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Original Article

No adverse effect of confirmation of ovulation by rectal palpation and ultrasonography after artificial insemination on formation, development, and function of the corpus luteum and conception rate in cows

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Abstract. The effect of confirmation of ovulation by rectal palpation and ultrasonography after artificial insemination (AI) on the development of the corpus luteum (CL) and conception rate was investigated in cows. A total of 90 clinically healthy Holstein-Friesian dairy cows were examined in this study. After AI, the cows were divided into three groups (30 cows per group). In Group I, ovulation was confirmed by rectal palpation at 24 h after AI. In Group II, ovulation was confirmed using transrectal ultrasonography 24 h after AI. In Group III, ovulation was not confirmed after AI. Day 0 was defined as the day when ovulation was confirmed in Groups I and II, and as the day after AI was performed in group III. Transrectal ultrasonography was performed on days 3, 5, 7, and 14 to measure the CL diameter, tissue area, and CL blood flow area, and the ratio of CL blood flow area to CL tissue area was calculated. On the day of CL measurement, blood samples were collected to determine the plasma concentrations of progesterone (P_4) and estradiol-17β (E_2). Pregnancy was diagnosed at 28 and 60 days after AI. A high conception rate of approximately 80% was achieved in Groups I and II, in which confirmation of ovulation was conducted. There were no differences in the diameter, tissue area, or blood flow area of the CL between the three groups. These results indicate that the confirmation of ovulation by rectal palpation and transrectal ultrasonography did not affect the formation and function of the CL or conception rate.

Key words: Artificial insemination (AI), Conception rate, Confirmation of ovulation, Cow, Development of corpus luteum (CL)

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In recent years, a worldwide decline in dairy cow reproductivity has become a problem [1–8]. Various factors have been considered as the cause or etiology of this decrease in reproductivity, and the main factors suspected are that estrus and estrous signs have weakened and the duration of estrus has shortened in dairy cows [9, 10]. Indistinct estrus and estrous signs, as well as a shortened duration of estrus, might lead to missed or poorly timed artificial insemination (AI). The easiest and most effective way to confirm whether AI was performed at the optimal time is to confirm ovulation by rectal palpation or transrectal ultrasonography. However, in clinical practice, confirmation of ovulation has not been sufficiently carried out because of concerns about adverse effects on fertility and the labor required to visit farmers.

The optimal time for AI that is generally accepted in dairy cows is based on the confirmation of the state of standing estrus and is considered to be a time span of 6–24 h after the onset of standing estrus [11, 12]. According to subsequent studies conducted 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 estrus, which is earlier than the traditional optimal time for AI [13, 14]. Even with the results of more recent studies using automated activity monitoring systems (AAM), the optimal interval from the onset of estrus based on AAM to AI is 5 to 17 h [15], which is similarly shorter than the traditional optimal time for AI. In recent years, synchronization of estrous and fixed-time AI protocols has been developed and has become more popular in dairy cows [16–18]. However, this requires labor and hormonal drug costs, and is not a breeding technology that can be applied immediately when requesting AI from farmers. Therefore, it is necessary to determine the optimal time for AI and confirm the ovulation to determine whether the timing of AI is appropriate. On the other hand, in cows for which standing estrus cannot be observed, AI should be performed at the assumed optimal time based on observations of estrous signs, such as changes in behavior, hyperemia and swelling of the vulva, mucus discharge from the vulva, follicle and regressed corpus luteum (CL) findings of the ovary, and contraction of the uterus by rectal examination and transrectal ultrasonography. In particular, for dairy cows kept in tie-stall barns, we demonstrated that the optimal time for AI could be determined using the external and internal estrous signs [19, 20]. However, the timing of AI is often inappropriate timing because of weakened and indistinct estrous signs, as described above. In such cases, it is necessary to confirm the ovulation within the period during which semen retains sufficient conception ability to be confident that AI has been performed at a suitable time to improvement of the conception rate. At present, it is generally recommended to confirm ovulation approximately 24 h after AI, which is considered to be the optimal time for insemination of dairy cows, as mentioned above, and the lifespan of the functionally viable sperm [21], if ovulation has not occurred, AI may have been performed earlier than the optimal time for AI in which case, the conception rate decreases. In this case, supplementary second AI at the optimal time may increase the conception rate [22]. After ovulation, the CL develops by hypertrophy and luteinization of the

