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Analysis of follicular events in owl monkeys (*Aotus azarai infulatus*) using B-mode and Doppler ultrasound

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ABSTRACT

Ultrasound (B-mode) was used to analyze follicular events in 12 trained female owl monkeys (*Aotus azarai infulatus*). The animals were examined every 48 hours for over 90 days to measure and map follicular growth in both ovaries and to measure (using Doppler velocimetry) local hemodynamic changes during the peri-ovulatory stage. There were 44 follicular growth events, each with two or three follicular waves, and a mean ± SEM interval between events of 17 ± 1.13 days. There were various hemodynamic changes during follicular growth; both vascular resistance index and pulsatility index decreased during the time when the follicle diameter peaked. Thus, both B-mode and Doppler ultrasound were useful for monitoring ovarian follicular events in owl monkeys.

1. Introduction

Nonhuman primates (NHPs) are extremely important animals for biodiversity because they contribute to a variety of species in the ecosystem [1]. A primary goal of research in primate reproduction is preventing the disappearance of endangered NHP species. Studies on animals under controlled conditions provide information regarding the physiological basis for reproductive events.

The Brazilian National Primate Center (Centro Nacional de Primatas—CENP) at the Evandro Chagas Institute and the Health Surveillance Office (the Brazilian Ministry of Health) are responsible for performing biomedical studies in the Amazonian region and maintaining a colony of owl monkeys for reproductive studies. Mating this species in captivity has been considered difficult [2], but it can be enhanced by closely simulating the social structure of their natural habitat [3].

Studies on the reproduction of captive animals from the *Aotus* genus have been increased recently [4–11]. However, reproductive success in captivity is generally not accompanied by basic studies of the reproductive physiology of the genus. Although monitoring the estrous cycle is a basic procedure, performing this on wild animals can be more complex.

Ultrasound technology is an excellent tool for studying primate gynecology [7,9] as it allows the internal organs to be monitored noninvasively [12]. In addition to other technologies, such as Doppler velocimetry, which has been used for monitoring the gestational and estrous cycle in both domestic and wild animals [13], ultrasound can be used to monitor ovarian follicular events in owl monkeys.

The objective of this study was to monitor (B-mode and Doppler ultrasound) ovarian follicular events in owl

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monkeys and to increase our understanding of the reproductive physiology of this species.

2. Materials and methods

The project was carried out at the Brazilian National Primate Center (CENP/Ananindeua, PA) between April and August 2011 and was approved by the Ethical Committee for Animal Research at the Evandro Chagas Institute (Comitê de Ética de Pesquisa com Animais do Instituto Evandro Chagas—CEPAN/IEC, 036/2008).

Twelve adult females (six multiparous, four primiparous, and two nulliparous animals) from the owl monkey (*Aotus azarai infulatus*) reproductive colony at CENP were used. These females were isolated from males and were kept in individual brick pens with tiled walls and floors and metal screens covering the top, back, and front sides. The monkeys were maintained in a closed system in the mating facility number I and were not mated.

All animals were evaluated physically in the laboratory by using direct examinations: the flotation technique [14], the sedimentation technique [15], and the coproparasitological examinations.

Animals were fed various fruits, vegetables, roots, tubers, milk, eggs, and commercial feed for Cebidae (Cebidae P18 Megazoo, Megazoo Feed, Betim-MG, Brazil) in accordance with the care practices adopted by CENP. The animals also received daily supplements containing amino acids (0.5 g/kg of their body weight), vitamins, and macro- and microminerals (Aminomix Pet—Vetnil Indústria e Produtos Veterinários Ltda, Louveira-SP, Brazil) and had *ad libitum* access to water.

2.1. Training animals to be examined with ultrasound

Females were trained for ultrasound examinations using daily food rewards for over a 30-day period [10]. Females were later taken to the reproductive facility clinic at CENP in an animal transport cage, removed from the cage, and physically restrained using leather gloves to perform the examination. After the animals became acclimatized to being restrained, their training proceeded efficiently. The females were highly cooperative during the examinations. Vocalization, urination, and defecation only occurred during their capture in the enclosure and their removal from the transport carrier. Each ultrasound examination lasted for an average of 10 minutes.

2.2. Ultrasound analysis of follicular events

The B-mode and triplex Doppler ultrasounds were performed every 48 hours for over a 90-day period (45 times per animal) to monitor the ovarian cycle.

Females were restrained and placed in a supine position while their abdominal areas were shaved. A water-based gel was used to aid the ultrasound. During the examination, females were rewarded with several types of fruit, as described by Monteiro et al. [7,10].

A MyLab30 VET Gold (Esaote, Genoa Via A. Siffredi, 58 16153 Genova, Italy) ultrasound machine equipped with a linear multifrequency transducer (LA435, 6–18 MHz) was used to perform the ultrasounds. Images were blindly analyzed on a 15″ LCD high-resolution monitor by an experienced technician who located and evaluated the ovaries for size, shape, position, follicular growth pattern, ovulation, and corpus luteum (CL) formation.

