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—Original Article—

Evaluation of criteria for optimal time AI postulated by estrous signs in lactating dairy cows kept in tie-stalls

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Abstract. Relaxation of the intravaginal part of the uterus is obvious around 6 to 18 h before ovulation, and this is considered the optimal time for artificial insemination (AI), as demonstrated in recent studies. Estrous signs have been suggested as useful criteria for determining the optimal time for AI. Therefore, this study evaluated the usefulness of estrous signs, particularly the relaxation of the intravaginal part of the uterus, as criteria for determining the optimal time for AI. A Total of 100 lactating Holstein-Friesian cows kept in tie-stall barns were investigated. AI was carried out based on the criterion for the optimal time for AI (optimal group), and earlier (early group) and later (late group) than the optimal time for AI, determined on the basis of estrous signs. After AI, ovulation was assessed by rectal palpation and ultrasonographic observation at 6-h intervals. For 87.5% (35/40) of cows in the optimal group, AI was carried out 24-6 h before ovulation, which was previously accepted as the optimal time for AI. AI was carried out earlier (early group) and later (late group) than optimal time for AI in 62.1% (18/29) and 71.0% (22/31) of cows, respectively. The conception rate for the optimal group was 60.0%, and this conception rate was higher than that for the early group (44.8%) and late group (32.2%), without significance. Further, the conception rate of the optimal group was significantly higher than the sum of the conception rates of the early and late groups (38.3%; 23/60) ($P < 0.05$). These results indicate that the criteria postulated, relaxation of the intravaginal part of the uterus and other estrous signs are useful in determining the optimal time for AI. Furthermore, these estrous signs enable the estimations of stages in the periovulatory period.

Key words: Artificial insemination (AI), Conception rate, Dairy cows, Estrous signs, Ovulation

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Over the past half-century, the productivity of the dairy industry in Japan has been improving through genetic improvements, by artificial insemination and embryo transfer, and improvements in feeding and housing of cows. As a result, the average 305-day milk production per cow has increased remarkably from 7,798 to 9,225 kg in the 21 years from 1990 to 2011. On the other hand, the average calving interval of dairy cows has increased from 403 days to 438 days over the same 21-year period [1,2]. This decline in the reproduction of cows has been observed not only in Japan but also worldwide [3–10]. Recently, it has been suggested that one of the main factors for this decline in reproduction is the weakened and shortened estrus and estrous signs [11, 12]. These factors might lead to failed artificial insemination (AI) or missing of the optimal time of insemination, leading to a decrease in the conception rate and a consequent increase in the calving interval.

It is generally accepted that cows standing to be mounted, which is called "standing estrus", is the most reliable criterion for determining estrus in cattle [6, 13–18]. Therefore, the observation of standing estrus is important to determine the optimal time for AI in dairy cows. Previous studies have investigated the optimal time for AI. Trimberger and his co-workers postulated the optimal time for AI in cattle, and this has been widely accepted to date [19–21]. The index for AI is also based on observations of standing estrus and ranges between 6 and 24 h [19, 20] and between 6 and 28 h [21] after the onset of standing estrus.

However, observing standing estrus in dairy cows kept in tie-stall barns is quite difficult. Therefore, AI is conducted at the assumed optimal time, when a certified, professional inseminator has determined the time to be optimal, based on observations of the external and internal estrous signs. However, definite criteria to determine the optimal time for AI based on estrous signs have not been established. We previously examined the changes in 11 estrous signs, plasma profiles of ovarian steroids, and luteinizing hormone (LH), from luteolysis to ovulation, in dairy cows kept in tie-stall barns [22]. The results showed that most of the estrous signs appeared with an increase of estradiol-17β (E_2), became most remarkable around the peak of E_2 and initiation of the LH surge, and then decreased toward ovulation. In addition, the changes in estrous signs were more distinct in the vagina, as observed by vaginoscopic examination, than in the vulva when observed by the naked eye or in the uterus by rectal palpation. Thus, estrous signs more distinctly reflected the blood estrogen dynamics in the intravaginal part of the uterus and the external uterine orifice than in the vulva or uterus. Furthermore, among the estrous sings observed by vaginoscopic examination, relaxation of the intravaginal part of the uterus became most obvious 6, 12, and 18 h before ovulation. This period, during which the

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relaxation of the intravaginal part of the uterus was most obvious, is consistent with the previously accepted optimal time for AI [22]. Based on these observations, we hypothesized that the relaxation of the intravaginal part of the uterus could be used as a criterion to determine the optimal time for AI. The results of our preceding study indicated that precise observation of 8 estrous signs, including the relaxation of the intravaginal part of the uterus, was essential for determining the periovulatory stage and optimal time for AI.

