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Original Research

Assessing the Pulsatility of Luteinizing Hormone in Female Vervet Monkeys (*Chlorocebus aethiops sabaeus*)

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Specific alterations in the pulsatility of luteinizing hormone (LH) are linked to obesity-related subfertility in ovulatory women. Vervet monkeys (*Chlorocebus aethiops sabaeus*) are an Old World nonhuman primate that develops obesity and has a menstrual cycle similar to humans. We evaluated follicular-phase LH pulses in 12 adult normal-weight female vervets. Serum was collected every 10 min for 4 h by using a tether device in conscious, freely moving monkeys on menstrual cycle days 2 through 5. Serum estradiol was collected daily during the follicular phase to identify the luteal–follicular transition. For comparison, we used data from 12 ovulatory normal-weight women who had undergone frequent blood sampling of early-follicular LH. LH pulse frequency was similar, with 2.8 ± 0.7 LH pulses during 4 h in vervets compared with 2.3 ± 0.7 LH pulses during 4 h in women. The LH pulse mass (percentage change in the pulse peak over the preceding nadir) was 123.2% ± 27.4% in vervets and 60.9% ± 14.9% in humans. The first day of low serum estradiol after the follicular-phase peak was denoted as the day of the luteal–follicular transition. Luteectomy was performed on luteal days 7 through 9, and corpora lutea were confirmed by histology. We demonstrate that follicular LH patterns in vervets are similar to those in humans and that the luteal phase is easily identified by monitoring daily serum estradiol. These findings demonstrate that vervet monkeys are a suitable animal model for evaluating LH pulse dynamics longitudinally in studies of diet-induced obesity.

Abbreviations: CL, corpus luteum; LH, luteinizing hormone.

Nonhuman primates have been used in biomedical research for decades and have enabled advancements in many areas, including HIV–AIDS, Alzheimer disease, diabetes, asthma, and endometriosis.[23] Neuroendocrine research in menstruating nonhuman primates, such as rhesus and cynomolgus macaques, have provided valuable information regarding the hypothalamic–pituitary–ovarian axis, including modulating factors of pulsatile gonadotropin-releasing hormone secretion and the negative and positive feedback mechanisms of sex steroids.[20,25,33]

Normal reproductive physiology in women involves highly coordinated communication between the hypothalamus, pituitary gland, and the end organ of female reproduction, the ovary. These processes are governed by the magnitude and frequency of secretory outbursts (pulses) of gonadotropin-releasing hormone from the hypothalamus. The activity of gonadotropin-releasing hormone results in a pulsatile mode of secretion of follicle-stimulating hormone and luteinizing hormone (LH) from the anterior pituitary. In females, follicle-stimulating hormone drives ovarian follicle growth during the follicular phase of the menstrual cycle. The midcycle LH surge results in ovulation and the subsequent formation of a corpus luteum (CL). Secretion of estradiol, produced by the developing follicles, progressively increases over the course of the follicular (proliferative) phase of the menstrual cycle and peaks prior to ovulation. Progesterone, secreted by the CL, is the dominant sex steroid during the luteal (secretory) phase.[12] Both estradiol and progesterone exert tightly regulated negative feedback on the hypothalamus and pituitary and affect gonadotropin release. Alterations in this intricate system can result in anovulation or infertility.

Obesity is a growing worldwide hazard that has many adverse health outcomes, including subfertility. Endocrine alterations associated with obesity include relative hypogonadotropic hypogonadism[29,34] and selective impairment of LH pulse amplitude.[14] Progesterone metabolite excretion in morbidly obese women is reduced by 70% compared with that in normal-weight women,[29] and pulsatile LH amplitude is suppressed by half in frequent blood-sampling studies.[14] However, despite the recent advances in understanding the endocrine pathophysiology of obesity-related subfertility,[15] its molecular mechanisms are poorly understood.

