FULL PAPER *Theriogenology*

Delay of Ovulation Due to Diets Containing Levonorgestrel in Cynomolgus Monkeys (*Macaca fascicularis*)

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ABSTRACT. This study was undertaken to develop a simple and practical method to control the time of ovulation in cynomolgus monkeys. Diets containing a synthetic gestagen, levonorgestrel (LNG) were given daily to normally cycling female monkeys for 2 weeks, and plasma concentrations of estradiol-17β and progesterone were determined by EIA in order to estimate the time of ovulation. Doses of LNG (0, 3.2, 8, 20, 50, or 125 μg) were given from Day 2 (Day 0 =the first day of menstruation) through Day 15. The numbers of days from the last administration of LNG to the estimated ovulation in the groups treated with LNG at 20 μg and above were significantly greater than those in the controls, and the values in the group treated with LNG at 50 μg were within a narrow range. In a second experiment, LNG was administered at 50 μg in different phases of the menstrual cycle (Days 9–22, 16–29, and 23–36), and the results indicated that ovulation occurred more than 12 days after the last administration in all monkeys, and the number of days from the last administration of LNG to the estimated ovulation in the group treated on Days 16–29 (luteal phase) was significantly greater than that in the group treated on Days 23–36. These results indicate that daily provision of a diet containing 50 μg LNG could be applicable for delaying ovulation, and suggest that the total level of (exogenous and endogenous) progestins is critical for determining the length of ovulation delay in cynomolgus monkeys.

KEY WORDS: control of ovulation time, levonorgestrel, monkey.

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Monkeys, whose physiological characteristics are close to those of humans, are indispensable experimental animals. In particular, recent safety research using monkeys has contributed to the development of new drugs. Although there is much demand for monkeys as experimental animals, their reproductive efficiency is generally very low.

Although estrous cycle manipulation has been used as a research and management tool for studying and maximizing reproductive performance in domestic livestock species [1, 2, 7], there is little information about control of the ovarian cycle in monkeys. Menstrual cycle synchronization was attempted by orally administering a progestin, altrenogest, to 4 rhesus monkeys and the possibility of achieving ovulation synchronization was suggested [8]. Daily intramuscular injection with a commonly used progestin contraceptive, levonorgestrel (LNG), inhibited follicular maturation and ovulation in cynomolgus monkeys [6]. A single subcutaneous administration of a synthetic progestogen, danazol, induced a prolonged suppression of ovarian cyclicity in cynomolgus monkeys [3]. Although these findings suggest that the use of progestogens is effective for control of ovulation in monkeys, suitable doses and effects of the treatment at different phases during the menstrual cycle had not been studied.

This study was performed to evaluate the effects of treatments with LNG, especially at different doses and phases of the menstrual cycle, on the time of ovulation in cynomolgus monkeys. As a practical and simple application, the compound was infused into feeding pellet and orally administered, thus eliminating the need for daily injections.

MATERIALS AND METHODS

Animals: Twenty-two female cynomolgus monkeys (*Macaca fascicularis*) (4–12 years of age; 2.5–5.0 kg BW) were used for the present study after establishment of at least 2 normal menstrual cycles (25–35 days) by daily examination of vaginal discharge. The monkeys were housed in individual cages with a 12 hr:12 hr light-dark schedule at 23–29°C, and provided with solid feed (Teklad Global Certified 25% Protein Primate Diet, Harlan Sprague Dawley Inc., Madison, WIS, U.S.A., 12 g, 9 pieces per day) once per day at 15:00 with water available *ad libitum*. The menstrual status of the animals was visually checked daily. The first day of menstruation was designated Day 0 of the menstrual cycle. Blood samples were drawn by femoral venipuncture with a syringe containing heparin sodium,while the animals were unanesthetized. The heparinized blood was immediately cooled on ice and then centrifuged (4°C, 1710 × g, 3,000 rpm, 15 min), and the supernatant was transferred to screw-cap tubes. The plasma was stored in a deep freezer at −80°C

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until being assayed for estradiol-17β (E_2) and progesterone. Based on the report by Saldarini *et al*. [9], ovulation was estimated to occur at 2 days after reaching peak E_2 concentration.