Thus, in the present study, the utility of 8 such estrous signs that could be observed clearly: swelling of the vulva, hyperemia, swelling and relaxation of the intravaginal part of the uterus, opening of the external uterine orifice, viscosity of cervical mucus, contraction of the uterus, and diameter of the uterine horn when relaxed were evaluated as criteria for determining the optimal time for AI in lactating dairy cows kept in tie-stall barns. Relaxation of the intravaginal part of the uterus was also mainly incorporated into these criteria. AI was carried out during 3 stages: the assumed optimal time for AI in cows, and before and after the optimal time for AI, as determined by the criteria of the 8 estrous signs. Thereafter, the ovulation time and conception rate were examined.

Materials and Methods

Animals

This study was carried out in a total of 100 Holstein-Friesian cows. These 100 cows included the repeatedly subjected cows and were kept in three tie-stall style farms, i.e., 45, 39, and 16 cows at Farms A, B, and C, respectively, in Kanagawa prefecture between March 2012 and October 2013. The cows were confirmed beforehand to be clinically healthy. AI was performed on most of the cows based on observations, made by farmers, of the appearance of estrous signs (i.e., roaring, restlessness, hyperemia and swelling of the vulva, mucus discharge from the vulva, and decrease in milk production), the predicted day of estrus from the last AI, and the last postestrual bleeding. In addition, the cows determined to be in a pre-ovulatory period, based on postpartum periodical inspection of the genitalia, were also used in the present study. These cows were more than 60 days postpartum and had no genital abnormality, as confirmed by rectal and vaginoscopic examinations and transrectal ultrasonography. At the beginning of the study, the average age, parity, and days after parturition of the cows were 4.2 ± 1.7 years (mean ± SD); (4.3 ± 1.6, 3.8 ± 1.5, and 5.2 ± 1.9 years at Farms A, B, and C, respectively), 2.2 ± 1.3 times (2.4 ± 1.4, 1.9 ± 1.1, and 3.0 ± 1.8 times at Farms A, B, and C, respectively), and 201.4 ± 119.9 days (173.6 ± 68.6, 234.9 ± 133.9, and 161.5 ± 76.2 days at Farms A, B, and C, respectively), respectively. The body condition score (BCS) of the cows was 2.5 ± 0.3 (2.4 ± 0.2, 2.6 ± 0.3, and 2.5 ± 0.1 at Farms A, B, and C, respectively), based on a 5-point scale [23]. The cows were milked twice a day, and the average milk yield per lactation (305 days) was around 9,000 kg per cow on every farm. The cows were fed a total mixed ration (TMR), or dry hay and concentrate supplements separately, according to the Japanese feeding standards for dairy cattle (2006). The study was not performed during July to September, when high temperature might have had an influence on the conception rate. All experimental procedures were approved by the University Committee for the Use and Care of Animals of Tokyo University of Agriculture and Technology (No. 25–36).

Outline of the experimental procedure

The cows scheduled for AI, as requested by the farmers, and those determined to be in a pre-ovulatory period by the postpartum periodical inspection of genitalia were subjected to the following clinical inspections. Observation of estrous signs was carried out; in addition, rectal palpation and transrectal ultrasonographic observations of the ovary and uterus were conducted for these experimental cows. The 8 estrous signs were evaluated in detail and AI was carried out depending on the criteria for determining the time for AI. Ovulation was examined at 6-h intervals after AI. Pregnancy diagnosis was carried out 60 days after AI by rectal examination and transrectal ultrasonography. The following are the details of each procedure and criteria.

Observation of estrous signs

In this study, 8 estrous signs, i.e., swelling of the vulva, hyperemia, swelling and relaxation of the intravaginal part of the uterus, opening of the external uterine orifice, viscosity of cervical mucus, contraction of the uterus, and diameter of the uterine horn when relaxed were selected and evaluated. Three estrous signs, i.e., hyperemia of the vulva, mucus discharge from the vulva, and accumulation of fluid secreted into the uterus, were excluded because these were indistinct, inconsistent, and required ultrasound equipment, respectively, in accordance with the findings of a preceding study [22]. With regard to the observation order, first, swelling of the vulva was observed. Second, after cleaning and sterilizing the vulva with a disinfectant, a vaginoscope (sterilized by soaking in an antiseptic solution) was inserted into the vagina and hyperemia, swelling, relaxation of the intravaginal part of the uterus, and opening of the external uterine orifice were observed. Third, vaginal mucus around the external uterine orifice was aseptically collected using a sterilized tampon, and the viscosity of the mucus was examined. Finally, rectal examination and ultrasonography were carried out to evaluate the diameter of the uterine horn, contraction of the uterus, and the diameter of the dominant follicles and degenerated corpus luteum (CL) to confirm the cows were in a periovulatory period. The estrous signs were classified as no, moderate, or obvious signs. The viscosity of the cervical mucus was classified as starchy, sticky, or watery, and the diameter of the uterine horn when relaxed was classified as < 1.5 fingerbreadths, 1.5–2 fingerbreadths, or > 2 fingerbreadths. The classified estrous signs were given 0, 1, or 2 points depending on their obviousness [22], and the observed estrous signs were assigned a total score based on the given points (Table 1).