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granulosa cells lining the follicle. Enlargement is rapid, and 48 h after ovulation, it attains a diameter of approximately 1.4 cm. At this stage, the developing CL is soft and yields upon palpation [18]. However, it has been suggested that rough and careless confirmation of ovulation by rectal palpation causes exfoliation of granulosa cells from the dominant follicle and hinders the development or formation of the CL, resulting in lower CL function and infertility [23]. However, there are no reports investigating in detail whether the confirmation of ovulation affects the development or formation of the CL and conception rate. Therefore, the objectives of this present study were to investigate the effect of confirmation of ovulation by rectal palpation and ultrasonography after AI on subsequent CL development formation, and conception rate.

Materials and Methods

Animals

This study was conducted in a total of 90 clinically healthy Holstein-Friesian dairy cows, of which 52 cows were kept in Farm A and 38 cows were kept in Farm B in tie-stall style barns in Kanagawa prefecture in Japan for 32 months, between October 2014 and October 2017. These cows were determined to be in the peri-ovulatory period based on estrous signs observed at the time of postpartum periodical inspection of the genitalia or the farmer's request for AI owing to the detection of estrous signs. In addition, these cows were more than 60 days postpartum and had no genital abnormalities on rectal and vaginoscopic examinations or transrectal ultrasonography. At the time of the study, age, parity, and days after parturition of the cows were 4.1 ± 1.9 (mean ± SD) years, 2.3 ± 1.5 times, and 155.7 ± 74.4 days, respectively. The body condition score (BCS) of the cows was 2.5 ± 0.3, based on a 5-point scale [24]. The cows were milked twice daily, and the average milk yield per lactation (305 days) was approximately 9,000 kg per cow from both the farms. The cows were fed a total mixed ration (TMR) in accordance with the Japanese feeding standards for dairy cattle (2006). This study was suspended for 3 months in July, August, and September, when high temperatures might have influenced the conception rate. All experimental procedures were approved by the University Committee for the Use and Care of Animals of the Tokyo University of Agriculture and Technology (No. 25-36).

AI procedure

AI was performed at the optimal time based on the examination of the estrous signs (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), size and consistency of the follicles and CL by rectal palpation, and ultrasonography by an experienced veterinarian [19, 20]. In accordance with a previous study [20], the observed estrous signs were classified into three categories. The classified estrous signs were assigned 0, 1, or 2 points depending on their obviousness, and the estrous signs were assigned a total score based on the given points. Based on a previous study [20], the criteria for determining the optimal time for AI were defined as relaxation of the intravaginal part of the uterus was obvious and the sum of the other 7 estrous signs ranged from 5 to 10 points for the 8 estrous signs examined. Observations with transrectal ultrasonography were performed using a B-mode Color Doppler ultrasound scanner (S6V; Sono Scape, Shenzhen, China) equipped with a 5.0–10.0 MHz linear array probe. AI was carried out using the recto-vaginal method, and commercially available frozen semen containing approximately 30×10^6 sperms in 0.5 ml straws was used. The semen was thawed by plunging the straw into lukewarm water at 38ºC for 15 sec. After thawing, the straw was placed into the instrument for AI (semen injector) (A1-450; FUJIHIRA INDUSTRY CO., LTD., Tokyo, Japan) as soon as possible. The AI procedure was performed as follows. First, the vulva was cleaned with water and sterilized using an antiseptic solution. Second, a sterilized vaginoscope was inserted into the vagina and opened, and then the instrument for AI was inserted into the vagina to avoid contamination, and the tip of the semen injector was placed into the external uterine orifice. Third, 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. Semen was deposited into the uterine cavity when the tip of the semen injector reached 1–2 cm inside the uterine body. All AI procedures, observation, and classification of the estrous signs were performed by one veterinarian, who is the first author with approximately 15 years of experience.