Procedure: LNG (Sigma Chemicals, St. Louis, MO, U.S.A.) was dissolved with 0.1 m*l* corn oil and infused into the centers of monkey diet pellets by an injection syringe pump with a needle (22G). During feeding time, the monkeys were first supplied with the LNG diet, and the remaining eight pellets to make up 1-day's food provision were given after confirming that the LNG pellets had been completely eaten. To compare the time of ovulation after treatment between groups, the number of days from the last administration of LNG to the estimated ovulation was determined by counting from the last day of LNG treatment to the estimated first day of ovulation.

Experiment 1: The animals were given pellets containing 0, 3.2, 8, 20, 50 or 125 μg LNG daily from Day 2 through Day 15 of the menstrual cycle (n=4 in each group). Three animals dosed at 0, 8, or 50 μg LNG (one at each level) were administered LNG again, at 3.2 μg. These animals were re-used after a long interval (11–14 months from the last administration at the first dose level) and after at least two normal menstrual cycles (25–35 days) had been confirmed. The menstrual status of the animals was visually checked daily during and after the treatment period. Blood samples were drawn every third day from Day 0 through the day of the second menstruation after the start of LNG treatment.

Experiment 2: The experiment was performed to evaluate the effects of LNG administration for 2 weeks at different phases of the menstrual cycle on the time of ovulation. The animals were given pellets containing 50 μg LNG daily on Days 9–22, 16–29 or 23–36 of the menstrual cycle (n=4 in each group). Eleven animals used in Experiment 1 were used again for this experiment. These animals were re-used after a long interval (at least 2 years after the last administration in Experiment 1) and after at least 2 normal menstrual cycles had been confirmed. The menstrual status of the animals was visually checked daily during and after the administration period. Blood samples were drawn every third day from the first day of LNG administration through the day of the second menstruation after the start of the administration.

Hormone assay: Duplicate plasma samples were assayed. E_2 and progesterone were measured using a commercially available enzyme immunoassay kit (E_2: Cayman Chemicals, Ann Arbor, MI, U.S.A.; progesterone: Neogen, Lexington, KY, U.S.A.) using a processed standard curve. Plasma treated with dextran-coated charcoal was used for the standard. The samples (0.1 m*l*) underwent extraction with diethyl ether (1.0 m*l*) for E_2 and progesterone assay. Specificity of the antiserum for E_2 assay was 100.00% with E_2, 1.00% with testosterone, 0.41% with estriol and 0.10% with estrone. Specificity of the antiserum for progesterone assay was 100.0% with progesterone, 2.50% with deoxycorticosterone, 2.00% with corticosterone and 2.00% with pregnenolone. The respective intra-assay and inter-assay coefficients of variations were 2.3–6.7 and 1.3–8.4% for E_2, and 3.4–10.0 and 2.7–7.8% for progesterone. The detection limits were 7.8 *p*g/m*l* and 0.4 *n*g/m*l* for E_2 and progesterone, respectively.

Animal welfare: This study was conducted after approval by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories. This committee approves studies that accord with the NIH Guide for the Care and Use of Laboratory Animals and are to be conducted in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Data analysis: The days from the last administration to the estimated ovulation in Experiments 1 and 2 were analyzed by Bartlett's test (significance level: 5%) to determine homogeneity of variance. Scheffé's multiple comparison test (significance level: 5%) was applied to mean values.

RESULTS

All monkeys showed an increase in the progesterone level after the peak E_2 concentration was reached, and the progesterone level decreased to the base line level thereafter.

Experiment 1: Figure 1 shows an example of plasma E_2 and progesterone concentrations in controls which were given pellets containing 0 μg LNG daily from Day 2 through Day 15 of the menstrual cycle. Plasma E_2 concentration increased during the administration period, and the estimated day of ovulation was Day 11 of the menstrual cycle. The number of days from the last administration to the estimated ovulation was −4, suggesting that ovulation might have occurred 4 days before the last day of LNG treatments. After estimated ovulation, the plasma progesterone level showed an increase which was followed by a decrease, and thereafter menstruation occurred in the monkey. Similar hormonal and menstrual patterns were repeated thereafter. All four control animals showed these patterns.

