Plasma LH and Progesterone Levels before and after Ovulation and Observation of Ovarian Follicles by Ultrasonographic Diagnosis System in Dogs

Masayoshi HASE, Tatsuya HORI, Eiichi KAWAKAMI and Toshihiko TSUTSUI

Department of Reproduction, Nippon Veterinary and Animal Science University, 7-1, Kyonan-cho 1 Chome, Musashino-shi, Tokyo 180-8602, Japan

(Received 8 June 1999/Accepted 11 November 1999)

ABSTRACT. Recently, canine frozen semen has been attracting attention for breeding purposes, and methods of judging ovulation and optimum timing for insemination have become important. As methods of predicting the canine ovulation, vaginal smear, plasma sex hormone levels and ultrasonographic diagnosis system (US) have been investigated in combination, but a standard technique has not yet been established. Therefore, we investigated a method of predicting canine ovulation in dogs by US, and by measuring plasma LH and progesterone (P) levels three times a day. Ovulation could be observed by detecting irregularly shaped ovarian follicles by US in six of 11 dogs (54.5%). In these dogs, the time between the LH peak and ovulation was 24–48 hr, 38.0 hr on average. The P level on the ovulation day was 1.88–2.81 ng/ml, 2.34 ng/ml on average. A value of 1.88 ng/ml was detected in one dog, but the other five dogs showed P levels of 2 ng/ml or higher. The P level on the day before ovulation was 0.8–1.56 ng/ml, 1.12 ng/ml on average. Assuming that ovulation occurred two days after the LH peak in the 11 experimental dogs, the P level was 2.12–4.06 ng/ml, 2.78 ng/ml on average. The period of a high LH level, not less than 10 ng/ml, continued for 12 hr around the LH peak. Based on these findings, to predict ovulation using US and LH level, it would be necessary for the tests to be performed several times a day. In contrast, it was shown that the day on which a plasma P level of 2 ng/ml or higher was detected by the test performed once a day corresponded to the ovulation day.—KEY WORDS: canine, LH, ovulation, progesterone, ultrasonography.

J. Vet. Med. Sci. 62(3): 243–248, 2000

The mating period with possible conception in dogs continues as long as approximately seven days, from initiation of estrus to 108 hr after ovulation [17, 20]. Ova at ovulation are immature primary oocytes and go through meiosis during descent in the oviduct, then become fertilizable within approximately 60 hr [17, 18]. Ova at this point are located in the middle to lower oviduct [16, 17]. The fertilizable ova maintain fertilizability for approximately 48 hr [16, 18]. Since the duration of the fertilizable period of ova and sperm is long in dogs, methods of judging appropriate mating period have attracted little attention. However, canine frozen semen has recently been attracting interest in breeding studies, and judgment of the appropriate insemination period has become important.

Although various methods of predicting ovulation in dogs have been investigated, only a few reports have been based on direct observation of ovarian follicular maturation and ovulation. In our previous study [15, 17], female dogs at estrus underwent laparotomy after copulation and their ovaries were directly observed. It was shown that ovulation occurs on the 3rd day of estrus. Concannon *et al.* [4, 5] reported that they measured plasma LH and P levels three times a day, and days were ovariectomized to observe maturation and ovulation of the ovarian follicles. They reported that ovulation occurred approximately 44 hr after the LH peak. Recently, observations of development and ovulation of canine ovarian follicles by the ultrasonographic diagnosis system (US) have been reported [3, 6–9, 13, 14, 21]. According to these studies, although observation of ovarian follicle development is possible using US,

determining the ovulation is difficult, and a technique to predict accurately ovulation in dogs has not yet been established.

In this study, therefore, we investigated a method of predicting the ovulation in dogs by thrice daily observation by US and thrice daily measurement of blood LH and P levels. Since the mating period in dogs is 3–5 days after ovulation [17, 18, 20], a method of predicting the ovulation during the mating period was investigated.

MATERIALS AND METHODS

Experimental animals: Eleven in-bred beagle dogs aged 2 to 5 years maintained at Nippon Veterinary and Animal Science University were used in the experiments. These dogs were known to be reproductively normal from previous estrous cycles and consisted of nine multiparous dogs and two nulliparous dogs. The dogs were maintained in cages measuring 160 × 75 × 65 cm and were fed commercial dog foods once a day. The dogs were given drinking water three times a day, in the morning, afternoon and evening.

Observation of estrus: The female dogs were observed every day for pudendal enlargement and the presence or absence of vulval bleeding.

