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## An investigation of the time period within which frozen-thawed semen delivers a high conception rate in lactating dairy cows

Toshiaki SUMIYOSHI<sup>1, 2)</sup>, Tomomi TANAKA<sup>1, 2)</sup> and Hideo KAMOMAE<sup>1, 2)</sup>

<sup>1)</sup>Laboratory of Veterinary Reproduction, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

<sup>2)</sup>United Graduate School of Veterinary Science, Gifu University, Gifu 501-1193, Japan

**Abstract.** We determined the length of time within which frozen-thawed semen delivered a high conception rate in present-day lactating dairy cows. The cows utilized were a total 100 milking Holstein-Friesian cows kept in tie-stall style farms. We carried out artificial insemination (AI) during the periovulatory period at a predicted time based on ovulation, and checked ovulation at 6-h intervals after AI. The period from AI to ovulation ranged from 48 h (i.e., 48 h before ovulation) to -12 h (i.e., 12 h after ovulation). High conception rates averaging 63.0% were obtained by carrying out AI > 6–30 h before ovulation, significantly higher than the conception rates of 30.0% ( $P < 0.05$ ) and 26.9% ( $P < 0.01$ ) from AI carried out earlier than 30 h before ovulation and later than 6 h before ovulation, respectively. It was concluded that frozen-thawed semen delivers a conception rate of  $\geq 60\%$  for > 24–30 h after AI, and that a conception rate of  $\geq 60\%$  can be achieved by carrying out AI 6–30 h before ovulation using frozen-thawed semen.

**Key words:** Artificial insemination (AI), Conception rate, Dairy cows, Frozen semen, Ovulation

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To improve the reproductive outcome of dairy cows that continue to decreasing, reproductive technology such as synchronization of the estrous cycle and fixed-time artificial insemination (AI) protocols have been developed [1–3]. However, the reproductivity of dairy cows is still decreasing globally [4–11]. To take measures against this decreasing reproductivity in dairy cows, it is important that the detection of estrus should be accurate and that AI should be carried out at the proper time.

In relation to the optimal time for AI in dairy cows, some studies had been conducted with AI times before and after the onset of estrus or ovulation by reference to the conception rate. In these studies, Trimberger and co-workers postulated the optimal time for AI in cows, and these results are still widely accepted (the so called AM-PM guideline) [12, 13]. According to this index, the optimal time for AI is considered to be a period of 6–24 h [12, 13] or 6–28 h [14] after the onset of standing estrus. However, this index of the optimal time for AI was based on the results of studies using fresh semen. Currently, AI is primarily carried out using frozen-thawed semen, and the AI coverage rate is 95.7% (1991), including dairy cows and beef cows, in Japan [15]. In a recent study that examined the relationship of AI timing after the onset of standing estrus and the conception rate using frozen-thawed semen, it was found that when standing estrus was detected using a radiotelemetric estrus detection system, a high conception rate of 50.9–51.1% was achieved when AI was carried

out between 4 and 12 h after the onset of standing activity, which is earlier than the conventional optimal time for AI [16, 17]. However, few studies have examined the relationship between conception rate and AI time after the onset of estrus or ovulation time after AI using the current types of frozen-thawed semen.

We investigated the usefulness of the criteria of the optimal time for AI, which was determined by detailed observation of the estrous signs in the dairy lactating cows kept in tie-stall barns in our previous study [18] and found that a high conception rate of 60% was achieved in the group which was judged to be optimal for AI by the criteria [19]. In the present study, to determine a factor of the frozen semen side that participating the optimal time for AI, we investigated the relationship between ovulation time after AI and conception rate and identified the time span during which frozen semen retains high conception rate after deposition in the uterus. Our subjects were the dairy lactating cows kept in tie-stall barns that were used in our previous study [19].

The cows used in this study were kept in three farms, A, B and C. Conception rates at A, B, and C were 42.2%, 51.3%, and 50.0%, respectively. No significant difference was detected among the conception rates at the three farms.

The conception rate of all 100 cows was 47.0% (47/100) (Table 1). The highest conception rates of 66.7% were achieved when AI was carried out > 24–30 h and > 18–24 h before ovulation. In addition, high conception rates of > 60%, specifically 63.2% (12/19) and 60.6% (12/20), were achieved with AI was carried out > 12–18 h and > 6–12 h before ovulation, respectively. The conception rate was 63.0% (34/54) for AI carried out 6–30 h before ovulation. By comparison of conception rates, the conception rate was 63.0% (34/54) when AI was carried out > 6–30 h before ovulation, which was significantly higher than the conception rates of 30.0% (6/20) for AI carried out earlier than 30 h before ovulation and 26.9%

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Correspondence: H Kamomae (e-mail: Kamomae@cc.tuat.ac.jp)

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**Table 1.** Time interval from artificial insemination (AI) to ovulation and conception rate