Figure 2 shows an example of plasma E_2 and progesterone concentrations in the group treated with LNG at 3.2 μg from Day 2 through Day 15 of the menstrual cycle. The hormonal and menstrual patterns were similar to those in controls. In this group, 3 out of 4 monkeys showed these patterns, and one monkey exhibited the hormonal and the menstrual patterns similar to those shown in Fig. 3

Figure 3 shows an example of plasma E_2 and progesterone concentrations in the group treated with LNG at 8 μg from Day 2 through Day 15 of the menstrual cycle. Increase in E_2 concentration during the administration period was inhibited, and the estimated day of ovulation was delayed to Day 32 of the menstrual cycle. The number of days from the last administration to the estimated ovulation was 17. Menstruation was observed 3 days after the last administration of LNG. In this group, 3 out of 4 monkeys showed these patterns, and 1 monkey exhibited the hormonal and menstrual patterns similar to those in controls.

Figure 4 shows an example of plasma E_2 and progesterone concentrations in the group treated with LNG at 20 μg from Day 2 through Day 15 of the menstrual cycle. The hormonal and menstrual patterns were similar to those in Fig. 3. Increase in E_2 concentration during the administration period was inhibited, and the estimated ovulation was delayed to

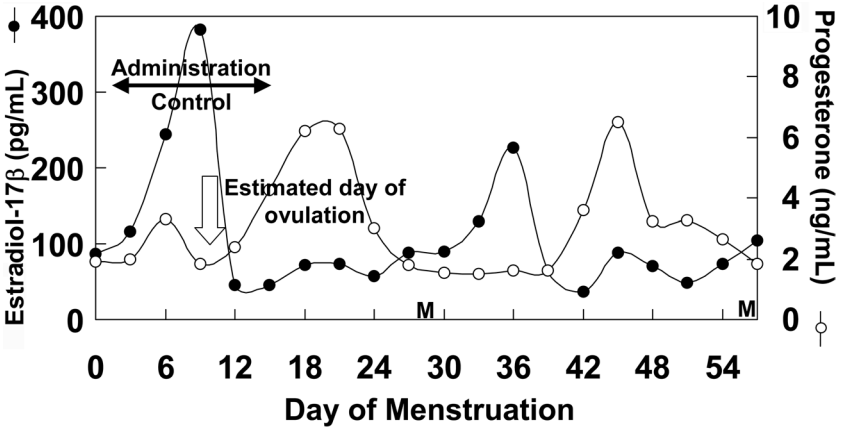

Fig. 1. Plasma concentrations of estradiol-17β and progesterone during the vehicle treatment cycle in a cynomolgus monkey; vehicle was administered daily from Day 2 through Day 15 of the menstrual cycle. Menses (M) are indicated on the abscissa.

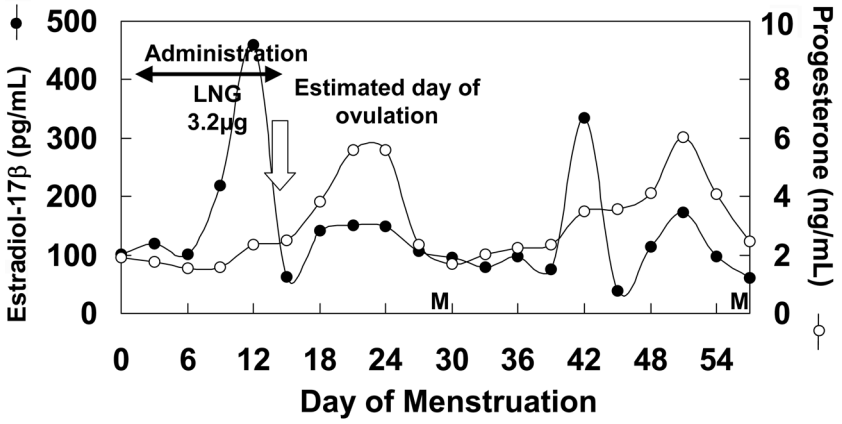

Fig. 2. Plasma concentrations of estradiol-17β and progesterone in a cynomolgus monkey treated with 3.2 μg LNG from Day 2 through Day 15 of the menstrual cycle. Menses (M) are indicated on the abscissa.