Experimental group

The cows were randomly divided into three groups by block randomization, with 6 cows in one block and each group consisting of 30 cows. The number of cows in Farm A and B was 16 and 12 in Group I, 18 and 11 cows in Group II, and 18 and 15 cows in Group III, respectively. There were no significant differences in age, parity, days after parturition, or BCS among the cows in the three groups. In Group I, ovulation was confirmed by rectal palpation 24 h after AI. If ovulation did not occur, an additional AI was performed. Ovulation was confirmed again by rectal palpation 24 h after AI. In Group II, ovulation was confirmed using transrectal ultrasonography without palpating or touching the ovaries 24 h after AI. If ovulation was not assured, a subsequent AI was performed, and 24 h later, ovulation was confirmed again using transrectal ultrasonography. In Group III, ovulation was not confirmed after AI. Three cows in Groups I and II received the second insemination. In addition, there were 3 cows in Group I and 4 cows in Group II, in which ovulation could not be confirmed, or was recognized, but without the development or formation of a CL. Similarly, no CL formation was observed on day 3 in the 4 cows in Group III. These cows were excluded from the analysis. The day on which ovulation was confirmed was designated as day 0 in Groups I and II. In Group III, considering the lifespan of the functionally viable sperm (approximately 24 h) [13] and the optimal time for AI (6–24 h after the onset of estrus, which corresponds to 6–24 h before ovulation, because ovulation occurs approximately 30 h after the onset of estrus) [11, 12, 25], if AI is performed at an optimal time, ovulation should occur 24 h after AI; therefore the day after AI was set as day 0. Rectal palpation and transrectal Color Doppler ultrasonography of the ovaries were performed on days 3, 5, 7, and 14 to examine the condition of the CL and the follicles. The diameter, tissue area, and blood flow area of the CL were measured, and the ratio of the blood flow area to the tissue area was calculated. For the measurement of the tissue area and diameter of the CL, three different recorded images that maximized the cross-sectional area of the CL, and the mean value of the major and minor axes was calculated as the diameter, and the mean value of the three images was adopted as the diameter (mm) and area (mm^2) using Image J (version 1.51, the US National Institutes of Health, USA). Similarly, for the blood flow area of the CL, imaging was switched to color flow Doppler mode, which maximized the

cross-sectional area of the CL and depicted the luteal blood flow area. Three images were recorded and the areas were calculated. The mean value of the area was considered as the CL blood flow area (mm^2). The settings for ultrasonography imaging (B-mode frequency: 7.0 MHz; B-mode gain value: 47; color flow Doppler frequency: 6.0 MHz; pulse repetition frequency: 1.0 MHz) were standardized for all examinations. In addition, blood samples were collected by coccygeal venipuncture into heparinized vacutainers (Venoject II, Terumo, Tokyo, Japan) along with rectal palpation or transrectal ultrasonography on days 3, 5, 7, and 14 to analyze the plasma concentrations of progesterone (P_4) and estradiol-17β (E_2).

Confirmation of ovulation

Ovulation was confirmed by the following findings; marked depression on palpation or a clear disappearance of the follicle at its presenting site, or a clear decrease in the follicle diameter due to the disappearance of follicular fluid as determined by rectal palpation or transrectal ultrasonography [20, 22]. The ovaries were not palpated when ovulation was confirmed by transrectal ultrasonography. All ovulations were confirmed by a veterinarian who is the first author with approximately 15 years of experience.

