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Serum Progesterone and Estradiol Levels Throughout the Endoscopy-Observed Ovarian Cycle in Captive Formasan Macaques (*Macaca cyclopis*)

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Abstract.- The Formosan macaque is endemic to Taiwan. It is considered one of the least known among the 19 extant species of the genus *Macaca*. Although studies of reproductive hormones and the ovarian cycle have been extensively conducted on other macaques, there is little information of Formosan macaques. The purpose of the present study was to establish information about the reproductive physiology of Formosan macaques. From February 2013 to November 2013, 11 wild adult female Formosan macaques were selected for the study. Blood sampling from anesthetized macaques started on the day of first menstrual bleeding and was performed on days 4, 7, 10, 11, 12, 13, 14, 15, 16, 18, 20, 22, 24, and 27 afterwards. Laparoscopic examination was carried out every six days after the first day of menstrual bleeding, and daily during the periovulatory stage. The duration of the ovarian cycle, follicular phase and luteal phase was 28.04 ± 3.0 days, 13.3 ± 2.1 days and 14.1 ± 1.3 days respectively. In addition, changes in serum levels of sex hormones were compared with endoscopic observations; the peak serum level of estradiol was 290 ± 12.7 pg/ml, and predicted an ovulation episode within 24–48 hours. Serum progesterone rose to a peak of 9.13 ± 1.78 ng/ml eight days after ovulation and then declined gradually. The data may be considered as reference values associated with the ovarian cycle in Formosan macaques. Hopefully, the study will be beneficial to further studies and the conservation of this endemic species in Taiwan.

Key words: Endocrine, laparoscopy, primate, reproduction

INTRODUCTION

As a consequence of their close phylogenetic relation to humans, higher primates have attracted considerable interest in a wide range of research areas. The similarities in the biological mechanisms of human and non-human primates underlie the value of the latter for research across a broad range of disciplines. In particular, studies on the reproductive biology of non-human primates have been increasing in recent decades (Shimizu, 2008). Macaque models have been useful specifically for research on ovarian cycle control, endometriosis, anovulatory infertility, osteoporosis, stress-associated infertility, menopausal changes in physiology, and related metabolic disorders (Weinbauer *et al*., 2008). In addition, to solve the human–macaque conflict which causes financial loss, structure destruction and rises the concern of zoonotic disease transmission that occurs in Asia and Africa (Priston and McLennan, 2013), numerous contraception strategies have also been proposed, based on fundamental studies of the reproductive biology of macaques (Shek and Cheng, 2010).

The Formosan macaque is endemic to the island of Taiwan. It is considered one of the least known among the 19 extant species of the genus *Macaca* (Hsu and Lin, 2001). Although studies of reproductive hormones and the ovarian cycle have been extensively conducted on Japanese macaques (*Macaca fuscata*), long tail macaques (*Macaca cynomolgus*) and rhesus macaques (*Macaca mulatto*) (Shimizu, 2008), there is little information about the reproductive hormones of Formosan macaques. Therefore, the purpose of the present study was to clarify the hormonal changes occurring throughout the ovarian cycle by measuring the serum progesterone and estradiol in serial samples from individual animals. Meanwhile, with pre-

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determined ovarian cycle by vaginal cytology, we observed the ovarian cycle by endoscopy and correlated the findings with the hormonal changes. This fundamental study will provide more information on reproductive physiology and will be of benefit to the conservation of this endemic species in Taiwan.

MATERIALS AND METHODS

Animals

From February 2013 to November 2013, 11 wild adult female Formosan macaques were sheltered in the Wildlife Conservation Center, I-Lan, Taiwan. The sources of the animals included wild casualties, those resulting from government confiscation, and orphans. All methods proceeded according to the wildlife conservation law of Taiwan announced in 2011, and were approved by Application to the Endangered Wild Animals Committee. All macaques were housed in separate cages measuring 1 × 1.8 × 1.2 m, maintained in a semi-open outdoor space. The average ambient temperature and average humidity were between 19.1% to 28.6% and 76% to 87% respectively, which were identical to the outdoor environment and were not controlled manually. Physical examination and blood tests, including complete blood cell count and serum biochemistry, were performed to confirm that all the macaques were clinically healthy. The macaques were fed monkey chow, toast, fruit and seasonal vegetables twice a day. Water was provided *ad libitum*.