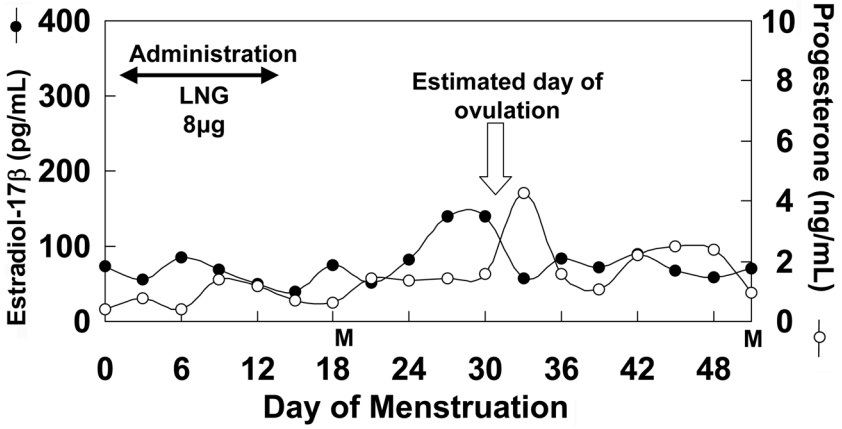

Fig. 3. Plasma concentrations of estradiol-17β and progesterone in a cynomolgus monkey treated with 8 μg LNG from Day 2 through Day 15 of the menstrual cycle. Menses (M) are indicated under the abscissa.

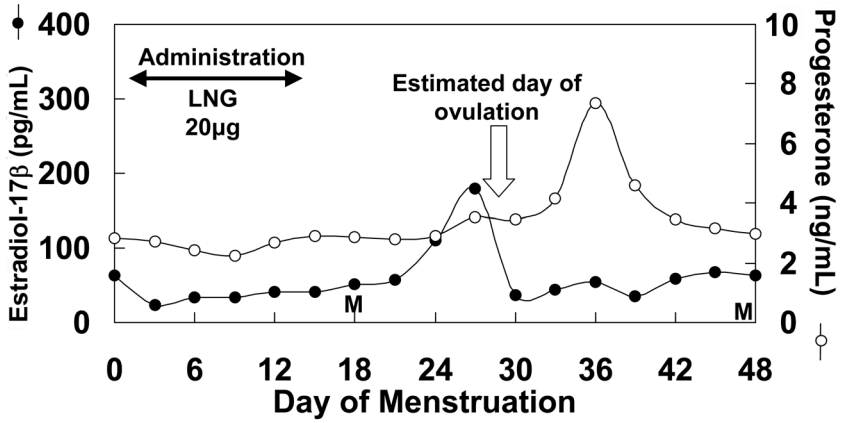

Fig. 4. Plasma concentrations of estradiol-17β and progesterone in a cynomolgus monkey treated with 20 μg LNG from Day 2 through Day 15 of the menstrual cycle. Menses (M) are indicated on the abscissa.

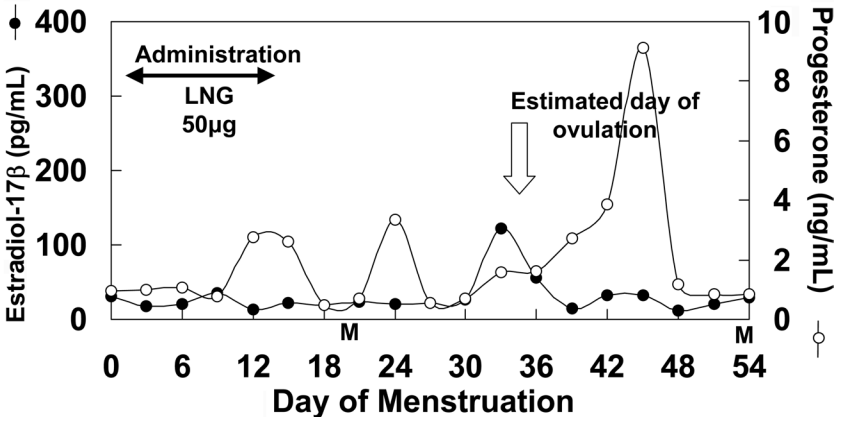

Fig. 5. Plasma concentrations of estradiol-17β and progesterone in a cynomolgus monkey treated with 50 μg LNG from Day 2 through Day 15 of the menstrual cycle. Menses (M) are indicated under the abscissa.