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Animal models for obesity-related subfertility are needed for mechanistic studies but are currently unavailable. The hormonal control of the menstrual cycle has been extensively studied in rhesus and cynomolgus macaques and is similar to that of humans.[12,22,26] These nonhuman primates have also been shown to develop obesity and resultant metabolic disturbances.[1] However, demand for rhesus and cynomolgus macaques is high, and the NIH has espoused the need to identify other species of nonhuman primate that are suitable for research.[6]

Vervet monkeys (*Chlorocebus aethiops sabaeus*) are a small, Old World nonhuman primate with an ovarian cycle similar to that in humans; therefore vervets may be an appropriate alternative species in which to do neuroendocrine research. LH pulsatility in this species has not been assessed comprehensively. Our objective in the current study was to characterize the follicular LH pulse pattern in vervet monkeys, to establish the feasibility of using this model in future studies to assess the effect of body mass on pituitary function.

Materials and Methods

Animal housing, subject selection, and study design. Monkeys were pair-housed in cages that were equipped with perches and hanging mirrors. The monkeys were fed a commercial nonhuman primate diet (Purina Monkey Chow, PMI, St Louis, MO) once daily in the afternoon, were provided water ad libitum, and received enrichment feedings and foraging opportunities 3 to 4 times a week. All procedures and policies were in accordance with state and federal laws regarding animal welfare. The study protocol was approved by the Wake Forest University Institutional Animal Care and Use Committee. The Wake Forest University Primate Center is accredited by AAALAC.

Twelve adult female vervet monkeys (*Chlorocebus aethiops sabaeus*) were selected randomly from the middle of distribution of body mass from the Vervet Research Colony at the Wake Forest University Primate Center (Winston-Salem, NC). The presence of monthly menstruation was confirmed by daily vaginal swabbing for 2 mo. Body weight, length, and height and waist circumference were measured; and dual energy X-ray absorptiometry was performed. Frequent blood sampling during the early follicular phase of the menstrual cycle was performed to assess LH pulsatility. Serum estradiol levels were measured daily from cycle day 7 until a peak was seen (defined by a subsequent drop in estradiol concentration).

To verify the ovulatory status of study subjects, all vervets underwent minilaparotomy for collection of a CL during the same cycle as for the follicular-phase estradiol monitoring. Prior to surgery, monkeys were premedicated with atropine and then sedated with ketamine (15 mg/kg IM). Isoflurane anesthesia was used during the procedure. By using aseptic technique, a ventral midline abdominal incision was made to enable visualization of both ovaries. The ovary with the CL was identified, and the CL was removed by using blunt dissection. A small piece of the CL was placed in 10% neutral buffered formalin for histologic confirmation, and the rest of the tissue was snap-frozen in RNA Later (Invitrogen, Life Technologies, Grand Island, NY) for future gene expression analysis. All vervets recovered from surgery without complications.

Sample collection. Frequent blood sampling was conducted by using a remote catheter and tether system in awake vervets. Anesthetic procedure for the placement of indwelling femoral catheters was identical to that described for the laparotomy procedure. A catheter was inserted into the femoral vein, sutured in place, and tunneled subcutaneously to exit the skin on the left thorax. The external portion of the catheter then was threaded through a jacket and metal tether system that attached to the back of the cage. The catheter exited the cage and was passed through an aperture in the wall, allowing blood samples to be collected from an adjacent room while monkeys were awake and unaware of blood collection. The monkeys were allowed to recover from line placement for at least 1 wk prior to blood sampling. Indwelling catheters were used to avoid potential suppression of LH release due to stress associated with blood sampling using a restraint system.[28]

Blood sampling for LH pulsatility was performed during the early follicular phase (cycle days 2 through 5). Blood (0.5 mL) was collected every 10 min for 4 h, starting at 0800. Serum samples were frozen until ready to be processed.

Samples for determining follicular-phase serum estradiol were collected daily at 0800 beginning on menstrual cycle day 7 and continued until the estradiol concentration peaked. The day of lowest estradiol (at least 30% to 50% drop) after the midcycle peak was designated as luteal day 1.[7] The ovulatory status during the studied cycles in vervets was confirmed by CL histology.