Anesthesia

After 12 h of fasting, all macaques were submitted to light anesthesia or heavy sedation via intramuscular injection of dexmedetomidine hydrochloride (Dexdomitor®, Pfizer Animal Health, NY, USA) at 0.02 mg/kg and zoletil (Zoletil®, Virbac, Carros, France) at 1 mg/kg. If laparoscopy was scheduled on the same day, the macaques were submitted to anesthesia via intramuscular injection of dexmedetomidine hydrochloride at 0.03 mg/kg and zoletil at 3 mg/kg. The dosages were adjusted according to the author's personal experience. After intubation and intravenous catheterization, anesthesia was maintained using propofol (Lipuro 1%, B.Braun Melsungen AG, Melsungen, Germany) at 0.4mg/kg/min when necessary. Intermittent positive pressure ventilation was provided by ambu bag only when voluntary ventilation was absent or the peripheral capillary oxygen saturation (SpO_2) was lower than 90%. Monitoring included assessments of withdrawal reflexes in the four limbs and ventral muscle tone, jaw tone, heart rate, pulse oximetry, indirect blood pressure measurement by Doppler, and body temperature. After the procedures, the animals were injected intramuscularly with atipamizole hydrochloride (Antisedan®, Orion Corporation, Espoo, Finland), using the same volume as the dexmedetomidine, for reversal of anesthesia.

Ovarian cycle monitoring

The cyclic pattern was determined initially from vaginal smears for two cycles. Subsequently, the hormone pattern was assessed by blood sampling, which was starting on the day of first bleeding and performed on days 4, 7, 10, 11, 12, 13, 14, 15, 16, 18, 20, 22, 24, and 27 afterwards (Weinbauer *et al*., 2008). Blood samples (1.0–2.0 ml) were taken from the femoral vein of the anaesthetized animals using a 2.5-ml syringe with a 25-gauge needle. The samples were centrifuged at 3,000 rpm for 15 min immediately after sampling, and the serum collected was stored at –20°C until assay within 2 months. Ovulation was observed by laparoscopy and analyzed in conjunction with the hormone data in order to interpret the cyclic changes.

The follicular phase is defined from the first day of menstrual bleeding to the day of ovulation; the luteal phase is defined from the day after ovulation to the next menstrual cycle. In some cases, the monkeys were kept under continuous anesthesia for several hours so that the moment of ovulation could be observed. If ovulation did not occur on the day of laparoscopic observation, the day of ovulation was defined as one day before laparoscopic observation of a hemorrhagic plaque on the ovary. The durations of the complete cycle, menstrual bleeding, the follicular phase and luteal phase were recorded.

Hormone assays

Hormone assays for progesterone and

estradiol were performed on sera using the ADVIA Centaur® Progesterone assay (Siemens Corporation, Malvern, PA, USA) and Cayman Chemical® Estradiol EIA Kit (Cayman Chemical company, Ann Arbor, MI, USA). Determination of the hormone concentrations was performed in triplicate at two different dilutions according to the manufacturers' instructions without any modification.

Table I.- Endoscopic equipment used for ovarian cycle observation.

