

## GnRH analogue treatment on LH surge day 0 followed by single transvaginal artificial insemination with frozen semen on day 5 in bitches

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**ABSTRACT.** Reproductive parameters were evaluated in 19 and 14 estrous beagles that received 100 µg of gonadotropin-releasing hormone (GnRH) and saline treatment, respectively, on the day of luteinizing hormone (LH) surge (Day 0; estimated by serial progesterone assay) and balloon catheter-aided single transvaginal artificial insemination of frozen semen on Day 5. Although the conception rate and litter size were similar between the GnRH and saline groups, the concentration of LH peak was significantly higher in GnRH-treated bitches ( $P < 0.01$ ). In addition, the actual LH surge did not occur on the estimated Day 0 in one saline-treated bitch. In clinical practice that daily progesterone assay is difficult, administration of GnRH on estimated Day 0 would be recommended to induce or enhance the LH surge for timely and successful insemination.

**KEY WORDS:** bitch, Foley catheter, frozen semen, gonadotropin releasing hormone, transvaginal insemination

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Canine artificial insemination (AI) using frozen semen has been increasingly in demand in recent years. The pregnancy rate after transvaginal AI of frozen-thawed semen, however, is very limited, because canine spermatozoa are viable only for a day after thawing [8, 19]. To achieve good pregnancy rates, surgical intrauterine deposition is often recommended for frozen sperm insemination, but many dog owners express disinclination toward the procedure due to its invasive nature. Alternatively, intrauterine AI can be performed transcervically with the aid of a rigid endoscope or a metal catheter, but these devices can be costly and require a level of technical skill [21]. In contrast, transvaginal AI using a balloon catheter is a simple, noninvasive procedure [23]. However, transvaginal AI requires 2 to 3 times more sperms per insemination compared to other methods [14–16], and ideally it should be performed at least twice within the most fertile period if conducted with frozen semen [22]. This imposes a financial burden on owners, as they have to purchase multiple doses of high-quality frozen semen often from overseas. As such, the development of a single insemination protocol is a pressing challenge in order to make the simple balloon catheter-aided transvaginal AI more practical.

In the reproductive cycle of the bitch, luteinization of

follicular granulosa cells and increase of the progesterone level at the time of luteinizing hormone (LH) surge are characteristic physiological events before ovulation. Generally, detection of the LH surge is an accurate diagnostic tool for determining the best time for breeding, but in Japan a more convenient progesterone assay is often used to indirectly estimate the timing of the LH surge by the change in the progesterone levels, which is easy to assay, since in-clinic LH surge detection kits are not widely available [1, 3, 5, 9, 10, 17]. However, the LH surge estimated from progesterone levels does not always correlate with the actual LH surge [10].

In the present study, a gonadotropin-releasing hormone (GnRH) analog was administered on the day of the LH surge (Day 0), which was estimated as the day the progesterone level started to increase, in order to ensure that the LH surge would occur on that day, and balloon catheter-aided single transvaginal AI of frozen semen was carried out on Day 5. Then, reproductive performances were evaluated to examine the efficacy of this AI protocol.

Thirty-three female and 16 male beagles kept at the Laboratory of Theriogenology at the Nihon University were used in this study. The animals were maintained in an animal room and housed in individual cages. They were supplied with dry food once a day and drinking water *ad libitum*. Unrelated dogs were used to prevent inbreeding. All procedures used in this experiment were approved by the Ethical Committee for Animal Experimentation at the College of Bioresource Sciences, Nihon University.

The frozen semen was prepared by a method described by Hayashi *et al.* [11].

The timing of insemination in relation to the LH surge

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Table 1. Comparison of hormone levels and reproductive performances between 19 GnRH- and 14 saline-treated bitches in Foley catheter-assisted transvaginal artificial insemination of frozen semen