Pregnancy diagnosis and conception rate

Early pregnancy diagnosis was performed 28 days after AI for early detection of non-pregnant cows and the confirmative diagnosis was carried out 60 days after AI. Early pregnancy diagnosis was confirmed by the presence of a fetal sac, embryo, and embryo heartbeat using transrectal ultrasonography. The Second definitive pregnancy diagnosis was confirmed by the presence of enlarged uterine horn with fetal fluids, existence of a functional CL in the ovary, and fetal membranes using the slipping method by rectal palpation. In addition, the placental fluid, fetus, and fetal heartbeat were observed using transrectal ultrasonography. The conception rate reflected the results of the confirmative diagnosis performed 60 days after AI and was expressed as the ratio of the number of conceived cows among the experimental cows (24, 23, and 26 cows in Groups I, II, and III, respectively).

Hormone assays

The plasma concentration of P_4 was measured using an enzyme immunoassay according to the method described previously [26]. The assay was performed using rabbit anti-progesterone antibody (7720-0496, UCB Bioproducts, Brussels, Belgium) and anti-rabbit goat IgG antibody (111-005-003, Jackson Immuno Research, West Grove, PA, USA). Progesterone (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard. The intra- and inter-assay coefficients of variation were 13.8% and 12.9%, respectively, and sensitivity was 0.2 ng/ml. Plasma concentration of E_2 was measured using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). The intra- and inter-assay coefficients of variation were 10.2% and 13.8%, respectively, and sensitivity was 0.98 pg/ml.

Statistical analysis

All data were analyzed using the Statcel 4 software (OMS Publishing, Tokorozawa, Japan). A total of 6 cows in Group I, 7cows in Group II, and 4 cows in Group III were excluded from the analyses because they received the second insemination (3 cows in Group I and 3cows in Group II), ovulation was not confirmed or was recognized, but without the development or formation of a CL (3 cows in Group I, 4 cows in Group II), and formation of CL was not observed on day 3 (4 cows in Group III). All the experimental data, except the conception rate, which was calculated overall, are presented as the mean and SD (mean ± SD). The statistical differences in age, parity, days after parturition, and BCS between the 2 farms were determined using the chi-square test for goodness of fit and two-sided Mann-Whitney *U* test. Differences in conception rates between the 2 farms were analyzed using the Fisher's exact test. Bartlett's test, one-way ANOVA, and the Tukey-Kramer test were used to compare age, parity, days after parturition, BCS, conception rate, diameter, tissue area, blood flow area, the ratio of the blood flow area to the tissue area of the CL, and plasma concentrations of P_4 and E_2 among the three groups. Differences were considered statistically significant at $P < 0.05$.

Results

Comparison of the cows raised in the 2 farms and 3 groups

The conception rates of the cows in farm A and B were 61.5% (32/52) and 63.2% (24/38), respectively. There were no significant differences in conception rate, average age, parity, days after parturition, and BCS between the cows of the 2 farms and no significant difference in average age, parity, days after parturition, and BCS among the cows of the 3 groups.

Diameter, tissue area, blood flow area and ratio of blood flow area to tissue area of the CL

For the CL, the diameter and tissue area increased from days 3 to 7 to the maximum and maintained their maximum values until day 14 (Fig. 1). The blood flow area increased with advancing days after ovulation in all the three groups and reached a maximum on day 14 (Fig. 1). No significant differences were observed among the three groups in terms of diameter, tissue area, and blood flow area of the CL. The ratio of the blood flow area to the tissue area of the CL was significantly lower in Group I than in Group III on day 7 ($P < 0.05$); however, no significant difference was observed among the three groups on other days (Fig. 1).

Plasma concentration of P_4 and E_2

The plasma concentration of P_4 was significantly higher in Group II than in Groups I ($P < 0.05$) and III ($P < 0.01$) on day 5. No differences were observed among the groups on the other days (Fig. 2). There was no difference in the plasma concentration of E_2 among the three groups at any time point (Fig. 2).