Observation of the ovary and uterus by rectal palpation and ultrasonography

Transrectal ultrasonographic observations were carried out using a B-Mode ultrasound scanner (Tringa-V 50S; Esaote Pie Medical B.V., Maastricht, Netherlands) equipped with a 5.0-MHz linear array probe. All follicles and CLs that grew larger than 6 mm in diameter were recorded for confirming the dominant follicle and being in a periovulatory period. The sizes of the follicles and CLs were evaluated as the mean lengths across the major and minor axes [24]. The diameter of the uterine horn and contraction of the uterus were observed by rectal palpation and ultrasonography.

Table 1. Scoring scale for the 8 observed estrous signs

Region	Estrous signs	Scale
Vulva	• Swelling	0 = no signs, 1 = moderate signs, 2 = obvious signs
Intravaginal part of the uterus	• Hyperemia	0 = no signs, 1 = moderate signs, 2 = obvious signs
	• Swelling	0 = no signs, 1 = moderate signs, 2 = obvious signs
	• External uterine orifice	0 = closing, 1 = opening, 2 = conspicuous opening
	• Relaxation	0 = no signs, 1 = moderate signs, 2 = obvious signs
	• Viscosity of the mucus	0 = starchy, 1 = sticky, 2 = watery
Uterus	• Contraction	0 = no signs, 1 = moderate signs, 2 = obvious signs
	• Diameter of uterine horn [a)]	0 = under 1.5 fingerbreadths, 1 = 1.5−2 fingerbreadths, 2 = 2 fingerbreadths and over

[a)] While relaxed.

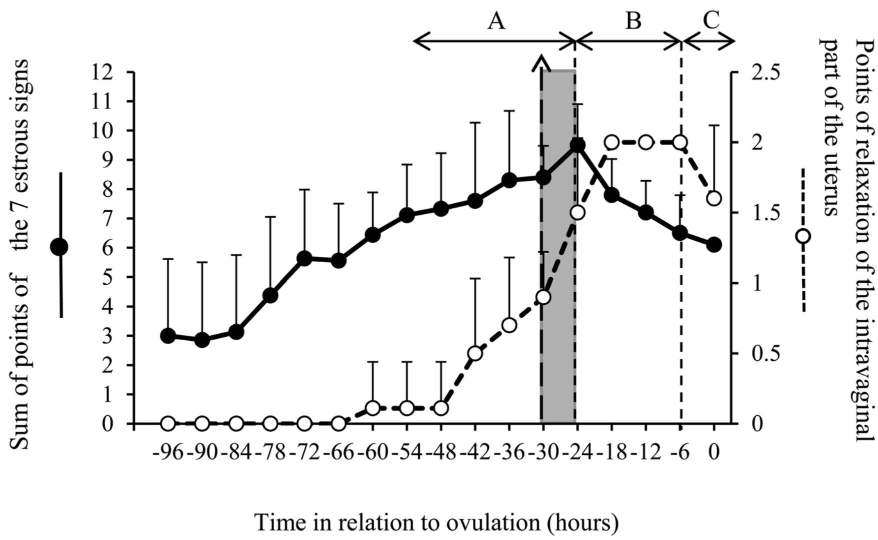

Fig. 1. The estrous sign criteria for determining the timing of AI, as postulated in our previous study [22]. A, B, and C in the upper right of the figure, above the arrows, indicate the 3 AI groups: A, early group, which was inseminated before the optimal time; B, optimal group, which was inseminated at the optimal time; C, late group, which was inseminated after the optimal time. Changes in the relaxation of the intravaginal part of the uterus and the sum of the points of the other 7 estrous signs (i.e., swelling of the vulva, hyperemia and swelling of the intravaginal part of the uterus, opening of the external uterine orifice, viscosity of the cervical mucus, contraction of the uterus, and diameter of the uterine horn) are utilized for determining the index and criteria. The vertical dashed arrow indicates the E_2 peak. The shaded area indicates the LH surge period.