Rigid endoscopy equipment (Karl Storz GmbH & Co. Tuttlingen, Germany)
64018BSA, Hopkins telescope, 2.7 mm x 18 cm, 30°
30114GK, 3.5-mm graphite and plastic cannula with valve and stopcock, and trocar
30322MDS, 3-mm short curved Kelly dissecting and grasping forceps, plastic handle without ratchet
495NVL, Fiber optic light cable, with 90° deflection to the instrument, 3.5 mm, 300 cm
62120J, Veress pneumoperitoneum needle, 10 cm
20043101-020, Tele pack NTSC
26430508-1, Electronic Endoflator Set

Endoscopy

Laparoscopic examination (Table I) was performed every six days after the first day of menstrual bleeding, and daily during the periovulatory stage. Under anesthesia, each macaque was placed in dorsal recumbency with the head facing the rear end of the surgery table. Both hind legs and the tail were stretched out over the front end of the table, with the pelvis padded higher so that the reproductive system would fall down into the abdominal cavity. The endoscopic tower was located at the very front end of whole surgical unit. The surgical entry site of the telescope port was on the midline just below the umbilicus; the instrument port was caudolateral to the telescope port and cranial to the fallopian tube on each side. A 0.2 cm incision was made on the midline just below the umbilicus. A Veress needle was directed caudally and pushed through the linea alba. Care was taken to avoid puncturing underlying organs and large vessels.

Once the Veress needle was in place, the abdominal cavity was inflated with carbon dioxide to a pressure of 12 mmHg. The Veress needle was then removed and a trocar for the telescope was inserted through the same opening. The telescope was inserted through the cannula until a view of the abdomen was seen on the monitor. After a rapid scan of the abdomen, the entry site for the instrument trocar was selected and the skin and abdominal wall incised. The entry of the trocar was observed on the monitor. After the trocar was in position, Kelly grasping forceps were inserted through the cannula. The ovary was isolated with the Kelly grasping forceps, and the stage of the ovarian cycle was recorded. The contralateral side was observed in the same manner.

After the procedure, the trocars were removed and the wound was observed internally with the telescope. Finally, the telescope was withdrawn; the pressure relieved, and the last trocar removed. The portal site was double checked to ensure that no mesentery had been pulled into the subcutaneous space. Upon completion of the procedure, only the skin was closed by use of a single 4-0 polydioxanone bury knot (Martelli, 2009). All macaques were permitted to recover from anesthesia and were provided with postoperative analgesia (0.1 mg/kg meloxicam, IM) and antibiotics (40000 IU/kg penicillin G, IM).

Statistical analysis

Day 0 was taken as the day of maximum estradiol concentration upon data presentation and the duration of the follicular and luteal phases was counted relative to this time point. Serum hormone concentrations were recorded on the same day. Outliers were deleted if the difference between the outlying value and the adjacent value exceeded 1/3 of the total range of all values. In addition, values over 3 times the standard deviation (SD) were deleted (Lumsden and Mullen, 1978; Healy, 1979; Harris and Boyd, 1995; Solberg, 1999).

Descriptive statistics and distributions for each data variable were examined using SAS, vers. 8.2 (SAS Institute, Cary, NC, USA). Mean values and the SD were calculated for each day. A Kolmogorov–Smirnov test ($p < 0.05$) was used to determine whether the data were from a Gaussian distribution (Lumsden and Mullen, 1978). When the

data were from a Gaussian distribution, reference intervals were defined by minimum and maximum values for groups of fewer than 40 samples and by central 95% percentiles (mean ± 2 SD) for groups of more than 40 samples (Solberg, 1999).

RESULTS

The average body weight of macaque in the study was 5.78±0.99 kg with normal body condition. There was no abnormality detected through physical examination and blood examination.

Ovarian cycle

During the experimental period, 57 complete ovarian cycles were observed in the 11 females. Each macaque had 4 to 6 ovarian cycles during this time period. The duration of the ovarian cycle was 28.04 ± 3.0 days, with longest cycle 34 days and the shortest 22 days. Menstrual bleeding lasted 3.4±0.6 days, with a range between 3 and 5 days. The laparoscopically observed follicular phase lasted 13.3 ± 2.1 days; the longest and shortest duration was 18 and 9 days respectively. The luteal phase was 14.1 ± 1.3 days; the longest and shortest duration was 17 and 12 days respectively (Fig. 1).