Body composition. Body composition was assessed by dual energy X-ray absorptiometry (Hologic, Bedford, MA). Vervets were sedated with ketamine HCl (15 mg/kg) intramuscularly and transported to the dual energy X-ray absorptiometry scanner for measurement of body composition. Vervets were placed in the supine position, with the tail secured to one of the animal's thighs to ensure that the tail was included in the scan. A whole-body scan was performed to assess lean mass, fat mass, and bone density. Scans were analyzed by using APEX 3.3 software (Hologic).

Menstrual cycle documentation. Due to the small volume of menstrual blood characteristic for this species,[4] vaginal swabbing was performed daily to evaluate for menses. Vervets were trained to present for vaginal swabbing by positive reinforcement with food treats. A cotton-tipped swab was inserted once daily into the vagina until it reached the cervix. The presence and amount of blood was recorded by using a scoring system of 0 (no blood), 1 (light), 2 (moderate), or 3 (heavy).

Assays. LH radioimmunoassays were performed by the Endocrine Technology and Support Lab (Oregon National Primate Research Center, Beaverton, OR) by using a modified double-antibody radioimmunoassay[24] that the laboratory routinely uses for LH measurement in cynomolgus, rhesus, and Japanese macaques as well as vervets.[21]The LH radioimmunoassay kit was purchased from Dr Albert Parlow (NHPP, Harbor–UCLA Medical Center, Los Angeles, CA) and is a homologous cynomolgus macaque assay with recombinant cynomolgus LH (AFP-6936A) for both iodination and standards. The rabbit anticynomolgus LH antibody (AFP-342994) was used at a final dilution of 1:972,973. The standard curve ranged between 0.005 and 10 ng per reaction. The detection limit of the assay was 0.005 to 0.1 ng per reaction. At sample volume of 100 µL, the sensitivity of the assay was 0.05 to 0.1 ng/mL. The assay shows minimal or no crossreactivity with hormones other than the cynomolgus LH provided in the kit. The intraassay variation was less than 8%, and the interassay variation was less than 12%.

Follicular-phase serum estradiol was assayed daily by using chemiluminescence (Immulite 1000, Siemens, Deerfield, IL).