Criteria for determining AI timing

In the present study, the optimal time for AI was generally defined as 6–24 h after the onset of estrus, which was retrospectively accepted [19, 20]. This time period corresponds with 24-6 h before ovulation, because ovulation occurs about 30 h after the onset of estrus [25]. In addition, we indicated in our preceding study that most of the estrous signs became most remarkable when the E_2 peak and LH surge were achieved and then weakened as the ovulation time approached [22]. Furthermore, the estrous signs in the intravaginal part of the uterus were more obvious than those in the vulva and uterus. The E_2 concentration increased, the intravaginal part of the uterus become hyperemic and swollen, and the external uterine orifice opened. After the E_2 peak and LH surge, the hyperemia and swelling diminished, and the relaxation of the intravaginal part of the uterus, which appeared when other estrous signs started to diminish, became most obvious during 18-6 h before ovulation. This duration of remarkable relaxation of the intravaginal part of the uterus corresponded with the retrospectively accepted optimal time for AI, i.e., 24-6 h before ovulation [22] (Fig. 1). Based on these findings, the criteria for determining the optimal time for AI and times earlier or later than the optimal time for AI were established as follows (Table 2 and Fig. 2).

Criteria for determining a time earlier than the optimal time for AI (early group): Most of the estrous signs were obvious or moderate. In particular, hyperemia and swelling of the intravaginal part of the uterus were obvious, but relaxation was not apparent in most cases. In addition, the sum of scores of the other 7 estrous signs, excluding relaxation of the intravaginal part of the uterus, was at least 8 points.

Criteria for determining the optimal time for AI (optimal group): Relaxation of the intravaginal part of the uterus, observed by vaginoscopic examination, was obvious and the other 7 estrous signs were diminishing, from obvious to moderate, and the sum of the scores

Table 2. Criteria for determining the timing of AI on the basis of scores of estrous signs [a] in cows

Timing of AI	Relaxation of the intravaginal part of the uterus	Total scores of the other 7 estrous signs [b] (points)
Early	0/(1) [c]	8 ≤
Optimal	2	5–10
Late	1	< 8

Numerical values are the sums of the points for the estrous signs. [a] Estrous signs were scored on a 3-point scale: none (0), moderate (1), and obvious signs (2) depending on their obviousness. [b] The 7 estrous signs are: swelling of the vulva, contraction of the uterus, diameter of the uterine horn (when relaxed), hyperemia of the intravaginal part of the uterus, swelling of the intravaginal part of the uterus, opening of the external uterine orifice, and viscosity of cervical mucus. [c] Most were 0/(some were 1).

of the other 7 estrous signs ranged from 5–10 points.

Criteria for determining the time later than the optimal time for AI (late group): Most of the estrous signs had changed to moderate and were not obvious. In particular, hyperemia and swelling of the intravaginal part of the uterus, observed by vaginoscopic examination, had disappeared, and relaxation of the intravaginal part of the uterus had changed to moderate, and was not obvious. In addition, the sum of the scores of the other 7 estrous signs was < 8 points.

Experimental groups

A total of 100 cows were divided into the following 3 groups according to AI time. Early group: AI was performed in 29 cows at a time determined to be earlier than the optimal time for AI. The average age, parity, days after parturition, and BCS in the early group were 4.8 ± 2.2 years, 2.6 ± 1.6 times, 209.6 ± 122.6 days, and 2.4 ± 0.3, respectively. Optimal group: AI was performed on 40 cows at a time determined to be the optimal time for AI. The average age, parity, days after parturition, and BCS in the optimal group were 4.1 ± 1.4 years, 2.2 ± 1.2 times, 215.3 ± 127.6 days, and 2.6 ± 0.3, respectively. Late group: AI was performed on 31 cows at a time determined to be later than the optimal time for AI. The average age, parity, days after parturition, and BCS in the late group were 4.0 ± 1.7 years, 2.3 ± 1.3 times, 167.5 ± 84.6 days, and 2.4 ± 0.3, respectively. Among the 3 groups, homogeneity of variances and means were analyzed statistically.

AI procedure and examination of ovulation and pregnancy

AI was carried out by the general recto-vaginal method. Commercially available frozen semen that contained about 30×10^6 sperm in 0.5 ml straws and was kept cryopreserved at –196ºC was used. The semen was thawed by plunging the straw into lukewarm water at 38ºC for 15 sec and then placed into the instrument for AI. The AI procedure was performed as follows. First, the vulva was cleaned with water and then sterilized with an antiseptic solution. Second, a vaginoscope, sterilized with an antiseptic solution, was inserted into the vagina, and it was opened in the same manner as the procedure for observing the intravaginal part of the uterus. Third, the instrument for AI (semen injector) was inserted into the vagina, and the tip of the semen injector was inserted into the external uterine orifice. Then, the instrument was held in position with one hand. Fourth, after removing the vaginoscope, with the semen injector still inserted, the other hand was inserted into the rectum to fold the cervix. The tip of the semen injector was guided into the uterus through the cervical canal by manipulating the cervix, through the rectal wall, with the hand inserted in the rectum. Then, semen was deposited in the uterus when the tip of the semen injector was 1–2 cm inside the uterine body. All the AIs were performed by one experienced veterinarian having good skill.