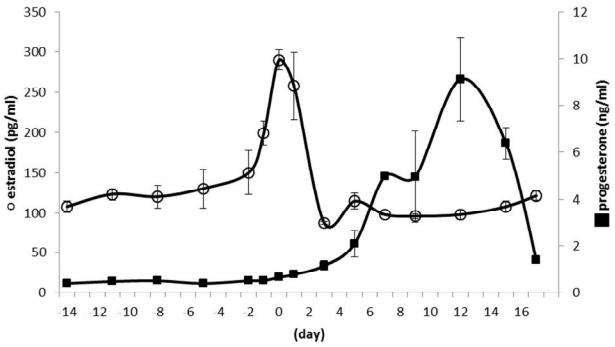

Fig. 1. Endocrine profiles of estradiol and progesterone during the ovarian cycle in the Formosan macaque. The data were present as the form of average ± standard deviation. The day of the estrogen peak is denoted as day zero, and the durations of the follicular and luteal phases are counted relative to that time point.

Serum sex hormone concentrations

During the early follicular phase, estradiol concentrations were low (50–100 pg/ml) until day –8, followed by an increase to 100–150 pg/ml. Subsequently, they peaked at concentrations of approximately 290 pg/ml on day 0. After the peak, they declined rapidly to a nadir on day 3 (Fig. 1). During the follicular phase, minimal progesterone levels (<0.6 ng/ml) were found in the peripheral circulation. On day –1 to day –2, progesterone concentrations began to increase. Maximum concentrations of 5–10 ng/ml were reached on about day 8 and they remained relatively constant for approximately 7 days, before gradually declining to follicular phase levels before menstruation (Fig. 1).

Endoscopic examination

The activity of undeveloped follicles (Fig. 2A) could be observed on day –6 to day –7 as 2–3 mm semi-transparent circular structures protruding from the surface of ovary (Fig. 2B). On day –3 to day –4, the follicle started to grow rapidly until 24–48 hours before ovulation, when the top of the follicle became translucent and it contained follicular fluid. At this time, the diameter of follicle was 5–6 mm and it was surrounded with dense surface capillaries (Fig. 2C). Upon ovulation, the apex of the follicle showed scattered red spots, which gradually expanded and cracked. The follicular fluid drained out; it first appeared clear and turned red afterwards (Fig. 2D-2E). In some cases, the outflow of follicular fluid happened abruptly and in others it took about 30 min. After ovulation, the follicle collapsed and the blood vessels observed in the pre-ovulatory follicle reappeared. An ovulation point through which ovulation had occurred became distinguishable.

Forty-eight hours after ovulation, the fresh corpus luteum showed a hemorrhagic appearance (corpus hemorrhagicum). It was red, round, jelly-like and surrounded by capillaries (Fig. 2F). The size of the corpus hemorrhagicum varied and this depended on the crack in the follicle upon ovulation. Seventy-two hours after ovulation, some partial luteinization began at the base of the corpus luteum. The corpus luteum became pink and then yellow (Fig. 2G). A developed corpus luteum had a more yellowish appearance with well defined blood vessels.

On day 7 to day 8, the corpus luteum was still prominent and protruded from the surface of the