Follicles were defined as structures that were filled with anechoic fluid and surrounded by a smooth outline in the ovarian stromal. Ovulation was considered to have been occurred when a follicle with large diameter was detected during one examination but was not observed during the following examination. The interval between the two follicular growth periods was defined as one cycle.

The hemodynamic characteristics of the right and left internal iliac arteries (RIIA and LIIA, respectively) were measured in triplex Doppler mode, and the arteries were identified and analyzed in a longitudinal section. The blood flow velocity waveforms were obtained by measuring a sample of the volume with colored Doppler, followed by spectral Doppler [16]. The equipment automatically calculated the vascular resistance index (RI) and pulsatility index (PI) following adequate measurements of the frozen image using calipers.

The results of the gynecological measurements were summarized in ultrasound examination reports that were saved in the equipment and were later recorded in a database on a portable external 250-GB hard drive (Samsung Eletrônica da Amazônia Ltda, Av. Itauba, BR 319 km 03, Manaus-AM, Brazil).

2.3. Statistical analysis

Basic descriptive statistics were calculated for all the analyzed variables, including the average duration of follicular events. An analysis of the Doppler ultrasound data was performed by comparing the averages of the periovulatory stage with the average of the whole cycle.

3. Results

3.1. Ultrasonography

Ultrasound provided an excellent visualization of ovaries, which are located cranial-laterally to the uterus, close to the internal iliac arteries and veins (Fig. 1). The ovaries were round in shape and slightly elongated with fine hypoechogenicity and homogeneous aspects. The mean ± SD volumes of the right and left ovaries were 0.32 ± 0.05 cm^3 and 0.38 ± 0.09 cm^3, respectively. The growing follicles were observed in various stages of development. The follicles were round, homogeneous, and finely anechoic and were located in the ovarian stromal (generally not extending beyond the organ's boundaries). The presence of a follicle made the ovarian stromal appear heterogeneous by ultrasound, which allowed differentiation of the stromal from the CL. Follicles were detected frequently with an average interval of 17 ± 1.13 days (average ± SEM). Forty-four events (22 in each ovary) that were compatible with follicular growth were observed. Two females had two events, four females had three events, two females had four events, and four had five events. Of these events, 21 (47%) had two or three follicular waves

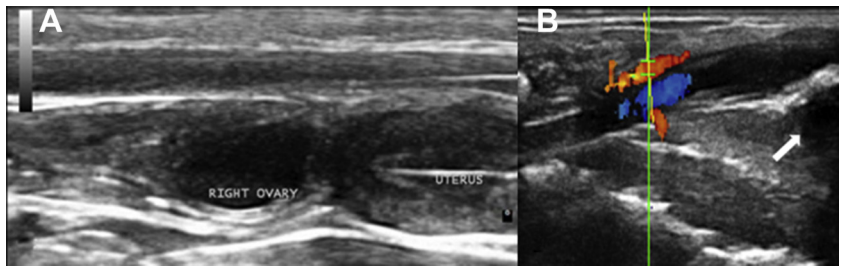

Fig. 1. (A) Ultrasound image of the uterus and right ovary of a primiparous owl monkey in sagittal section. (B) Ultrasound image of the left internal iliac artery and vein Doppler imaging and part of the ovary (arrow). (CENP—Ananindeua, PA, 2012).

identified by ultrasound, with the growth and regression of nondominant follicles being observed before the follicle with the largest diameter was formed (Fig. 2). All subjects had at least one cycle during the study period. The average diameter of the follicles was 0.37 cm (range, 0.2–0.8 cm). The CL was not visible; therefore, follicles >0.4 cm in diameter that disappeared before the next examination and did not reappear with a larger or developing follicle were considered ovulatory.

3.2. Doppler velocimetry of the iliac arteries

Ovarian and uterine arteries were difficult to identify. Therefore, the RIIA and LIIA, which are wider and more easily visualized, were used for Doppler velocimetry studies. The RIIA and LIIA are parallel to the internal iliac veins that are clearly shown in B-mode and exhibited a normal wave spectrum, which was readily observed in Doppler mode (Fig. 3). The RI varied significantly in the RIIA and LIIA ($P < 0.05$), with an average value of 0.84 ± 0.07 for the RIIA and 0.85 ± 0.06 for the LIIA, higher than the RI calculated in the same vessels during the examination performed close to the period when the largest diameter follicles (LDFs) were observed (up to 96 hours before or after), 0.79 ± 0.06 and 0.78 ± 0.04, respectively. The PI decreased rapidly during the ovulatory period and subsequently increased afterward. The average PIs of the RIIA and LIIA were 2.24 ± 0.52 and 2.31 ± 0.49, respectively. However, these indices decreased (~20%) to 1.80 ± 0.34 and 1.80 ± 031 ($P < 0.05$) when the LDF was observed.

4. Discussion

Unlike previous studies [4,7,9], which used three measurements for each ovary, the present study measured the volume of each ovary using only a single image. The use of more advanced equipment and a higher resolution transducer, than had been used previously, facilitated retrospective image analysis and contributed to the speed of the examination. Consequently, the duration of animal restraint required was decreased, with corresponding reductions in stress-related behaviors.