Day 29 of the menstrual cycle. The number of days from the last administration to the estimated ovulation was 14. Menstruation was observed 3 days after the last administration of LNG. All animals in this group showed these hormonal and menstrual patterns. However, the numbers of days from the last administration to the estimated ovulation in the other animals in this group were 14, 20 and 23, and the values were not constant.

Figure 5 shows an example of plasma E_2 and progesterone concentrations in the group treated with LNG at 50 μg from Day 2 through Day 15 of the menstrual cycle. Increase in E_2 concentration during the administration period was inhibited, and the estimated day of ovulation was delayed to Day 35 of the menstrual cycle. The number of days from the last administration to the estimated ovulation was 20. Menstruation was observed 4 days after the last administration of LNG. All monkeys in this group showed these hormonal and menstrual patterns. The numbers of days from the last administration to the estimated ovulation in the other animals in this group were 17, 20 and 20, and the values were relatively constant.

Figure 6 shows an example of plasma E_2 and progesterone concentrations in the group treated with LNG at 125 μg from Day 2 through Day 15 of the menstrual cycle. Increase in E_2 concentration was inhibited for a prolonged period, and the estimated day of ovulation was also delayed. The number of days from the last administration to the estimated ovulation was 47. Menstruation was observed 58 days after the last administration of LNG. The numbers of days from the last administration to the estimated ovulation in the other animals in this group were 17, 17 and 47, and the values were variable.

The results for the estimated day of ovulation in Experiment 1 are summarized in Table 1. The numbers of days from the last administration to the estimated ovulation in the groups treated with LNG at 20, 50, or 125 μg were sig-

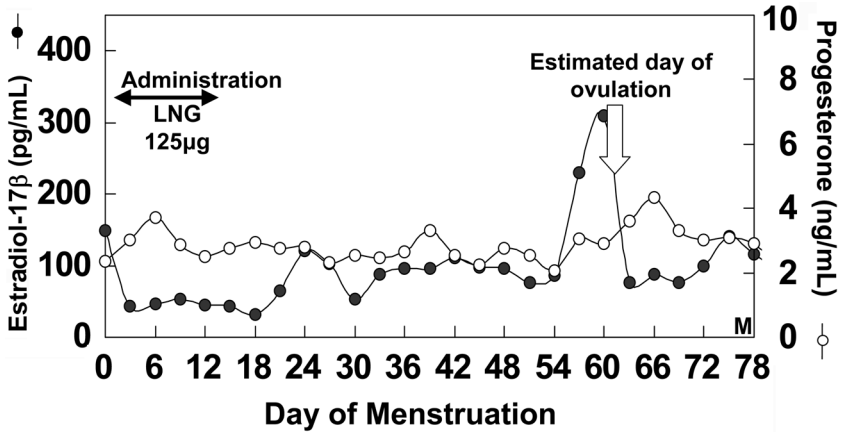

Fig. 6. Plasma concentrations of estradiol-17β and progesterone in a cynomolgus monkey treated with 125 μg LNG from Day 2 through Day 15 of the menstrual cycle. Menses (M) is indicated on the abscissa.