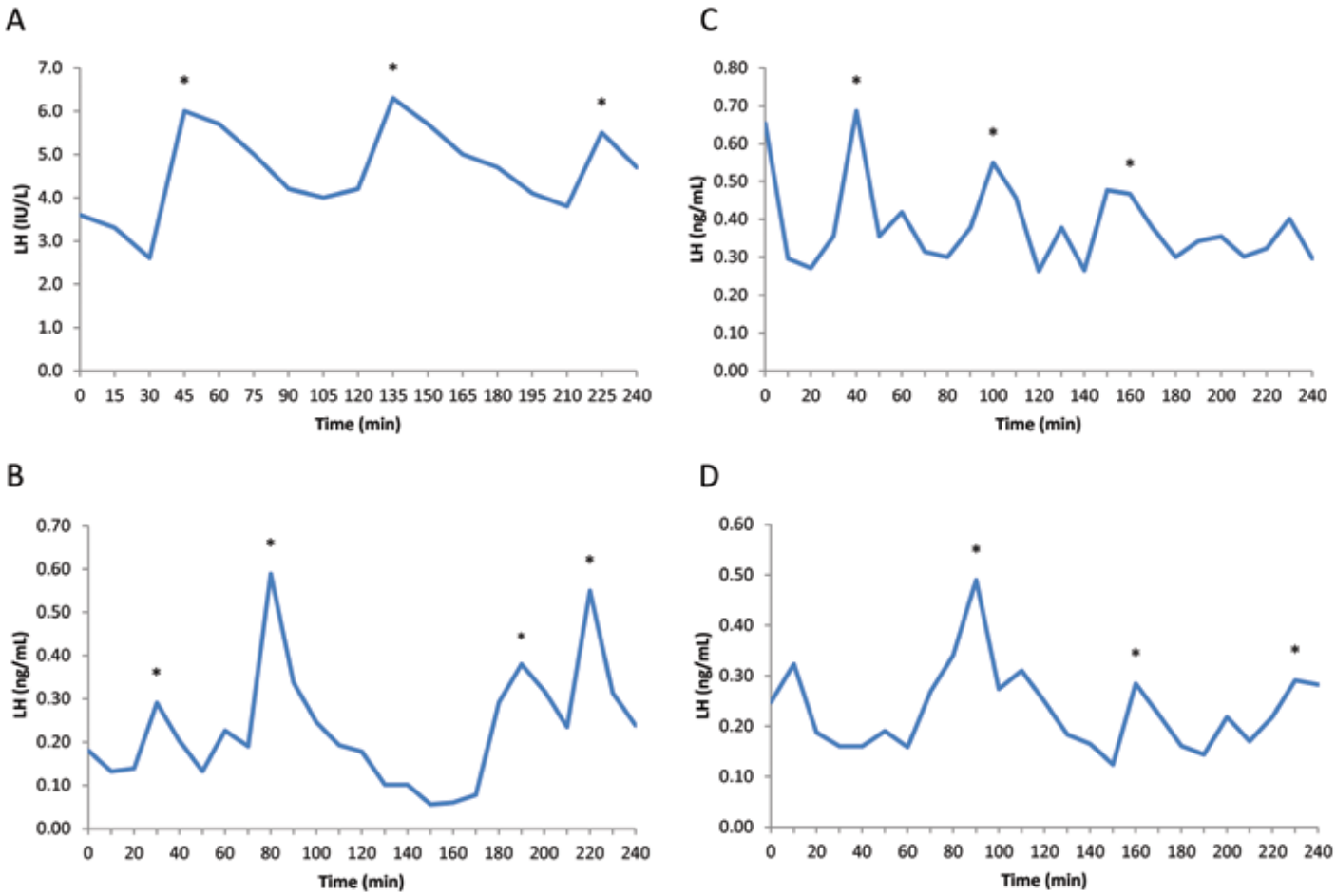

Figure 1. Representative pattern of LH pulsatility in (A) women and (B–D) vervet monkeys. Pulses marked with asterisks.

Data analysis. LH levels were evaluated for the following parameters: mean, peak (highest level during sampling), and baseline (mean of all troughs recorded before the peaks). According to a modified Santen–Bardin algorithm,[30] an increase of 20% from the preceding nadir for 2 consecutive time points defined an LH pulse. The LH pulse frequency was calculated for the 4-h study period. Pulse amplitude was calculated as the peak value of each pulse minus the preceding nadir. In addition, LH pulse mass, defined as the percentage change of the LH pulse peak over the preceding nadir for each pulse, was calculated to enable cross-assay comparison of human and vervet data.

Human comparison group. To assess the utility of using LH pulsatility in vervet monkeys as a model of human disease, vervet outcomes were compared with data previously collected from 12 healthy, normal-weight, ovulatory women.[2] Frequent blood sampling was performed through an indwelling IV catheter for LH pulsatility evaluation every 15 min starting at 1000 for 24 h on cycle days 2 through 7. LH was measured by using a solid-phase, 2-site specific immunofluorometric assay (Delfia, Perkin Elmer, Turku, Finland). Intraassay and interassay coefficients of variation for human serum LH were 2.3% and 5.5%, respectively. Pulse analysis methods were identical to those for vervet monkeys. Ovulatory status in women was confirmed by the presence of a midluteal progesterone greater than 9.43 ng/mL in the current and preceding menstrual cycle. Before data collection, this protocol was approved by the Individual Institutional Human Subjects Review Boards at the University of Pittsburgh School of Medicine and at Magee Women's Hospital.

In the human controls, LH pulse frequency was determined by dividing the mean pulse frequency during the first 12-h study period by 3. The group means (± 1 SD) were compared by using *t* tests (STATA 11, StataCorp, College Station, TX); a *P* value less than 0.05 was considered to be significant.