After AI, observation of estrous signs and determination of ovulation were performed at 6-h intervals. Ovulation was confirmed on the basis of the following findings: the follicles were indistinct or indefinite because of the disappearance of the follicular fluid, usually revealing a depression, as determined by palpation at the former site of the follicle, and a clear decrease in the follicle diameter by rectal examination and transrectal ultrasonography. If the cows did not ovulate until 48 h after AI, further observations of ovulation and estrous signs were not performed.

Pregnancy was confirmed 60 days after AI by rectal examination and transrectal ultrasonography.

Statistical analysis

All experimental data except the conception rate, which was calculated overall, are presented as mean and SD (Mean ± SD). Bartlett's test and one-way ANOVA or Kruskal-Wallis test was used to compare age, parity, time after parturition, and BCS among the 3 farms and 3 groups. Bartlett's test and one-way ANOVA was used to compare the conception rate among the 3 farms and 3 groups. Interactions among the 3 farms and 3 groups were tested using Bartlett's test and two-way ANOVA (age, parity, time after parturition, BCS, and conception rate). Differences in conception rate between 2 groups were analyzed using Fisher's exact test. Differences were considered significant at $P < 0.05$.

Results

Comparison of the cows raised in the 3 farms and 3 groups

The average age, parity, days after parturition, and BCS did not differ significantly among the cows of the 3 farms and 3 groups. The over-all conception rates of cows of the 3 farms (A, B and C) were 42.2% (19/45), 51.3% (20/39), and 50.0% (8/16), respectively. No significant difference was detected among cows of the 3 farms. With regard to the interaction among the cows of the 3 farms and 3 groups, significant differences were not detected for each item by Bartlett's test and two-way ANOVA (age, parity, time after parturition, BCS, and conception rate).

Conception rate, ovulation time after AI, and estrous signs in some incorrectly predicted cases (Tables 3 and 4)

Early group: The conception rate of all 29 cows in the early group was 44.8% (13/29). In the early group, 62.1% of the cows (18/29) were inseminated > 24 h before ovulation, which corresponded to a time earlier than the retrospectively accepted optimal time for AI. However, 27.6% (8/29) of the cows were inseminated 6 < - 24 h before ovulation, which is the optimal time for AI, and 10.3% (3/29) were inseminated ≤ 6 h before ovulation, which is later than the optimal