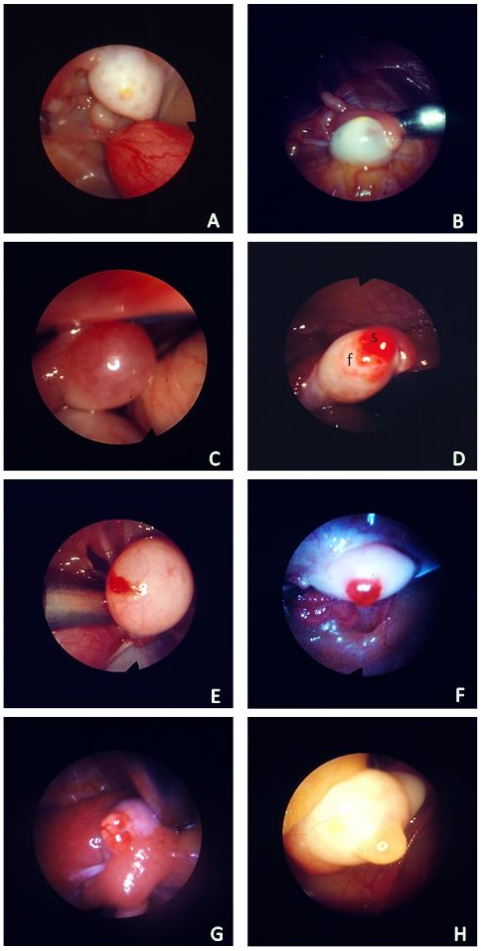

Fig. 2. Laparoscopically observed sequential changes in the ovary and follicle during the normal ovarian cycle of adult female Formosan macaques (*Macaca cyclopis*). A. During menstrual bleeding, the corpus luteum was still visible on the surface of the ovary, although smaller and lighter in color. A small and undeveloped follicle can also be seen. B. On the 7th day of the menstrual cycle, a developing, semi-transparent and protruded follicle was seen on the surface of the ovary. C. At 24–48 hours before ovulation, the follicle was pink and larger, with a dense distribution of surface capillaries. D–E. Ovulation: a remarkably prominent vascular pattern was observed on the mature follicle (f). A stigma (s) was seen protruding like a reddish bleb from the follicular surface. A hemorrhagic plaque will be formed after drainage of follicular fluid. F–G. At 48 hours after ovulation, a red and jelly-like corpus hemorrhagica was formed at the site of follicular rupture. H. At 7 days after ovulation, a mature corpus luteum has formed.

ovary. On day 13 to day 14, it began to degenerate, which continued with gradual regressive changes through the remainder of the cycle and eventually formed a scar. However, the scar of corpus luteum protruding from the ovarian surface was still detected throughout the next cycle (Fig. 2A).

DISCUSSION

The term “ovarian triad” refers to the follicle, oocyte, and corpus luteum in the primate menstrual cycle (Goodman and Hodgen, 1983). The ovarian cycle is the combination of follicular maturation during the follicular phase, ovulation, and the development of the corpus luteum during the luteal phase, followed by corpus luteum regression and menstruation. The first day of menstrual bleeding is designated as the first day of the ovarian cycle (Weinbauer *et al*., 2008). Analysis of a total of 647 menstrual cycles in cynomolgus monkeys revealed an average cycle duration of 29.2 days (range 22–37 days) (Shaikh *et al*., 1978). Other studies have reported average cycle durations of 30.2±1.4 (range 29–35) days and 35.9±1.6 (range 26–45) days (Butterstein *et al*., 1997; Attia, 1998).

The average cycle lengths reported for the rhesus macaque range from 25.5 to 29.5 days, and for the Japanese macaque from 26 to 31 days (Bosu *et al*., 1973; Johnson and Phoenix, 1983; Aso *et al*., 1976). In the Formosan macaques of this study, the average ovarian cycle was concluded to last for 28.04 ± 3.0 days (range 22–34 days). The duration of menstruation in cynomolgus monkeys varies from 1 to 8 days, with 85% of cycles exhibiting a duration of bleeding of 3 to 5 days (Shaikh *et al*., 1978). In Formosan macaques, the duration of menstrual bleeding was found to be 3.4 ± 0.6 days, with a range between 3 and 5 days. The cycle lengths are regularized at about one-month intervals, which approximate the lengths seen in women and other species of macaque. It should be noted that some non-human primate species exhibit distinct seasonal variation in reproductive functions, depending on the latitude of their habitat. The Japanese macaque and rhesus macaque are unique among higher primates in that they show a distinct seasonal pattern of breeding activity. During the out-of-season periods, reproductive hormone