Interval from AI to ovulation (h)	Number of cows	Conception rate (%)
> 48	5	0 (0/5) <sup>b</sup>
> 42–48	5	40.0 (2/5)
> 36–42	3	33.3 (1/3)
<hr/>		
> 30–36	7	42.9 (3/7)
> 24–30	6	66.7 (4/6)
> 18–24	9	66.7 (6/9)
> 12–18	19	63.2 (12/19)
> 6–12	20	60.0 (12/20)
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> 0–6	11	36.4 (4/11)
> –6 <sup>a</sup> –0	8	25.0 (2/8)
> –12––6	4	25.0 (1/4)
> –18––12	3	0 (0/3)
Total	100	47.0 (47/100)

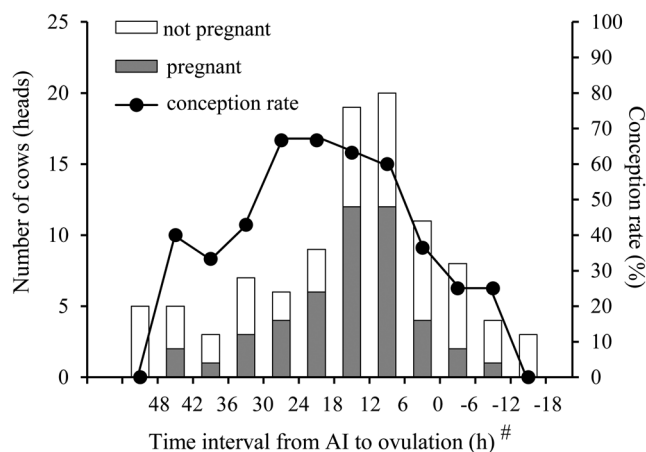
<sup>a</sup>) > –6 indicates more than 6 h after ovulation. <sup>b</sup>) The values in brackets indicates number of pregnant cows/number of inseminated cows are shown. The gray zone represents the generally accepted optimal time for AI. \* Significant differences ( $P < 0.05$ ) between the groups. \*\* Significant differences ( $P < 0.01$ ) between the groups.

(7/26) for AI carried out later than 6 h before ovulation ( $P < 0.05$  and  $P < 0.01$ , respectively). This result showed that, frozen-thawed semen possessed a high conception rate of 60% for 30 h after AI during the periovulatory period in cows (Fig. 1). This is equal to or slightly longer than the generally accepted time of 24–30 h [12, 13, 20], when fresh semen is used.

In view of the fact that, when AI was carried out 6–30 h before ovulation, frozen-thawed semen delivered its conception rate of  $\geq 60\%$ , this appears to be the optimal time for AI, when frozen-thawed semen is used. This time period is also longer than the time period of 6–24 h after the onset of estrus [12, 13], (which corresponds to 6–24 h before ovulation [21]), which was determined using fresh semen and retrospectively accepted as the optimal time for AI. Furthermore, our results confirm reports that a once-daily fixed time for AI, regardless of estrus confirmation, can be used as effectively as the guideline for the timing of AI because there is no difference in conception rates [22, 23]. In addition, this supports research in which a high conception rate of about 50% was achieved when the onset of estrus was determined precisely using an estrus detection system, and AI was performed with frozen-thawed semen between 4 and 12 h after the onset of estrus [16, 17].

From these findings, the time period in which a conception rate of  $\geq 60\%$  has been achieved in the present study is equal to the time period from just at the onset of estrus to 24 h after onset, which is equal to the time period in estrus [21, 24]. This means that the time period in estrus, the condition in which the female to permit mating by a bull, will be the optimal time for conception, and this is recognized as the reproductive mechanism of the animal.

From these results, it appears that the frozen-thawed semen delivers its conception ability as long as fresh semen. It is reasonable to conclude from this study that frozen-thawed semen has a conception



**Fig. 1.** Time intervals from artificial insemination (AI) to ovulation and subsequent conception rates. Columns represent the total number of cows inseminated and shaded columns represent the number of cows pregnant. Sequential line graph shows conception rates. # indicates the time after ovulation.

ability of  $\geq 60\%$  for 30 h, and a conception rate of  $\geq 60\%$  is achieved when AI was carried out 6–30 h before ovulation. In addition, to obtain a high conception rate using AI, it is necessary to confirm ovulation and to identify whether AI was carried out at the optimal time. The result of the present study shows that 30 h after AI is an appropriate time to check for ovulation. If ovulation has not occurred, an additional AI may be required to improve the conception rate.

## Methods

### Animals

The cows used in this study comprised 100 clinically healthy Holstein-Friesian dairy cows (45 at Farm A, 39 at Farm B, and 16 at Farm C) that were kept in tie-stall style barns in Kanagawa prefecture, Japan, between March 2012 and October 2013. Age (year), parity (time), and days after parturition (day) were  $4.2 \pm 1.7$ ,  $2.2 \pm 1.3$ , and  $201.4 \pm 119.9$  (mean  $\pm$  SD), respectively. The body condition score was  $2.5 \pm 0.3$ , based on a five-point scale [25]. The cows were milked twice a day, and the average milk yield per lactation (305 days) was around 9,000 kg per cow on all three farms. The cows were fed a total mixed ration (TMR), or dry hay and concentrate supplements separately, in accordance with the Japanese feeding standards for dairy cattle (2006). These cows were more than 60 days postpartum and had no genital abnormalities as confirmed by rectal and vaginoscopic examinations and transrectal ultrasonography. 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).