Blood collection for hormone measurements: Blood samples for measurements of plasma LH and P levels were collected once a day (19:00) between the first day of vulval bleeding and the first day on which ovarian follicles could be observed by US, then collected three times a day (7:00, 13:00 and 19:00) for several days after ovulation. Three ml

of blood were collected from the anterior brachiocephalic vein using a heparinized syringe. Blood samples were immediately centrifuged at a low temperature to separate the plasma, and stored at -40°C until used for hormone measurement.

Blood LH and P levels were measured by RIA and EIA, respectively.

The P level was measured by an enzyme immunoassay [11, 19]. The intra-assay coefficients of variation for samples at high, medium, and low concentrations were 4.9%, 6.2% and 10.5%, respectively, and the inter-assay coefficients of variation for the same sample pools were 8.7%, 10.9%, and 14.5%, respectively. The sensitivity of the assay was 0.25 pg/well . Plasma LH was measured using a double-antibody radioimmunoassay (RIA) in accordance with the procedure described by Nett *et al.* [12], except that radiolabelled porcine LH (LER-778) and anti-porcine LH serum were utilized, as reported by Kawakami *et al.* [10]. The intra-assay and inter-assay coefficients of variation were 3.2% and 16.0%, respectively. The highest LH level detected before ovulation was regarded as the LH peak.

Observation of ovarian follicles by ultrasonographic diagnosis system: Observation of ovarian follicles by US was started three days after initiation of vulval bleeding and performed once a day (19:00) until the follicles became detectable as a clear echo-free region. After ovarian follicles became detectable, the ovaries were observed by US three times a day (7:00, 13:00 and 19:00), then once a day (19:00) after deduced ovulation. The sizes of the observed ovarian follicles were estimated by measuring the largest inner diameters of the echo-free regions. US used in the experiment was a linear mode 7.5 MHz probe, ECHOVISION-SSD-500EV (Aloka Co., Japan). The time point after which large ovarian follicles began to shrink was regarded as ovulation.

RESULTS

Plasma LH and progesterone levels: Plasma LH and P

levels in the 11 experimental dogs are shown in Fig. 1. The LH levels around the plasma LH peak are shown in Table 1.

The peak value of plasma LH were $13.6\text{--}42.4\text{ ng/ml}$, 24.8 ± 3.1 (SE) ng/ml on average. The mean plasma LH levels around the LH peak were $7.0 \pm 2.1\text{ ng/ml}$ and $14.7 \pm 4.4\text{ ng/ml}$ at 12 hr and six hr before the peak, respectively, and $14.2 \pm 4.1\text{ ng/ml}$ and $5.8 \pm 2.4\text{ ng/ml}$ at six hr and 12 hr after the peak, respectively. The period during which the plasma LH level was 10 ng/ml or higher continued for 12 hr around the LH peak. The duration varied considerably among the animals.

The plasma P levels before and after the LH peak are shown in Table 2, with the day of LH peak being noted as day 0. Since there were almost no differences among the three values measured on a given day, the mean P levels are presented.

The plasma P levels were $0.22\text{--}0.80\text{ ng/ml}$, $0.53 \pm 0.07\text{ ng/ml}$ on average two days before the LH peak, $0.33\text{--}1.12\text{ ng/ml}$, $0.67 \pm 0.09\text{ ng/ml}$ on average on the day before LH peak, and $0.59\text{--}1.23\text{ ng/ml}$, $0.92 \pm 0.06\text{ ng/ml}$ on average on the day of LH peak. The plasma P levels were $0.99\text{--}2.26\text{ ng/ml}$, $1.56 \pm 0.15\text{ ng/ml}$ on average one day after LH peak, $2.12\text{--}4.06\text{ ng/ml}$, $2.78 \pm 0.20\text{ ng/ml}$ on average after two days, $3.29\text{--}6.01\text{ ng/ml}$, $4.79 \pm 0.33\text{ ng/ml}$ on average after three days, and $5.23\text{--}11.78\text{ ng/ml}$, $7.63 \pm 0.82\text{ ng/ml}$ on average after four days. These findings show that plasma P rapidly increased day by day after the LH peak.

The relationship between ovarian follicle development observed by ultrasonography diagnosis system and the plasma LH peak: The relationship between the size of ovarian follicles observed by US and the plasma LH peak is shown in Table 3.