Results

In our group of 12 adult female vervet monkeys, mean age (± 1 SD) was 7.13 ± 2.16 y, body weight was 4.8 ± 0.6 kg, and percentage body fat by dual X-ray absorptiometry scan was 16.4% ± 4.1%. Female vervets reach puberty at 2.5 y of age and attain adult size by 4 y.[8] The body weight and percentage body fat of the study population were within the normal range when compared with prior cross-sectional studies of animals in the Vervet Research Colony.[5,17]

All 12 vervets demonstrated LH pulses as calculated by the modified Santen–Bardin pulse algorithm (Figure 1); vervet LH pulse parameters are shown in Table 1. Daily serum measurements clearly revealed a unimodal rise in estradiol concentration during the follicular phase of the cycle (Figure 2). Daily assessment of serum estradiol indicated that the estradiol peak occurred

Table 1. Luteinizing hormone parameters

Parameter	Vervets (n = 12)	Women (n = 12)	P
Number of pulses per 4 h	2.8 ± 0.7	2.3 ± 0.7	0.43
Pulse amplitude	0.22 ± 0.05 ng/mL	1.6 ± 0.2 IU/L	not done
Pulse mass (% increase from baseline)	123.2 ± 27.4	60.9 ± 14.9	<0.01
Mean level during sampling	0.26 ± 0.05 ng/mL	3.2 ± 0.9 IU/L	not done
Baseline	0.18 ± 0.04 ng/mL	2.7 ± 0.7 IU/L	not done
Peak	0.49 ± 0.15 ng/mL	4.6 ± 1.3 IU/L	not done

Data are presented as mean ± 1 SD.

on menstrual cycle day 14.7 ± 3.0; the mean intermenstrual interval was 30 ± 4.1 d. All monkeys had distinct CL, which were confirmed by histology, during the monitored cycle (Figure 3).

The human comparison group comprised 12 women with a mean age 24.9 ± 4.8 y and normal average body mass index of 20.8 ± 0.5 kg/m^2.[36] All women volunteers had regular menstrual cycles (range, 25 to 35 d). Frequent blood sampling in the study women indicated approximately 2 LH pulses per 4 h, corresponding to 0.58 pulses hourly (Table 1), similar to the rate in vervets. The absolute increase in LH pulse mass (percentage increase from baseline) was significantly (P < 0.01) greater in vervets than in women.

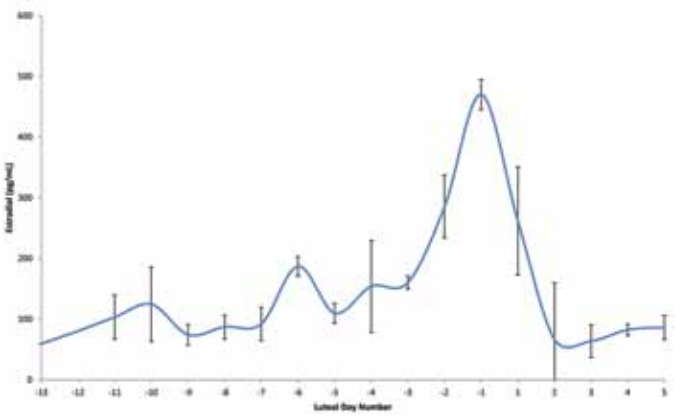

Figure 2. Composite mean serum estradiol in 12 female vervets during follicular phase of the menstrual cycle. Data was centered on the day of luteal transition (day 0) as defined by a drop in estradiol. Error bars represents standard error of mean

Discussion

To our knowledge, the current study is the first characterization of follicular-phase LH pulsatility in normal-weight adult vervet monkeys. We demonstrated regular LH pulses during early follicular phase in this species; and these pulses were easily determined by using a modified Santen–Bardin pulse algorithm.[30] In concert with our findings, others reported similar menstrual cycle length and follicular-phase duration in vervets.[4,18] Follicular-phase LH pulse frequency in rhesus monkeys has been reported to be 14 or 15 pulses during a 12-h period (approximately 5 pulses per 4 h),[25] which frequency is higher than those in our vervets as well as our normal-women comparison group. This finding suggests that vervets may represent a better animal model species for LH pulsatility than are rhesus macaques.