secretion and gonadal activity are at a complete halt (Nigi, 1975; Ghosh and Sengupta, 1992; Herndon *et al*., 1996). However, the long-tailed macaque shows reproduction which is unaffected by season. After menarche, normal ovulatory menstrual cycles persist throughout the year, and the gonadotropins and sex steroids show typical patterns (Shimizu *et al.*, 1996). The mating season of Formosan macaques is between September and February, the birthing season is between April and June (Hsu *et al*., 2000), but it is still controversial to consider them as seasonal breeders. In this study, we have demonstrated that the ovulatory cycle persists throughout the off season, rather than being completely silent. Therefore we suspect that the timing of birth may be related to the fruitful season, as in the cynomolgus monkey (Dang, 1977). However, further long-term research is needed.

Preovulatory follicular growth is initiated during the perimenstrual rise of follicular stimulation hormone (FSH) secretion (Zeleznik, 2001). Under laparoscopy, follicular development can be observed 7 days after menstrual bleeding. Meanwhile, the serum estradiol concentration was low, between 50 and 100 pg/ml. In Japanese macaques, follicular development starts on the 9th or 10th day of the menstrual cycle; the serum estradiol concentration is at about the same level as in Formosan macaques, and lower than that of long tail macaques (Shimizu, 2008). The duration of the follicular phase in rhesus, cynomolgus and Japanese macaques is 12 to 15 days (Shimizu, 2008); the duration in Formosan macaques falls in the same range. However, the peak estradiol level was variable among the species: 350 pg/ml in cynomolgus and rhesus macaques and 290–300 pg/ml in Japanese macaques (Shimizu, 2008). The peak serum estradiol value of the Formosan macaque is similar to that of Japanese macaque.

Luteinization of granulosa cells occurs within a few hours after ovulation (Weinbauer *et al*., 2008). Laparoscopically, luteinization can be observed 72 hours after ovulation in the Formosan macaque, which is similar to Japanese macaques. Similarly, the duration of the luteal phase is 14–17 days in the rhesus monkey (Shimizu, 2008), 10.5–15.1 days in Japanese macaques (Nigi, 1977), and 12.8–15.4 days in Formosan macaques. The secretion pattern of progesterone during the menstrual cycle of the Formosan macaque is nearly identical to that of other macaque species. However, quantitatively the peak value in the Formosan macaque is closer to that of the long tailed macaque and slightly higher than those of rhesus and Japanese macaques.

Laparoscopic observation of the ovarian cycle has been reported in various non-human primates (Dukelow, 1975; Nigi, 1977) and dogs (Wildt *et al*., 1977), and ovulation has been observed in some women in vivo (Lousse and Donnez, 2008). Both studies in non-human primates mentioned above concluded that repeat laparoscopic observation does not affect the normal ovarian cycle. However, if the corpus luteum is ruptured iatrogenically by the laparoscope the luteal phase will be shortened (Nigi, 1977). In addition, repeated anesthesia and blood sampling have been demonstrated to have no effects on the normal ovarian cycle (Hotchkiss *et al*., 1971; Stabenfeldt and Hendrickx, 1973; Channing *et al*., 1977, Aidara *et al*., 1981). In our study, no corpus luteum was accidentally ruptured during laparoscopic examination. Therefore we analyzed the hormone values and laparoscopically observed ovarian cycle together; the sequential changes are similar to those reported from a previous study in Japanese macaques (Nigi, 1977).

In conclusion, the hormonal study we have presented here was designed according to the suggested guidelines in macaques, and conditions that may influence the normal ovarian cycle were also considered (Weinbauer *et al.*, 2008). Although there is limitation that the study did not go through a complete breeding season, therefore, a statistical comparison of hormone values between breeding and non-breeding season was not possible. The data should be considered as reference values associated with the ovarian cycle in captive Formosan macaques, and they approximately match the general concepts regarding the reproductive physiology of other macaques. Hopefully, the study will be beneficial to further studies on human and non-human primates, and will aid the conservation of this endemic species in Taiwan.

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