2. Samples are aligned to the oestrone conjugate peak (Day 0). The average follicular-phase length, defined as the first day of menstruation to the peak oestrone conjugate value was 16.4 ± 1.4 days (range 13–24 days). The average luteal-phase length, calculated as the day after the oestrone conjugate peak to the next menstruation, was 14.0 ± 0.4 days (range 12–15 days). The total length of the cycle was then 30.4 ± 1.5 days (range 27–39 days). Hormone variation between individuals was greatest for PDG excretion. An example of high PDG excretion is illustrated in Fig. 3(a) (M12/24): PDG concentrations at ovulation (day 17) rose above baseline values ($1 \mu\text{g}/\text{mg}$ creatinine) to $2 \mu\text{g}/\text{mg}$ creatinine for 2 days, then between Day 3 and Day 10 after the LH peak a larger amount of PDG was excreted (range $5\text{--}18 \mu\text{g}/\text{mg}$ creatinine). Comparison of this profile to the profile in Fig. 3(b) illustrates the variability of luteal-phase PDG between individuals. A rise in PDG was not measurable in Gorilla H, cycle 11/15 until 3 days after the LH peak (range 1 to $2 \mu\text{g}/\text{mg}$ creatinine). The duration of the peak was 7 days.

To investigate the cause of the variability of PDG excretion rates, the height and duration of the PDG profile was compared to the height–duration of the LH profile by analysing the area under the curve for each hormone (Table 1). Variation in LH excretion within an individual (coefficient of variation range 7–30%) is less than the variation between individuals (CV = 48%). This is also true for PDG (within CV range 0–38%; between 42%), follicular-phase oestrone conjugate (within CV range 0–38%; between 42%), and luteal-phase oestrone conjugate (within CV 10–24%; between 29%). Correlation coefficients calculated by linear correlation indicated a significant ($P = 0.009$) relationship between increased LH excretion and increased PDG excretion ($r = 0.87$). When the area under the ovulatory LH profile was compared to the amount of oestrone conjugate excreted during and including the 4 days before ovulation the correlation coefficient ($r = 0.88$) indicated a significant ($P = 0.008$) relationship between the amount of oestrogen produced by the dominant follicle and the subsequent quality of the LH surge. The correlation between follicular oestrone conjugate and luteal phase PDG approached significance ($r = 0.73$; $P = 0.06$). The luteal excretion of oestrone conjugate significantly correlated with both LH ($r = 0.75$, $P = 0.05$), and follicular-phase oestrone conjugate ($r = 0.83$, $P = 0.05$), and tended to correlate with PDG ($r = 0.68$; $P = 0.09$).

Fig. 3. Daily measurements of immunoreactive urinary oestrone conjugate, PDG and LH for (a) Gorilla M (cycle 12/24) and (b) Gorilla H (cycle 11/15). Solid bars denote the beginning of menstruation. Cycle length is (a) 30 days and (b) 28 days.

Table 1. Areas under the curve for hormones measured in the urine of gorillas

Gorilla	Cycle	LH	PDG	Oestrone conjugate	
				Follicular phase	Luteal phase
P	7/84	10.3	4.8	334	1179
P	10/25	6.6	5.3	341	1011
H	9/24	6.1	1.3	187	715
H	11/15	6.8	1.3	326	905
M	1/24	16.6	6.9	715	1686
M	12/24	19.0	10.1	593	1188
K	8/14	9.4	2.7	467	867

The integrated area under the curve is shown for individual cycles for the ovulatory LH peak (baseline to baseline), luteal-phase PDG (oestrone conjugate peak to the day before the next menstruation), late follicular-phase oestrone conjugates (4 days before and including the peak ovulatory oestrone conjugate value), and luteal-phase oestrone conjugates (oestrone conjugate nadir following ovulatory oestrone conjugate peak to the next menstruation).

Discussion

Daily serum hormone measurements for prolonged periods are difficult to obtain in any situation and would be impossible to obtain from untractable animals like gorillas. Analysis of subtle endocrine differences in ovarian function, which may contribute to reduced fertility, however, require serial measurements through consecutive ovarian cycles. A sensitive enzymeimmunoassay for measuring gorilla LH in urine was therefore developed to assess ovarian and pituitary function. Previously, LH in the urine of gorillas was quantified by a lengthy and cumbersome bioassay. Two monoclonal antibodies against human gonadotrophins were found to cross-react specifically with gorilla LH which permits a more simple and rapid analysis of LH. This is the first study to use a rapid, simple and sensitive immunoassay for gorilla LH.

The results presented here suggest that each of the three urinary metabolic profiles reflect circulating hormone. The larger LH surge, associated with the increased preovulatory oestrogen production, is consistent with the relationship of the positive effects of oestrogen in eliciting the LH surge (Sherman & Korenman, 1974a). Similarly, the enhanced PDG profile after the larger LH surge suggests a causal relationship between the ovulatory surge of LH which has been induced by the antecedent oestrogen peak. In contrast, reduced oestrogen and LH profiles are associated with the lowered luteal-phase PDG profiles. These relationships provide the basis for the inference of an inadequate luteal phase and supports the contention of Mitchell *et al.* (1982a).

Sherman & Korenman (1974b) reported that an inadequate luteal phase in women has a normal luteal length with decreased serum progesterone values. This event is accompanied by decreased follicular and mid-cycle FSH concentrations and decreased luteal progesterone, but normal serum LH. Soules *et al.* (1984) report an increased serum LH pulse frequency during the early follicular phase (Days 4–6) in women with an inadequate luteal phase, with pulse amplitudes slightly lower than normal. Studies relating urinary concentration of LH and oestrogen in the human in this context have not been reported.

In the gorilla cycles examined in this study, through urinary hormone analysis, all had luteal phases of normal length, but varied from individual to individual in the quantity of progesterone metabolite excreted during the luteal phase. When comparing the ovulatory LH peak to the amount of PDG excretion, the high correlation ($r = 0.87$) indicates a positive relationship between increased LH and increased PDG excretion and conversely indicates that diminished PDG excretion is preceded by a lower ovulatory LH surge. In addition, the equally strong relationship between LH and follicular-phase oestrogen production would be consistent with the report of Sherman & Korenman (1974b) of depressed FSH values in the inadequate luteal-phase of women since oestrogen secretion is a direct result of FSH stimulation. It may be possible, therefore, to predict the quality of the forthcoming luteal phase by measuring oestrone conjugates and/or LH during the follicular phase. The high correlation of LH to luteal-phase oestrone conjugate concentrations ($r = 0.75$) additionally confirms the role of LH in stimulating the granulosa (luteal) cells during the luteal phase to produce progesterone and oestrogen. All other correlations support the contention that a marked follicular-phase oestrone conjugate rise precedes a larger LH surge and these are directly related to the amount of progesterone secreted in the luteal phase.

The present results support the claim of Mitchell *et al.* (1982a) that aberrant luteal excretion of PDG can be seen in captive gorillas which have reduced fertility. The inadequate luteal-phase cycles studied here also indicate a compromised follicular development, as judged by low preovulatory oestrone conjugate concentrations, which appear to have resulted in a lowered LH response by the pituitary. Therefore, urinary hormone metabolite values can be general indicators of ovarian function and may be indicators of fertility. Further studies should be designed to address the apparent imbalance in the pituitary–gonadal function and its relationship to reduced fertility in the captive gorilla.

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