Fig. 2. Ultrasound of the right ovary of primiparous owl monkey in transversal view, with preovulatory follicle (arrow). (CENP—Ananindeua, PA, 2012).

Minimizing stress-related behaviors during this type of study is essential to avoid neuroendocrine mechanisms that can alter the estrous cycle. Increased plasma concentrations of adrenocorticotrophic hormone and cortisol can inhibit the activity of reproductive hormones and consequently interrupt a cycle or induce anestrus [17]. In addition to reducing the duration of restraint by the use of the ultrasound technique, the inclusion of an adaptation period and offering fruit had positive results [7,9]. The efficacy of this technique was evident by the relative lack of behavioral changes throughout the course of the study and the frequent observation of follicles, even in the absence of data regarding blood concentrations of stress hormones.

The average period between observations of LDFs (17 ± 1.13 days) may indicate cycle length, which would be in agreement with the average cycle length in owl monkeys [18,19]. However, because we could not identify CL using this ultrasound technique, it was difficult to determine the exact follicular and luteal periods. Hormone concentrations in fecal samples from wild animals showed an average cycle length of 22 ± 3 days [20], which seemed longer than the cycle length observed in captive animals. This difference might be due to the controlled environment and diet of animals in captivity, although further studies are needed.

In owl monkeys, the uterus is the best anatomic landmark for locating the ovaries. Ultrasound imaging of ovaries in the present study provided similar results to previous reports [4,7,9]. The average ovarian volume in this study was larger than in previous studies of owl monkeys. However, it was noteworthy that individual follicles were observed in this study, but not in the previous studies.

The left ovary was significantly larger that the right ovary. This result is different than the results previously reported by Monteiro et al. [7], but is similar to what occurs in humans and the same species [9]. However, this increase in size does not indicate increased ovarian activity, unlike the results described for *Leontopithecus rosalia* [21].

Follicles were identified as described by Monteiro et al. [7] and had a diameter similar to those in other NHP

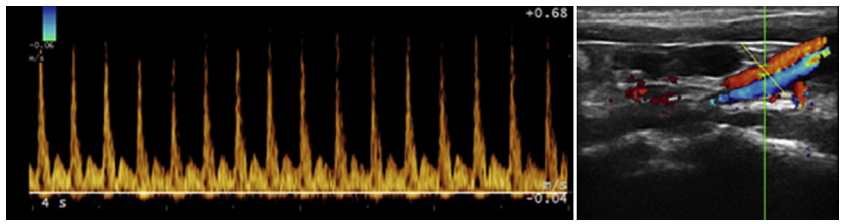

Fig. 3. Ultrasound image of the right internal iliac artery and vein with normal waves spectrum, visible in triplex Doppler mode in an owl monkey. (CENP—Ananindeua, PA, 2012).

species [12,13,21]. The follicular growth pattern observed in almost 50% of the follicular events was similar to the follicular waves observed in domestic mammals [22–25]. Follicular waves have not been described in *Aotus* sp., and only human women have been shown to have this characteristic among the primates [26]. However, the follicular waves did not have more than two simultaneously growing follicles.

Doppler velocimetry data, in association with assessment of ovarian function in *Aotus* sp., have apparently not been previously reported. This technique was performed on *Cebus apela* [16]. However, they used chemical restraints during their examinations, which may cause hemodynamic effects that may have altered the characteristics of the examination waves [27]. Therefore, another unique characteristic of the present study was the use of only physical restraint.

Previous studies have used the ovarian and uterine arteries to measure the RI and PI in *Cebus* [17] and humans [28,29]. In *Aotus*, these vessels are fairly small, making them difficult to locate using ultrasound; furthermore, with the animals' limited cooperation during Doppler examinations, it was impossible to take measurements of these arteries. The iliac artery is the largest of the vessels that perfuse the reproductive organs, making it the easiest to visualize by ultrasound. The shape of the Doppler signal or wave reflects the impedance of the vascularization downstream of the measurement location. Therefore, analyzing the iliac arteries is a valid method for tracing the hemodynamic behavior of the ovaries in relation to follicular events [28].

In women [30], the RI and PI progressively decrease when pre-ovulatory follicles are present at the beginning of the LH peak, consistent with our results. The activity of sex hormones and growth factors increases ovarian metabolism during follicular growth, ovulation, and CL formation by promoting neovascularization and dilating the local vessels. In the present study, ovarian blood perfusion exhibited cyclic characteristics with an increase during the peri-ovulatory period, which was confirmed by changes in the vascular indices, as described in studies conducted on dogs [31] and in *Cebus apela*, with changes in PI and RI near ovulation [16].

In conclusion, both B-mode and Doppler ultrasound modes were effective for monitoring the follicular events in owl monkeys. Furthermore, the mean RI and PI of iliac artery decreased near the time of ovulation and it is expected that these results can be used for future studies on the use of biotechnology on reproduction.

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