The Santen–Bardin method is a classic assumption-free methodology that has been used continuously since the 1970s. We opted to use this method for the vervets in the current study because the human comparison data were analyzed by using this technique.[2] In addition, the modified Santen–Bardin method has performed well in head-to-head comparison with other algorithms and has been demonstrated to be superior at increased LH pulse frequencies.[13]

Obesity in humans is associated with menstrual cycle irregularity, anovulation, and adverse pregnancy outcomes.[3] Reduction in LH pulse amplitude has been demonstrated to occur in obese women and represents a potential therapeutic target to combat the effect of obesity on fertility and reproductive ability.[14] Animal models for obesity-related effects on the hypothalamic–pituitary–ovarian axis would be useful for elucidating molecular mechanisms by which obesity impairs reproduction. Given the reported limited availability of historically used nonhuman primate species, such as rhesus macaques, and the need for alternative nonhuman primate models,[6] we chose to use a readily available, small nonhuman primate species. Compared with larger species, small nonhuman primates have been reported to be less prone to stress from handling due to their small size, making handling easier.[35] Therefore, vervets provide a less expensive, readily available, and trainable primate model of known genetic pedigree and thus are well suited for complex mechanistic experiments. In addition, the Vervet Research Colony has demonstrated utility as a promising candidate for an animal model of neuroendocrine changes associated with obesity.[17]

Vervet monkeys represent a potentially highly translatable animal model to elucidate the effect of obesity on various target organs, including the reproductive axis. A recent cross-sectional study of 295 adult vervets assessed the prevalence and heritability of obesity. Obesity, defined by a waist circumference exceeding 40.5 cm (corresponding to the upper 20th percentile) was identified in 25% of female and 16% of male vervets. Mirroring that in humans, obesity in vervets was associated with insulin resistance and triglyceridemia. Furthermore, visceral adiposity was highly heritable within the study population.[17] Therefore, the close degree of similarity between human and vervet obesity make vervets a particularly attractive tool to elucidate the mechanisms underlying obesity-related female reproductive impairment.

With the current study, we expand the literature by providing the first quantitative analysis of vervet LH pulsatility, which underscores the menstrual cycle mode of regulation in this nonhuman primate species. In contrast, species exhibiting estrous cycles demonstrate distinct physiologic regulatory mechanisms, exemplified by copulatory induction of ovulation in some species.[16] Moreover, whereas estrogens display a bimodal pattern during the menstrual cycle of humans, the estrous cycle in rats demonstrates only a single peak.[31] We recently reported a bimodal