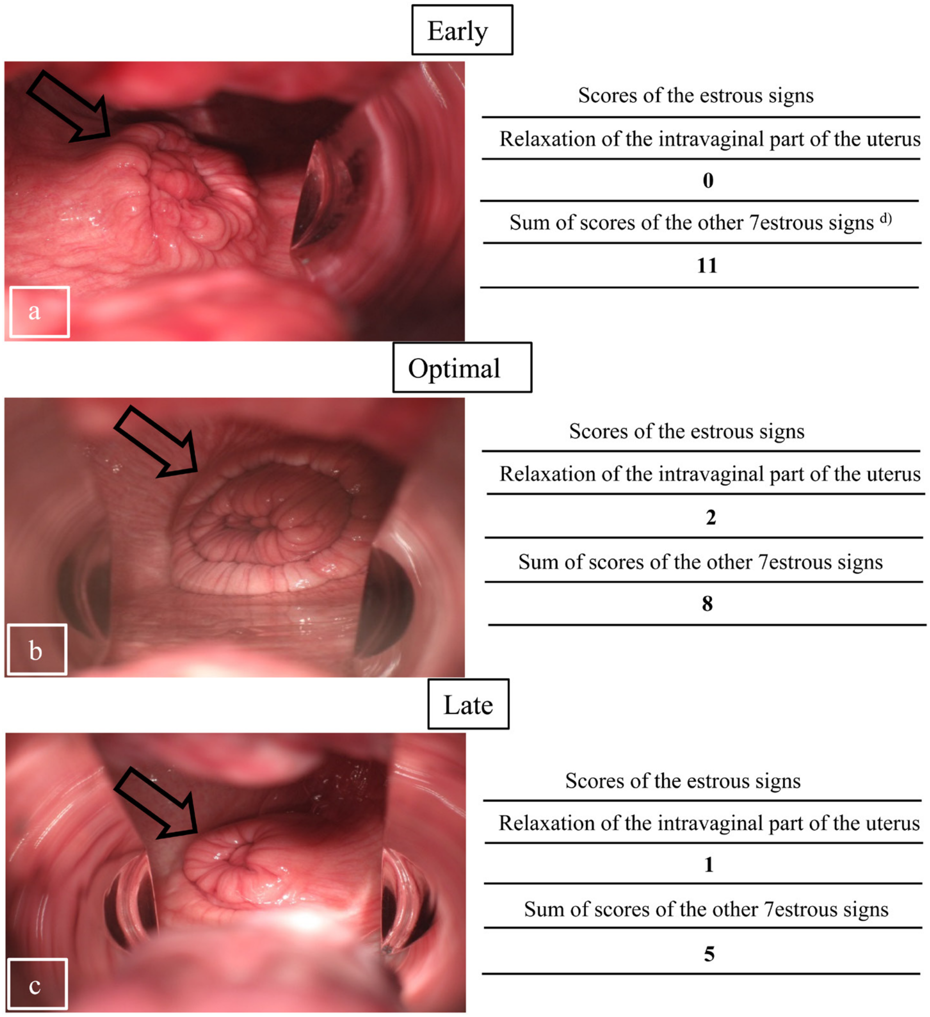

Fig. 2. Representative appearances of the estrous signs and scores of the intravaginal part of the uterus and sum of the scores of the other 7 estrous signs for early (a), optimal (b), and late (c) timing of AI. a) 30 < - 36 h before ovulation. The intravaginal part of the uterus (=>) is obviously swollen by hyperemia and edema. The external uterine orifice is conspicuously open. b) 24 < - 36 h before ovulation. The hyperemia and swelling of the intravaginal part of the uterus (=>) diminishes, and the intravaginal part of the uterus becomes obviously relaxed and, overall, dropped. The external uterine orifice is open. c) 0 < - 6 h before ovulation. The relaxation of the intravaginal part of the uterus (=>) decreases. [d)] Sum of the scores of swelling of the vulva, contraction of the uterus, diameter of the uterine horn (when relaxed), swelling of the intravaginal part of the uterus, hyperemia of the intravaginal part of the uterus, opening of the external uterine orifice, and viscosity of cervical mucus.

time for AI. Among these 8 cows that received AI, as a result, at the optimal time, 4 cows ovulated without exhibiting obvious relaxation of the intravaginal part of the uterus, and 4 cows ovulated after a short duration of 6 h with obvious relaxation of the intravaginal part of the uterus after the AI. The conception rate of the cows that were inseminated earlier than the optimal time for AI was 44.4% (8/18). The conception rates of the cows that were inseminated at the optimal time and later than the optimal time for AI were 50.0% (4/8) and 33.3% (1/3), respectively.

Optimal group: The conception rate of all 40 cows in the optimal group was 60.0% (24/40). In the optimal group, 2 cows (5.0%), 4 cows (10.0%), 19 cows (47.5%), 12 cows (30.0%), and 1 cow (2.5%) received AI at 24 < - 30 h, 18 < - 24 h, 12 < - 18 h, 6 < - 12 h, and –6 < - 0 h before ovulation, respectively. Consequently, the majority of cows in this group, 35 cows (87.5%), received AI 6 < - 24 h before ovulation, which is the retrospectively accepted optimal time for AI, and their conception rate was high, 62.9% (22/35). Among the remaining 5 of 40 cows, 1 cow received AI –6 < - 0 h before ovulation and 2 cows received AI 24 < - 30 h before ovulation. In these 3 cows, obvious relaxation of the intravaginal part of the uterus was observed during all observations until ovulation. However, 2 of the other 5 cows did not ovulate by 48 h after AI. In these 2 cows, relaxation of the intravaginal part of the uterus was obvious during all observations, and follicular cysts was developed subsequently.

Late group: The conception rate of all 31 cows in the late group was 32.2% (10/31). In the late group, 71.0% (22/31) of the cows received AI < 6 h before ovulation, which corresponded to a time later than the retrospectively accepted optimal time for AI. However, 16.1%

Table 3. Ovulation time and corresponding number of cows in the 3 groups inseminated on the basis of the criteria for determining the timing of AI by using the estrous sign scores

Ovulation time after AI (h)	Group					
	Early (n = 29)		Optimal (n = 40)		Late (n = 31)	
48 <	3		0		0	
42 < - 48	3		0		2	
36 < - 42	3	18(62.1) a)	0	2 (5.0)	0	4(12.9)
30 < - 36	5		0		2	
24 < - 30	4		2		0	
18 < - 24	5		4		0	
12 < - 18	0	8 (27.6)	19	35 (87.5)	0	5 (16.1)
6 < - 12	3		12		5	
0 < - 6	0		0		11	
–6 < - 0	3	3 (10.3)	1	1 (2.5)	4	22 (71.0)
–12 < - –6	0		0		4	
–18 < - –12	0		0		3	
No ov b)	0		2		0	

a) % of cows to the total number of cow in the group are shown in parenthesis. b) No ovulation; follicular cyst formation.