Ovarian follicles were detected by US at 3–8 days with an average of 5.1 ± 0.5 days after initiation of vulval bleeding. The sizes of ovarian follicles at this time point were $0.3\text{--}0.5\text{ cm}$, $0.40 \pm 0.02\text{ cm}$ on average. Ovarian follicles grew to their largest size 6–12 days, 9.1 ± 0.6 days on average, after the initiation of vulval bleeding. The largest sizes of ovarian follicles detected by US were 0.5--

Fig. 1. Changes in plasma LH and progesterone levels before and after LH peak.

Table 1. The LH levels around the plasma LH peak

Bitch No.	Time after LH peak								
	-24	-18	-12	-6	0	6	12	18	24
B178	1.5	—	7.4	17.3	21.0	—	9.5	10.0	4.6
B179	—	—	—	—	40.0	—	—	19.7	6.8
B193	2.2	4.7	—	32.8	42.4	26.8	—	—	2.6
B213	0.9	3.4	—	9.5	24.3	8.0	—	5.8	3.7
B214	1.7	4.2	7.1	—	23.9	13.3	9.2	—	7.9
B215	6.1	3.2	—	11.7	25.6	17.1	—	13.0	4.0
B216	3.2	2.0	—	4.5	32.2	6.1	—	7.5	3.1
B217	2.9	—	11.2	12.2	18.6	—	1.9	1.3	1.0
B222	1.9	—	—	—	14.1	—	—	—	8.4
B238	6.5	—	2.2	—	13.6	—	2.5	—	2.5
B247	4.8	—	—	—	16.7	—	—	—	3.4
mean	3.2	3.5	7.0	14.7	24.8	14.2	5.8	9.6	4.4
± SE	0.7	0.5	2.1	4.4	3.1	4.1	2.4	2.8	0.7

The period during which LH was 10 ng/ml or high. —: Not observed.

Table 2. The plasma progesterone level before and after the LH peak

Bitch No.	Days after LH peak						
	-2	-1	0	1	2	3	4
B178	0.39	0.33	0.85	1.88	2.41	5.52	8.81
B179	—	—	0.72	1.56	2.14	3.70	5.23
B193	0.74	0.90	0.93	0.99	2.80	3.29	10.27
B213	0.62	0.73	0.90	2.22	4.06	5.54	5.79
B214	0.56	0.47	1.05	1.05	2.81	5.99	11.78
B215	0.69	0.80	1.07	1.36	2.21	4.30	6.19
B216	0.30	0.31	0.59	1.01	2.88	3.70	5.39
B217	0.40	0.51	0.74	1.42	2.12	4.16	10.47
B222	0.80	0.84	1.23	1.34	2.30	4.50	6.80
B238	0.22	1.12	1.08	2.26	3.43	6.00	5.54
B247	0.61	0.64	1.01	1.02	3.44	6.01	—
mean	0.53	0.67	0.92	1.46	2.78	4.79	7.63
± SE	0.07	0.09	0.06	0.15	0.20	0.33	0.82

—: Not observed.

0.8 cm, 0.58 ± 0.03 cm on average. The number of days between the initiation of vulval bleeding and the LH peak was 6–10 days, 8.2 ± 0.4 days on average. The number of days between the initiation of vulval bleeding and the day on which ovarian follicles were the largest was within one to two days after the LH peak.

The relationship between the ovulation day determined by ultrasonographic diagnosis system and the plasma LH and P levels: The relationships between ovulation detected by US and the plasma LH and P values are shown in Table 4.

Ovulation could be detected in six of the 11 dogs (54.5%) by observing changes and shrinking of ovarian follicular shape by US (Fig. 2). From US observation during the few

days after ovulation, the ovarian follicle was the same shape as before ovulation and was detected as an echo-free region. In the other five dogs in which ovulation could not be detected by US, ovarian follicles were observed as stippled high echo regions similar to those observed in the six dogs in which ovulation could be detected.

The time between the LH peak and ovulation in the six dogs in which ovulation could be observed was 24–48 hr, 38.0 ± 4.4 hr on average. The plasma P level on the ovulation day was 1.88–2.81 ng/ml, 2.34 ± 0.17 ng/ml on average. Although the P level in B178 was 1.88 ng/ml, the ovulation day was the first day that the P level was 2 ng/ml or higher in the other five dogs. The P levels were 0.85–1.56 ng/ml, 1.12 ± 0.13 ng/ml on average, on the day before

Table 3. The relationship between the ovarian follicular development observed by ultrasonographic diagnosis system and the plasma LH peak