GnRH treatment						Saline treatment					
Bitches	Progressive motility of sperm (%)	P <sub>4</sub> concentrations (ng/ml)		Concentrations of LH peak (ng/ml)	Number of puppies	Bitches	Progressive motility of sperm (%)	P <sub>4</sub> concentrations (ng/ml)		Concentrations of LH peak (ng/ml)	Number of puppies
		Day 0	Day 5					Day 0	Day 5		
1	50	2.73	22.00	61.67	7	A	50	2.94	22.00	11.64	3
2	60	2.51	24.09	24.72	5	B	55	2.51	24.09	5.72	9
3	55	4.24	40.16	26.28	7	C	50	4.24	40.16	29.46	7
4	50	2.00	30.35	26.46	6	D	50	2.00	30.35	6.83	4
5	55	3.57	37.29	28.60	1	E	50	3.57	37.29	5.57	3
6	60	5.53	42.57	22.16	7	F	55	5.53	42.57	2.40	6
7	50	3.34	42.39	27.41	5	G	65	3.34	42.39	0.90	Non-conception
8	60	4.66	45.37	37.30	6	H	60	4.66	45.37	26.04	7
9	60	3.41	37.26	23.64	7	I	60	3.41	37.26	14.64	Non-conception
10	50	3.55	51.04	23.74	7	J	50	3.55	51.04	5.83	8
11	50	3.75	46.78	19.42	Non-conception	K	60	3.75	46.78	3.07	2
12	60	3.63	6.69	29.41	1	L	50	3.63	6.69	4.98	4
13	40	2.92	17.62	21.74	7	M	50	2.92	17.62	5.76	4
14	60	3.06	27.08	24.15	Non-conception	N	60	3.06	27.08	1.53	7
15	40	3.09	18.76	24.37	3						
16	60	4.49	14.74	27.79	8						
17	55	2.77	18.21	22.80	4						
18	70	3.54	36.66	21.80	8						
19	60	2.20	12.78	22.89	3						
Mean ± SEM	55.0 ± 1.8	3.06 ± 0.12	30.10 ± 3.02	27.18 ± 9.22*	5.4 ± 0.6	Mean ± SEM	54.6 ± 1.5	2.72 ± 0.13	29.78 ± 2.68	8.88 ± 8.82	5.3 ± 0.7

*P* < 0.01, in comparison with the Saline treatment.

was determined by monitoring the serum progesterone (P<sub>4</sub>) concentration using an automated chemiluminescence immuno analyzer (Spotchem Vidas SV-5020, Arkray, Kyoto, Japan) [1, 3, 5, 9, 10, 17]. The first day of proestrus was confirmed by daily observation of vulvar swelling and hemorrhagic discharge. Serum P<sub>4</sub> concentrations were measured daily after 5 days from the confirmed first day of proestrus or the appearance of >80% cornified cells in the vaginal smear. In principle, the day of the LH surge was estimated as the day when serum P<sub>4</sub> concentrations first reached 2–4 ng/ml. However, for bitches with a baseline serum P<sub>4</sub> concentration of <2 ng/ml, it was estimated that the LH surge occurred on the day when the serum P<sub>4</sub> concentration rapidly increased to >4 ng/ml within a day.

The bitches were divided into 2 groups: a GnRH group (n=19) and a control group (n=14). On Day 0 estimated from the P<sub>4</sub> concentration, the GnRH group received a single subcutaneous dose of 100 µg of fertirelin acetate (Conceral Injection, Nagase Medicals, Itami, Japan), while the control group received a single 2-ml dose of saline (Isotonic Sodium Chloride Solution, Terumo, Tokyo, Japan) subcutaneously. Blood samples were obtained at 0, 1, 2, 4, 6, 8, 10, 12, 16 and 24 hr after the treatment of GnRH or saline. Serum was separated and stored frozen at –20°C until LH assay. Serum LH concentration was measured by double-antibody radioimmunoassay as previously described [18]. The sensitivity of the assay was

10.7 pg/tube, and the intra- and inter-assay coefficients of variation (CVs) were 5.8% and 14.3%, respectively.