(5/31) of the cows were inseminated at 6 < - 24 h before ovulation, which is equal to the optimal time for AI. Moreover, 12.9% (4/31) received AI > 24 h before ovulation, which corresponded to a time earlier than the optimal time for AI. Among these 9 cows, 3 of 5 cows inseminated at the optimal time for AI ovulated without exhibiting obvious relaxation of the intravaginal part of the uterus after AI, and 2 of 5 cows ovulated after a short duration of 6 h with obvious relaxation of the intravaginal part of the uterus. In addition, among the remaining 4 cows, inseminated at a time earlier than the optimal time for AI, 1 cow ovulated without exhibiting obvious relaxation of the intravaginal part of the uterus and the other 3 cows ovulated after 6–12 h with obvious relaxation of the intravaginal part of the uterus. Regarding the relationship between ovulation and conception rate, the conception rate of the cows that received AI < 6 h before ovulation, which corresponded to the late group of this study, was 27.3% (6/22). The conception rate of the cows that received AI 6 < - 24 h before ovulation, which corresponded to the optimal group of this study, was 80.0% (4/5). The conception rate of the cows that received AI > 24 h before ovulation, which corresponded to the early group of this study, was 0% (0/4).

Comparison of conception rates between the groups

A significant difference was not found in the conception rates of the optimal group (60.0%; 24/40), early group (44.8%; 13/29), and late group (32.2%; 10/31) (Table 4). However, the conception rate of the optimal group was significantly higher than the sum of the conception rates of the early and late groups (38.3%; 23/60) ($P < 0.05$).

Discussion

This study was conducted to evaluate the usefulness of estrous signs, which were postulated in our previous study, as criteria for determining the optimal time of AI [22]. These criteria consisted of 8 estrous signs: swelling of the vulva; hyperemia, swelling, and relaxation of the intravaginal part of the uterus; contraction of the uterus; diameter of the uterine horn when relaxed; opening of the external uterine orifice; and viscosity of cervical mucus. Of these estrous signs, hyperemia, swelling and relaxation of the intravaginal part of the uterus were clear in expression and change. In particular, the relaxation of the intravaginal part of the uterus was regarded as a major criterion in this study. Moreover, the sum of the scores of the other 7 estrous signs was included in the criteria. Namely, the optimal time for AI, which was retrospectively accepted to be 24-6 h before ovulation, the time earlier than the optimal time for AI (early), and the time later than the optimal time for AI (late) were determined according to the criteria, and AI was carried out at each time period. Consequently, ovulation was confirmed at expected times in 87.5, 62.1 and 71.0% in the optimal, early, and late groups, respectively. In addition, obvious relaxation of the intravaginal part of the uterus was observed in many cows in the early and late groups at the time that corresponded with the retrospectively accepted optimal time for AI. These results demonstrate that the criteria are clinically useful for determining the appropriate stage and optimal time for AI in the periovulatory period. Furthermore, the highest conception rate of 60.0% was achieved in the optimal group. This conception rate was not significant but was higher than that of the early group (44.8%) and late group (32.2%) and significantly higher than the sum of the conception rates of the early and late groups (38.3%; 23/60) ($P < 0.05$). These results demonstrate that a high conception rate could be achieved using the criteria and that the criteria are useful for determining the optimal time for AI. These results support our previous hypothesis [22] that the relaxation of the intravaginal part of the uterus could be used as a superior index to determine the optimal time for AI.

In the present study, a high conception rate of 60% was achieved in the optimal group. This conception rate was higher than the conception rates achieved in other studies: 52.0% [26], 50.9–51.1% [27], 35.2% [28], and 34.7% [29]. The conception rates of 52.0%

Table 4. Conception rate referring to ovulation after AI in the 3 groups inseminated on the basis of the criteria for determining the timing of AI by using the estrous sign scores

Ovulation time after AI (h)	Group					
	Early (n = 29)		Optimal (n = 40)		Late (n = 31)	
48 <	0	0	0	0	0	0
42 < - 48	66.7	53.3 [a)]	0	100	0	0
36 < - 42	33.3		0		0	
30 < - 36	60.0		0		0	
24 < - 30	50.0		100		0	
18 < - 24	40.0	50.0	100	62.9	0	80.0
12 < - 18	0		66.7		0	
6 < - 12	66.7		50.5		80.0	
0 < - 6	0	33.3	0	0	36.3	27.3
–6 < - 0	33.3		0		25.0	
–12 < - –6	0		0		25.0	
–18 < - –12	0		0		0	
No ov [b)]	0	0	0	0	0	0
Total	44.8 [13/29]		60.0 [24/40]		32.2 [10/31]	

a) Percentage of pregnancy. b) No ovulation; follicular cyst formation.