Conception rate

The conception rates in Groups I and II were 79.2% (19/24) and 82.6% (19/23), respectively (Fig. 3). The conception rate in Group III was 50.0% (13/26). The conception rates of Groups I and II were non-inferior to those of Group III. The conception rate in Group II was significantly higher than that in Group III ($P < 0.05$). Three cows in each of the Groups I and II that received the second insemination were excluded from the conception rate data, and the conception rates of these cows in both the Groups I and II were 66.7% (2/3).

Discussion

In this study, the conception rate in Group II was significantly higher than that of Group III ($P < 0.05$). However, there were no significant differences in the conception rates among the other groups. Compared with the conception rate of 50.0% in Group III, in which ovulation confirmation was not conducted after AI, conception rates of approximately 80% were not inferior in Groups I and II, in

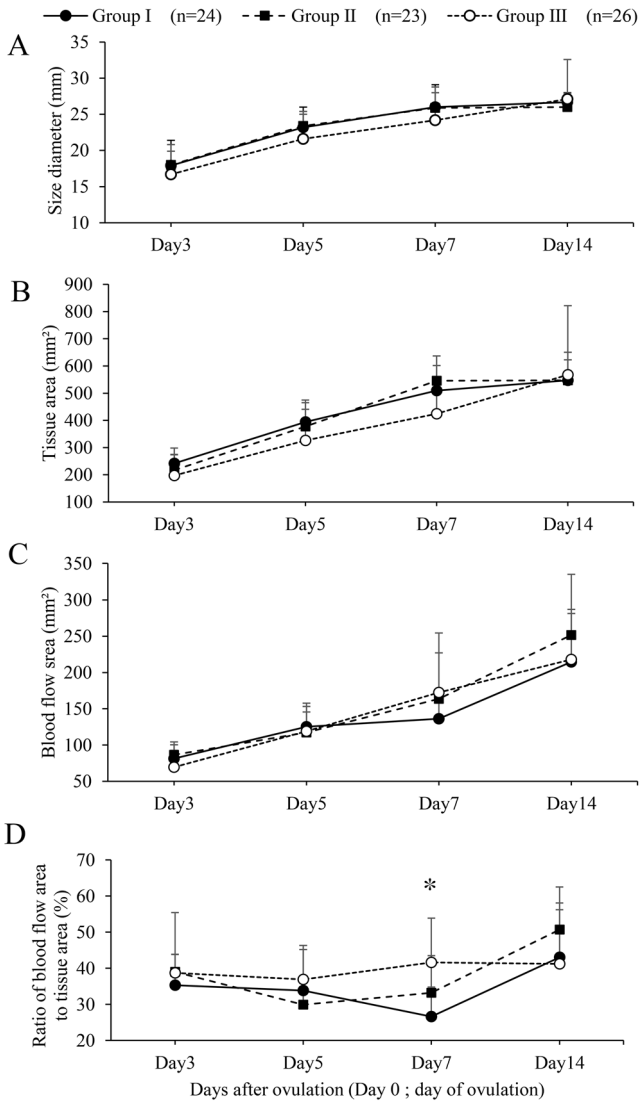

Fig. 1. Diameter (A), tissue area (B), blood flow area (C), and ratio of blood flow area to tissue area (D) of the CL in Groups I, II and III. * Significantly different between Group I and Group III ($P < 0.05$).