Bitch No.	Days after initiation of vulval bleeding				
	Days until follicles became detectable		Days until follicles reached the largest size		Days until LH peak
	Days	Follicular size (cm)	Days	Follicular size (cm)	
B178	4	0.3	8	0.5	8
B179	5	0.4	7	0.5	7
B193	5	0.4	11	0.5	9
B213	6	0.4	10	0.5	9
B214	4	0.3	10	0.6	8
B215	8	0.5	12	0.7	10
B216	3	0.4	9	0.5	9
B217	5	0.3	10	0.7	9
B222	5	0.4	8	0.8	6
B238	3	0.5	6	0.6	6
B247	8	0.5	9	0.5	9
mean	5.1	0.40	9.1	0.58	8.2
± SE	0.5	0.02	0.6	0.03	0.4

Table 4. The time of ovulation after LH peak determined by ultrasonographic diagnosis system and plasma progesterone levels

Bitch No.	Time (hr) between LH peak and ovulation	Plasma P level (ng/ml)		
		The day before ovulation	Ovulation day	The day following ovulation
B178	24	0.85	1.88	2.41
B179	42	1.56	2.14	3.70
B193	48	0.99	2.80	3.29
B213	30	0.90	2.22	4.06
B214	36	1.05	2.81	5.99
B215	48	1.36	2.21	4.30
mean	38.0	1.12	2.34	3.96
± SE	4.4	0.13	0.17	0.54

Fig. 2. US observation before and after ovulation

a: Mature ovarian follicles (five follicles)

b: Ovulation of the follicles shown in a) after 12 hr (follicles were distorted and shrunken). Scale in cm.

ovulation and the P level rapidly increased to 2.41–5.99 ng/ml, 3.96 ± 0.54 ng/ml on average, on the day following ovulation.

DISCUSSION

Silva *et al.* [14] investigated the ovulation in dogs by US and laparotomy, but an accurate ovulation could not be detected. They may not have detected ovulation because of the frequency of US examination and laparoscopy was not sufficient.

England and Yeager [8] reported that US observations before and after ovulation were similar and distinction by US is difficult. Ovulation could be observed in approximately half of dogs by US in this study. This may have been the result of thrice daily observation. The shape of the ovarian follicles may shrink during ovulation, but soon return to their former shape. England and Yeager [8] also reported that twice daily examination is required to observe ovulation.

As described above, there have been only a few reports in which the ovaries were directly observed to determine ovulation in dogs, and in this study the relationship between ovulation and plasma hormones was investigated based on the direct observation of the ovaries.

To predict ovulation in dogs more easily and apply knowledge to determine an appropriate mating period, the methods requiring systemic anesthesia such as laparotomy and laparoscopy are not recommended. Furthermore, application of US several times a day is also clinically difficult.

LH surge is widely regarded as a criterion to predict the ovulation in dogs [4, 5, 22]. However, the time between LH surge and ovulation has varied within a certain range among studies. This variation may be due to the number of times of LH measurement per day and how the ovulation was determined. As we also noted, the duration of the period with a high LH level is as short as one day. Therefore, to determine LH surge in the plasma, hormone measurement several times per day is necessary, which is clinically difficult.

On the other hand, the elevation of the P level on the ovulation day was determined with a daily measurement of the P level in six of eleven dogs in the present study. Although ovulation could not be observed by US in five other dogs, the P level increased to 2 ng/ml or higher on the second day after the LH peak in four dogs and on the day after the LH peak in one dog. The temporal relationship between the elevation of the P levels and LH peak was almost the same as that in the six dogs in which the ovulation could be observed by US, suggesting that ovulation occurred in all animals and that the elevation of the P levels after the LH peak was related to the ovulation. These findings indicate that a daily measurement of the P level is a useful parameter for the prediction of ovulation in dogs.

The possibility of predicting ovulation from the plasma P

level in dogs was suggested by Concannon *et al.* [4], Wright [23], Bouchard *et al.* [2], Renton *et al.* [13], Badinand *et al.* [1] and Silva *et al.* [14]. Among them, Concannon *et al.* [4] reported P levels on ovulation day and accurate observation of ovulation. According to their report, the plasma P level on ovulation day is approximately 4.4 ng/ml, which is a little higher than that noted in this study. This difference may be due to differences in the measurement method.

In this study, we determined that the day on which 2 ng/ml or higher plasma P level is detected by daily measurement is the ovulation day in dogs. In this study, prediction of the ovulation in dogs was investigated for the purpose of determining the optimal mating period. We found that the optimal mating period extends three to five days after ovulation, and the mating day can be determined after confirm a sharp elevation in plasma P level one to two days after ovulation.

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