[26] and 50.9–51.1% [27] were achieved in studies in which AI was carried out depending on the detection of standing estrus by human observation and the use of radiotelemetric pressure sensors, respectively. The conception rates of 35.2% [28] and 34.7% [29] were achieved by using a pedometer system to detect an increase in walking activity in relation to estrus. In the present study, the use of 8 estrous signs as criteria to determine the optimal time for AI helped achieve a higher conception rate of 60% than that in the above-mentioned studies, in which AI was carried out on the basis of the detection of standing estrus and pedometric walking activity; this reveals the effectiveness of the currently proposed criteria. The results also show that the criteria are effective for achieving a high conception rate in cows kept in tie-stall barns where the detection of standing estrus is usually difficult.

In the present study, AI was sometimes carried out at different stages than those planned in 12.5% (5/40), 37.9% (11/29), and 29.0% (9/31) of cows in the optimal, early, and late groups, respectively. As mentioned above, incorrect assessments had been made for the AI stage because of individual variation in the expression and duration of the 8 estrous signs, mainly the relaxation of the intravaginal part of the uterus, which was the major criterion for determining the optimal time for AI. Some of the cows, in which relaxation of the intravaginal part of the uterus was moderate, but not obvious, were not determined to be at the optimal time for AI. However, they were at the optimal time for AI and consequently ovulated without exhibiting obvious relaxation of the intravaginal part of the uterus or immediately after the short lasting period of 6–12 h with obvious relaxation of the intravaginal part of the uterus. In addition, regarding the determination of the earlier than optimal time for AI, particularly 30 or more h before ovulation, considerable variation was seen in the appearance of these estrous signs in some cows. Therefore, in such cases, determining the stage of proestrus, estrus, and metestrus was uncertain. We indicated in our preceding study that the relaxation of the intravaginal part of the uterus was initiated from 12 h before the E_2 peak and LH surge, reached the maximum at 12 h after the E_2 peak and LH surge, and remained for 12 h and decreased before ovulation (Fig. 1) [22]. Thus, in these cows, in which the relaxation of the intravaginal part of the uterus was noted as moderate, but not obvious, are nearly around the stage of LH surge and E_2 peak (i.e., commencement of estrus) or just before ovulation. In such cases, it is recommended that AI should be conducted immediately, if the other estrous signs such as swelling of the vulva, hyperemia, and swelling of the intravaginal part of the uterus are not obvious. When swelling of the vulva, hyperemia, and swelling of the intravaginal part of the uterus are obvious, subsequent observations of estrous signs should be performed again 6–12 h later to determine the optimal time for AI. In cases in which the relaxation of the intravaginal part of the uterus has not appeared and the other estrous signs are not moderate, AI should be postponed until the optimal time. In addition, if ovulation has not occurred within 24 h after AI, AI should be performed again.

However, in 2 cows that did not ovulate within 48 h after AI, the relaxation of the intravaginal part of the uterus was obvious. In these 2 cows, the relaxation of the intravaginal part of the uterus was continuously obvious at every 6-h interval observation for 48 h after AI. Consequently, these 2 cows developed follicular cysts. The characteristic signs of swelling of the vulva and estrous signs in the intravaginal part of the uterus were also observed in these 2 cows, but contraction of the uterus and changes in the diameter of the uterine horn were not noted at the time of AI; these signs were generally noted during the periovulatory period. We have previously reported that the scores of relaxation of the intravaginal part of the uterus started to increase 12 h before the E_2 peak and reached a high level (moderate) at 6 h after the E_2 peak [22]. Maximum scores (obvious) were reached by 12 h after the E_2 peak and remained until 6 h before the subsequent ovulation (for 12 h), and then began to decrease at the subsequent ovulation. In the 2 cases of follicular cysts, the relaxation of the intravaginal part of the uterus was obvious for a long period. Therefore, in cases in which the relaxation of the intravaginal part of the uterus lasts for a long period, ovarian diseases may develop. However, further investigations are needed to confirm this, because no studies have investigated the estrous signs during the course of transition to an ovarian follicular cyst.

The present study has shown that evaluation of the relaxation of the intravaginal part of the uterus and 7 other estrous signs provides useful information to determine the optimal time for AI, and that a high conception rate can be achieved when AI is performed according to the criterion of obvious relaxation of the intravaginal part of the uterus and the presence of the other 7 estrous signs. Furthermore, the stage of periovulatory period can be determined with high accuracy using the postulated criteria. However, it is occasionally difficult to determine the stage correctly by observing the estrous signs only once. In such cases, it is recommended that the estrous signs be observed again after 6–12 h and that AI be performed at the optimal time. Assessment of ovulation is recommended 24 h after AI, and if ovulation has not occurred by then, performing AI again could improve the chances of conception.

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