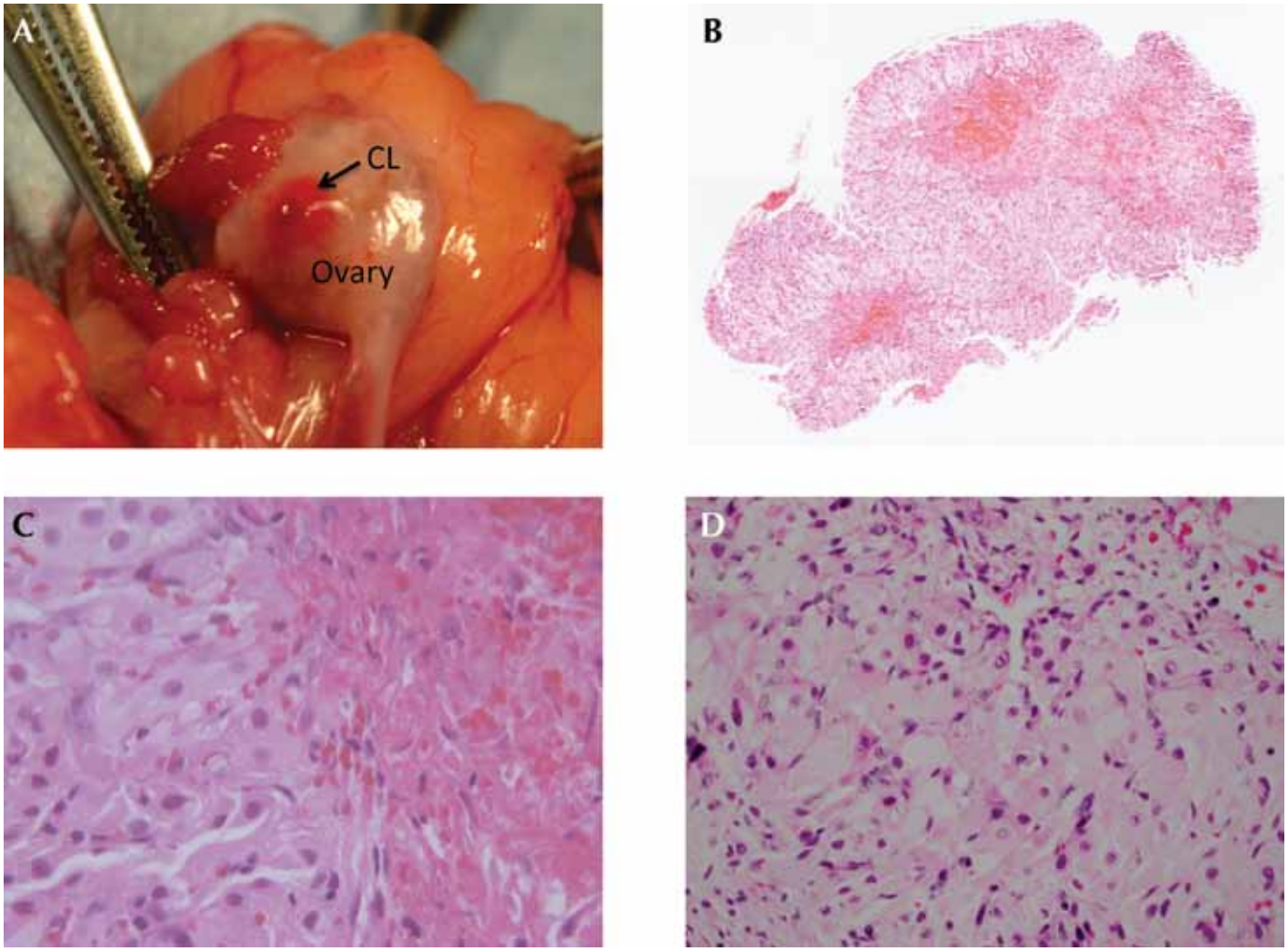

Figure 3. Vervet corpus luteum. (A) Ovary with corpus luteum (CL) exposed during luteectomy procedure. (B) Hematoxylin and eosin stained Section of vervet CL showing undulating shape. Hematoxylin and eosin stain; magnification 40×. (C and D) Sections of vervet CL showing characteristic steroid-producing cells with abundant granular eosinophilic cytoplasm and small, round, monomorphic nuclei with central dot-like nucleoli. Hematoxylin and eosin stain; magnification, 400×.

estrogen pattern in vervets by using urinary estrogen metabolite excretion surveillance.[19] Therefore, the differences in hormonal regulation between the menstrual and estrous cycles make it preferable to use menstruating animals (in our case, vervet monkeys) to model human disease affecting the hypothalamic–pituitary–ovarian axis and control of ovulation. Finally, as compared with larger animals, vervets provide a relatively less expensive, readily available, and trainable primate model with known genetic pedigree; therefore this species is well suited to use in complex mechanistic experiments.[9]

A limitation of our study is its small sample size. This report represents baseline assessment obtained from a study of the effects of diet-induced obesity on LH pulsatility in vervets. Sample size was calculated for 80% power to detect a 22% difference in LH pulse amplitude before and after the development of obesity and with an α error of 0.05. Another limitation is the difference in frequency of serum sampling between the human comparison and vervet groups; however, prior evaluation has shown 10- and 15-min sampling intervals to yield the same results.[11] Finally, our relatively brief session of frequent blood sampling in vervets may limit the ability to estimate pulse frequency reliably. However, in human studies, a 4-h session for measuring LH pulses has been used successfully by several groups[27,32] and yielded the same pulse frequency in our human comparison group as did the full 24-h period of frequent blood sampling used previously.[2]

In summary, our current findings further support the similarities between the hypothalamic–pituitary–ovarian axes in vervet monkeys and humans and provide a reference point for LH pulsatility in regularly cycling, adult, vervet monkeys. The similarities in follicular-phase LH pulsatility between female vervets and women further suggest that vervets are a promising model in which to study disorders of the hypothalamic–pituitary–ovarian axis and are well suited to the longitudinal evaluation of reproduction in the context of diet-induced obesity.

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