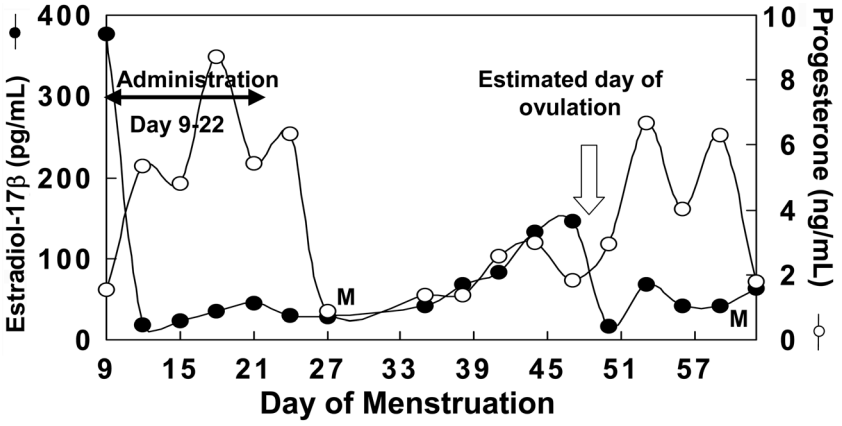

Fig. 7. Plasma concentrations of estradiol-17β and progesterone in a cynomolgus monkey treated with 50 μg LNG. from Day 9 through Day 22 of the menstrual cycle. Menses (M) are indicated on the abscissa.

Table 1. The effects of a range of doses of LNG on the time of ovulation in cynomolgus monkeys

Dose[1)] (μg/head/day)	No. of animals	Estimated day of ovulation[2)] (range)	
0	4	$-2.5 \pm 0.9^{a)}$	(−4.0~−1.0)
3.2	4	$-1.0 \pm 2.1^{a,b)}$	(−4.0~5.0)
8	4	$10.3 \pm 4.8^{a,b,c)}$	(−4.0~17.0)
20	4	$17.8 \pm 2.3^{b,c,d)}$	(14.0~23.0)
50	4	$19.3 \pm 0.8^{b,c,d)}$	(17.0~20.0)
125	4	$32.0 \pm 8.7^{d)}$	(17.0~47.0)

1) LNG was daily administered from Day 2 through Day 15 of the menstrual cycle.
2) Ovulation was estimated to occur two days after the peak plasma E_2 concentration was reached, and the values (mean ± SEM) are shown as days after the last administration of LNG.
a,b,c,d) Values with different superscripts differ significantly (P<0.05).

nificantly greater than those in controls, indicating that LNG treatments at these doses delay ovulation. The mean value in the group treated with LNG at 20 μg was close to that in the group treated with LNG at 50 μg. Individual values in the group treated with LNG at 50 μg showed a small range, while those in the group treated with LNG at 20 μg were not constant. Individual values in the group treated with LNG at 125 μg showed a wide range.

Experiment 2: Figure 7 shows an example of plasma E_2 and progesterone concentrations in the group treated with LNG at 50 μg from Day 9 through Day 22 of the menstrual cycle. In this case, plasma E_2 concentration might have shown its peak before or around the start of LNG administration, and ovulation might have occurred a few days after the start of administration. During the period of administration in the luteal phase, plasma progesterone concentration was high, and an increase in plasma E_2 concentration was not found. The estimated day of ovulation after administrations

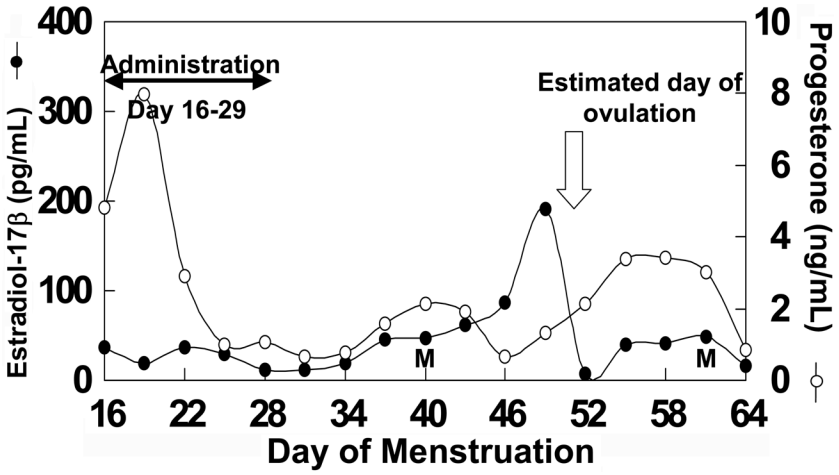

Fig. 8. Plasma concentrations of estradiol-17β and progesterone in a cynomolgus monkey treated with 50 μg LNG from Day 16 through Day 29 of the menstrual cycle. Menses (M) are indicated on the abscissa.