### Observation of estrous signs for AI

In conformity with the farmers' request for AI, depending on the appearance of estrous signs or the predicted day of estrus from the last AI and last post-estral bleeding, and through diagnosis for being in estrus or peri-ovulatory period by postpartum periodical inspection

of genitalia, estrous signs were checked as described below, and AI was performed only once. The cows in which estrus was induced by hormonal treatment such as PGF<sub>2</sub>α were not included in this study. Determination of the timing of AI used eight 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 the cervical mucus, contraction of the uterus, and diameter of the uterine horn when relaxed, which were observed and evaluated in accordance with the results of previous studies [18, 19]. The examination procedure for estrous signs were as follows. First, the cow was checked for, swelling of the vulva. Then, after cleaning and sterilizing the vulva with disinfectant, a vaginoscope, sterilized by soaking in antiseptic solution, was inserted into the vagina, and the following estrous signs were checked: opening of the external uterine orifice, hyperemia, swelling, and relaxation of the intravaginal part of the uterus. After that, 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 transrectal ultrasonography were carried out to evaluate the diameter of the uterine horn, contraction of the uterus, and the size and feel of the follicles and corpus luteum. In the present study, 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.

#### AI procedure

As shown in the previous study [18], criteria for determining an optimal time for AI, a time earlier than the optimal time for AI, and a time later than the optimal time for AI were established from estrous signs based on our previous study [19]. According to the criteria, AI was performed at the time determined to be earlier than the optimal time for AI in 29 cows, the optimal time for AI in 40 cows, and at a time later than the optimal time for AI in 31 cows. Using the criteria, the optimal time for AI was defined as 6–24 h after the onset of estrus, which was accepted retrospectively [12, 13]. This time period corresponds to 24–6 h before ovulation, because ovulation occurs about 30 h after the onset of estrus [21].

AI was carried out using the recto-vaginal method only once. Commercially available frozen semen that contained about  $30 \times 10^6$  sperm in 0.5 ml straws was used. The frozen semen was thawed by plunging the straw into lukewarm water at 38°C for 15 sec. The AI procedure was as follows. Firstly, the vulva was cleaned with water and then sterilized by washing with an antiseptic solution. Secondly, a vaginoscope, sterilized by dipping into antiseptic solution, was inserted into the vagina, and opened. Thirdly, the sterilized instrument for AI (semen injector) was inserted into the vagina, and the tip of the semen injector was put into the external uterine orifice. Fourthly, after removal of the vaginoscope, keeping the semen injector in position, 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 into the rectum. Then, semen was deposited into the uterine cavity when the tip of the semen injector reached 1–2 cm inside the cavity of the uterine body.

#### Confirmation of ovulation

After AI, checking for ovulation was performed at 6-h intervals. The confirmation of ovulation was performed by confirming a marked depression with palpation or a clear disappearance of the follicle at the presenting site of the follicle, or a clear decrease in follicle diameter due to the disappearance of follicular fluid by rectal examination and transrectal ultrasonography (Supplementary Fig. 1: online only). When ovulation was not confirmed until 48 h after AI, further examinations for ovulation were not performed.

#### Time from AI to ovulation

The time from AI to ovulation ranged from 48 h before ovulation to 18 h after ovulation. The classification of time from AI to ovulation is as follows and shown in Table 1: > 6–12 means ovulation was not confirmed in the first examination at 6 h after AI, but was ovulated in the second examination of another 6 h later. > –6– 0 means that AI was postponed as a result of the examination and AI was performed on those that had been ovulated as a result of the examination performed 6 h later. Similarly, > –12– –6 means that the ovulation was confirmed in the second examination, but AI was postponed another 6 h. As the results present study includes 15 cows that were inseminated after ovulation.

#### Examination of pregnancy

Pregnancy examinations were performed 60 days post insemination by confirming the increased diameter and fluctuating feel of the gravid uterine horn, presence of a functional corpus luteum in the ovary on the same side as the gravid uterine horn, slipping of the fetal membrane in the non-gravid uterine horn by rectal examination, and confirming fetal fluids, fetus, and fetal heart beats by transrectal ultrasonography.

#### Statistical analysis

All data were analyzed using the Statcel 4 (OMS Publishing, Tokorozawa, Japan). Age, parity, days after parturition, and BCS data were presented as mean and SD (Mean ± SD). Differences in conception rate were analyzed using Fisher's exact test and the Bonferroni / Dunn procedure. Differences were considered to be significant at  $P < 0.05$ .

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