Timing of ovulation was determined when P<sub>4</sub> levels reached ≥5 ng/ml based on quantitative serum P<sub>4</sub> assay performed on Day 5 [1, 3, 5, 17], and transvaginal AI was performed using a Foley catheter according to the method described by Tsumagari *et al.* [23]. Briefly, 0.5 ml straws were thawed in a water bath at 70°C for 8 sec just before AI. After thawing, the sperm progressive motility and sperm malformation rate were evaluated by direct microscopic examination. The semen gathered from four straws (total sperm count of 200 × 10<sup>6</sup>) was adjusted to a volume of 5 ml with a modified Tris-yolk-citrate extender, and a Foley catheter (24 Fr, 30 ml, Create Medic, Yokohama, Japan) was inserted into the caudal vagina and fixed with the balloon. Semen was inserted into the Foley catheter with a syringe and pushed with 7 ml of air in another syringe in order to insert the maximal amount of semen into the uterine tract. The Foley catheter was kept raised for 15 min after insertion of the semen. All AI procedures were performed by the same person. Pregnancy diagnosis was carried out by ultrasonography (Sonosite 180, SonoSite, Bothell, WA, U.S.A.) 30 days after AI [4, 13, 20], and the litter size was recorded after whelping.

All values are expressed in means ± standard errors of the mean (SEM), and mean values were compared by unpaired

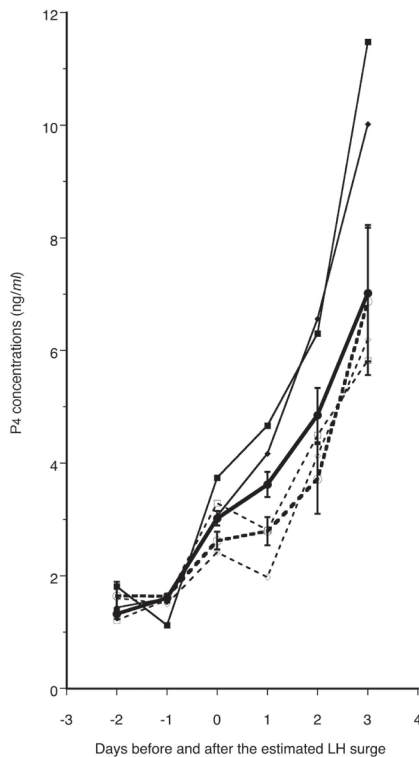


Fig. 1. Changes of P<sub>4</sub> concentration before and after estimated LH surge in GnRH- and saline-treated bitches. GnRH conception (—●—, n=17), Saline conception (·····○·····, n=17), GnRH non-conception (■or◆): solid line, Saline non-conception (□or◇): dashed line, Conception represents mean ± SEM.

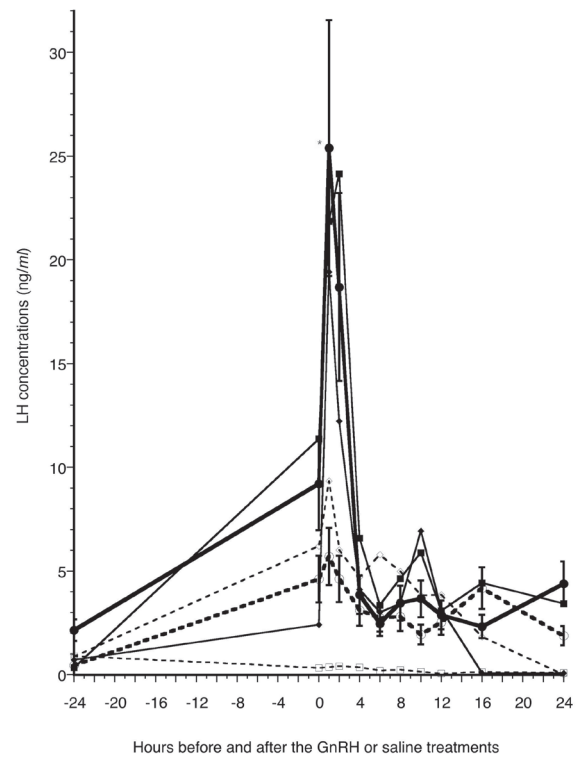


Fig. 2. Changes of LH concentration before and after GnRH- and saline-treated bitches. GnRH conception (—●—, n=17), Saline conception (·····○·····, n=17), GnRH non-conception (■or◆): solid line, Saline non-conception (□or◇): dashed line, Conception represents mean ± SEM.

two sample *t*-test. A level of significance less than 0.05 was considered statistically significant.