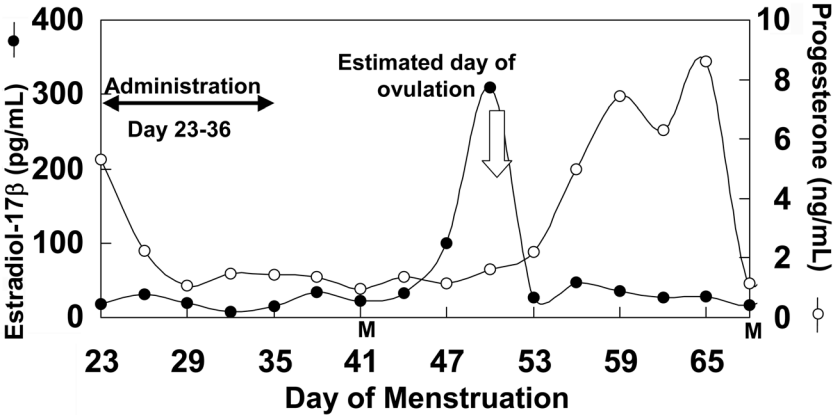

Fig. 9. Plasma concentrations of estradiol-17β and progesterone in a cynomolgus monkey treated with 50 μg LNG from Day 23 through Day 36 of the menstrual cycle. Menses (M) are indicated under the abscissa.

of LNG was Day 49 of the menstrual cycle. The number of days from the last administration to the estimated ovulation came to 27. Menstruation was observed 6 days after the last administration of LNG. All monkeys in this group showed these hormonal and menstrual patterns.

Figure 8 shows an example of plasma E_2 and progesterone concentrations in the group treated with LNG at 50 μg from Day 16 through Day 29 of the menstrual cycle. During the period of administration in the luteal phase, the plasma progesterone concentration was initially high, and this was followed by a decrease. Increase of plasma E_2 concentration was inhibited during the administration period. The estimated day of ovulation (Day 51) was delayed when compared with that in a normal menstrual cycle (around Day 41). The number of days from the last administration to the estimated ovulation was 22. Menstruation was observed 11 days after the last administration of LNG. All monkeys in this group showed these hormonal and menstrual patterns.

Figure 9 shows an example of plasma E_2 and progesterone concentrations in the group treated with LNG at 50 μg from Day 23 through Day 36 of the menstrual cycle. During the period of administration in the final part of the luteal phase and the early follicular phase, plasma progesterone concentration decreased and then stayed at a low level. The increase of plasma E_2 concentration was inhibited during administration. The estimated day of ovulation (Day 52) was delayed when compared with that in a normal menstrual cycle (around Day 41). The number of days from the last administration to the estimated ovulation came to 16. Menstruation was observed 5 days after the last administration of LNG. All monkeys in this group showed these hormonal and menstrual patterns.

The results for the estimated day of ovulation in Experiment 2 together with the data from the group treated with

Table 2. The effects of timing for LNG treatments on the time of ovulation in cynomolgus monkeys

LNG treatment[1)] (day of menstrual cycle)	No. of animals	Estimated day of ovulation[2)] (range)
Days 2–15	4	19.3 ± 0.8[*a,b)] (17~20)
Days 9–22	4	22.5 ± 1.9[a,b)] (18~27)
Days 16–29	4	25.8 ± 1.9[a)] (22~31)
Days 23–36	4	15.5 ± 1.3[b)] (12~18)

1) LNG was administered once at 50 μg on each of the indicated days.
2) Ovulation was estimated to occur two days after the peak plasma E_2 concentration was reached, and the values (mean ± SEM) are shown as numbers of days after the last administration of LNG.
* Values are the data from the group treated with LNG at 50 μg in Table 1.
a,b) Values with different superscripts differ significantly (P<0.05).