The progressive motility of sperm in the used semen was  $55.0 \pm 1.8\%$  in the GnRH group and  $54.6 \pm 1.5\%$  in the control group (Table 1). The P<sub>4</sub> concentration on Day 0 was  $3.1 \pm 0.1$  ng/ml in the GnRH group and  $2.7 \pm 0.1$  ng/ml in the control group and increased to  $30.1 \pm 3.0$  ng/ml in the GnRH group and  $29.8 \pm 2.7$  ng/ml in the control group on Day 5, when AI was performed (Table 1 and Fig. 1). In the GnRH group, 17 of 19 bitches became pregnant (89.5%), and the litter size was  $5.4 \pm 0.6$  puppies. In the control group, pregnancy was diagnosed in 12 of 14 bitches (85.7%), and the litter size was  $5.3 \pm 0.7$  puppies. P<sub>4</sub> concentrations on Days 0 and 5, conception rates and litter sizes were not significantly different between the two groups. The peak LH concentration, however, was significantly higher in GnRH-treated bitches than in controls ( $27.2 \pm 9.2$  vs.  $8.9 \pm 8.8$  ng/ml;  $P < 0.01$ ) (Table 1). Serum LH levels peaked at 20 ng/ml on average at 1 hr after GnRH treatment and then decreased to the basal levels by 4 hr regardless of the subsequent pregnancy status of the bitches (Fig. 2).

In the present study, the conception rates in the saline- and GnRH-treated groups were remarkably higher than the success rate of 75%, which was previously reported with 2 nonsurgical, transcervical intrauterine AI of frozen semen [22]. According to Tsutsui *et al.* [24], a single surgical in-

semination of frozen semen into the uterine horn resulted in a pregnancy rate of 90.0%, which is close to what we achieved in the present study. The litter sizes in our study,  $5.4 \pm 0.6$  and  $5.3 \pm 0.7$  puppies in GnRH- and saline-treated bitches, respectively, were also comparable to those reported by Thomassen *et al.* [22] after transcervical intrauterine AI ( $5.7 \pm 0.3$  puppies) and by Hori *et al.* [12] after surgical unilateral intrauterine AI ( $5.6 \pm 0.9$  puppies). Taken together, these results indicate that transvaginal single AI using a balloon catheter can be as efficient as surgical or nonsurgical intrauterine AI for frozen semen.

As shown in Fig. 1 and Table 1, serum P<sub>4</sub> levels on Day 0 were between 2 and 4 ng/ml in all bitches. In GnRH-treated bitches, the serum P<sub>4</sub> concentration increased markedly on Day 1 regardless of whether they later became pregnant or not. In the control group, on the other hand, 2 bitches (Bitches G and I) showed decreased P<sub>4</sub> levels on Day 1 and did not become pregnant. The mean P<sub>4</sub> concentration on Day 1 was  $3.7 \pm 0.2$  ng/ml in the GnRH group, which was significantly higher than  $2.71 \pm 0.2$  ng/ml in the control group ( $P < 0.01$ ).

In the control group, one bitch (Bitch G), which did not become pregnant, showed no detectable LH surge, and her LH concentrations remained below 1 ng/ml throughout the observation period, indicating that the LH surge did not occur as estimated in this bitch. This result is consistent with

the previous observation by Hase *et al.* [10] that the estimated day of the LH surge does not always coincide with the actual LH surge.

Although the conception rates were similar in both GnRH-treated and untreated bitches, GnRH did induce and/or enhance the LH surge on Day 0, as demonstrated by the significantly higher LH peak concentration in GnRH-treated bitches and possibly contributed to pregnancy. Since frozen-thawed semen has poorer quality, the timing of AI must be predicted precisely. The present study proved that high success rates could be achieved by single transvaginal AI with a balloon catheter when combined with daily serial progesterone assay. In the clinical setting, however, daily blood sampling for progesterone assay is not always possible. Concerning LH surge and ovulation, it has been reported that LH surge can be predicted at follicle diameter by ultrasound [2, 6, 7]. There appeared to be a shift in the population from small follicles to large follicles (>4 mm diameter) approximately 2 days prior to the LH surge [7]. In addition, it becomes clearer by using color Doppler [2]. Nonetheless, if transvaginal AI with frozen semen is still the technique of choice, administration of GnRH on estimated Day 0 is recommended to effectively induce the LH surge and to prevent poor success rates resulting from inaccurate estimation of the LH surge.

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