LNG at 50 μg in Experiment 1 are summarized in Table 2. The results indicate that ovulation occurred more than 12 days after the last administration in all monkeys. The numbers of days from the last administration to the estimated ovulation in the groups treated daily with 50 μg LNG from Day 16 through Day 29 were significantly greater than those in the group treated from Day 23 through Day 36 of the menstrual cycle.

DISCUSSION

Although reproductive technology for monkeys, such as artificial insemination and embryo transfer has been improved [4, 5, 10–12], control of the ovarian cycle has not been established. There are a few reports showing that progestins are useful for control of ovulation in monkeys. Three out of 4 rhesus monkeys showed ovulation synchrony after daily administration of a progestin, altrenogest, mixed with fruit juice [8], and daily intramuscular injections with LNG [6] or a single subcutaneous injection with danazol [3] inhibited ovulation in cynomolgus monkeys. Although these studies suggested the possibility for that progestins control ovulation, the suitable doses and the effect of the treatment at different phases of the menstrual cycle on the time of ovulation had not been studied. A simple and reliable method to control ovulation remained to be established. This study used pellets containing LNG, and time of ovulation was determined by examining plasma E_2 and progesterone concentrations in cynomolgus monkeys. Administration was performed by providing feed pellets containing LNG at the start of feeding time, without the need for any animal restraint, which would have been required for injection.

Saldarini *et al*. [9] showed that a preovulatory surge of estrogen occurs approximately two days prior to ovulation in cynomolgus monkeys. Based on this finding, in this study, ovulation was estimated to occur two days after reaching the peak E_2 level. The results was that all monkeys showed an increase in progesterone level after reaching the peak plasma E_2 level, from which the occurrence of ovulation could be confirmed.

The progesterone concentration during the LNG administration period increased or decreased depending on the functional state of corpora lutea regardless of LNG treatment, suggesting that LNG treatment may affect neither the function of the corpus luteum nor the assay system of progesterone in this study. Consistently, Pope and Gould [8] showed that in monkeys which had an active corpus luteum at the beginning of altrenogest treatment, luteolysis occurred at the expected time in the ovarian cycle, and Heikinheimo *et al.* [6] reported that LNG treatment does not interfere with progesterone assays in monkeys.

In Experiment 1, the effective and most suitable dose of LNG for control of ovulation was investigated. Monkeys were administered LNG during the follicular phase for 14 consecutive days, the expected term of the luteal phase. The results showed that daily administration of LNG at 20 μg and above delayed ovulation in all monkeys, and at 125 μg, LNG inhibited ovulation for a prolonged period. Since the length of the ovulation delay was constant within a narrow range following administration of LNG at 50 μg, this dose was used in Experiment 2. The mechanisms of the actions by which LNG inhibits ovulation are not clear. However, Heikinheimo *et al*. [6] showed that in cynomolgus monkeys, LNG injection was associated with significant suppression of serum E_2 in spite of increased levels of both follicle stimulating hormone and luteinizing hormone, suggesting that LNG inhibits ovulation via direct effects on both folliculogenesis and the hypothalamus.

In Experiment 2, monkeys were treated daily with 50 μg LNG at different phases of the menstrual cycle, and the results show that ovulation was delayed in all groups and occurred more than 12 days after the last administration. The delay of ovulation was significantly longer in the group treated in the luteal phase (Days 16–29 of the menstrual cycle) than in the group treated in the final part of the luteal phase and the early follicular phase (Days 23–36 of the menstrual cycle). Furthermore, treatment in a phase with high progesterone levels (Days 9–22 and 16–29 of the menstrual cycle) tended to delay ovulation for a longer time than that in phases with low progesterone levels (Days 2–15 and 23–36 of the menstrual cycle). These results suggest that the total level of (exogenous and endogenous) progestins is critical for determining the length of ovulation delay in cynomolgus monkeys. This suggestion is supported by the fact that a high dose of LNG (125 μg) inhibited ovulation for a prolonged period in Experiment 1.

In conclusion, administration of a diet containing 50 μg LNG for 2 weeks was found to be a practical, reliable, and simple method for the delaying of ovulation in cynomolgus monkeys. The findings of this study could contribute to the practical application of a diet containing LNG for control of ovulation, which is required for improvement of reproductive technology in monkeys.

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