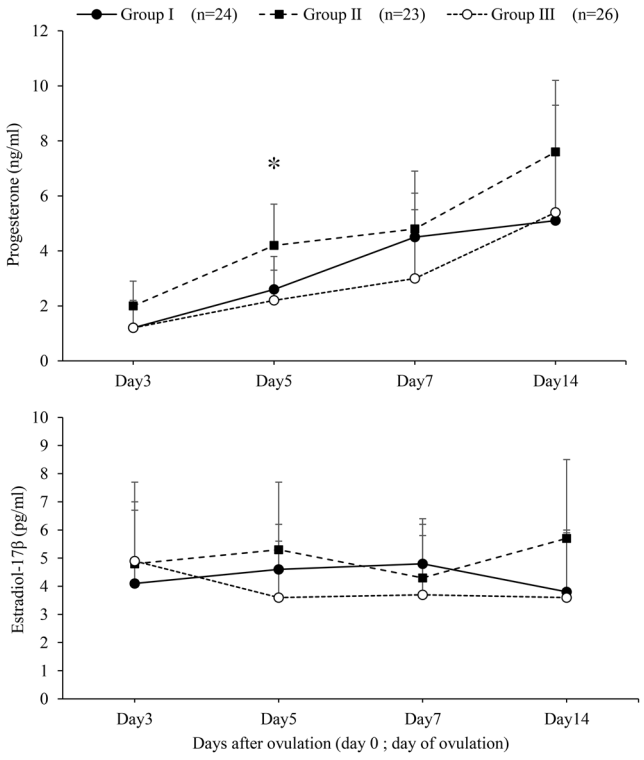

Fig. 2. Plasma concentrations of progesterone and estradiol-17β in Groups I, II and III. * Significantly different between Group II and Group I ($P < 0.05$) and between Group II and Group III ($P < 0.01$).

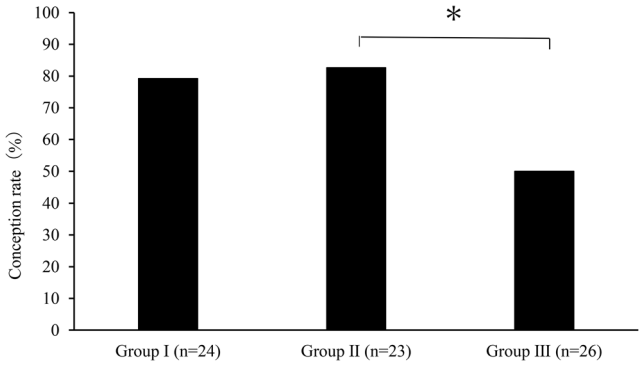

Fig. 3. Conception rates of Groups I, II and III. * Significantly different between Group I and Group II ($P < 0.05$). Group I: Ovulation was confirmed by rectal palpation at 24 h after AI. Group II: Ovulation was confirmed by transrectal ultrasonography at 24 h after AI. Group III: No confirmation of ovulation was conducted.

which ovulation was confirmed using rectal palpation and transrectal ultrasonography, respectively. These results indicate that ovulation confirmation by rectal palpation and transrectal ultrasonography did not negatively affect the conception rate. In addition, there were 3 cows in in Groups I and II that received the subsequent second AI, and the conception rates of these cows in Groups I and II were both 66.7% (2/3). These data were excluded from the conception rate because they may have been affected by the first AI; however, it is possible that they would not have conceived without confirmation of ovulation and received the subsequent second AI. Therefore, in Group III, if ovulation was confirmed or if ovulation was not assured, subsequent second AI was carried out, the conception rate may have further increased. These results suggest that a high conception rate may be achieved using subsequent supplemental secondary AI in cases where ovulation is not confirmed 24 h after AI.

Moreover, there was no significant difference in the diameter, tissue area, or blood flow area of the CL among the three groups. In this study, the ratio of luteal blood flow area to luteal area was calculated to evaluate the luteal blood flow area. There was a significant difference between Groups I and III in the ratio of the blood flow area to the tissue area of the CL only on day 7 ($P < 0.05$); however, there was no difference among the groups on day 7 in the diameter, tissue area, or blood flow area of the CL. Therefore, it is unclear whether this difference was due to the method used to confirm ovulation.

Regarding the plasma concentration of P_4, there were some significant differences among the groups on day 5 during the early stages of CL. Regarding the significant differences in this study, as ovulation was confirmed 24 h after AI, it is possible that the ovulation time differed by nearly 1 day depending on the cow. Thus, in the early stages of CL, this difference of nearly 1 day in confirmation

of ovulation was reflected in the P_4 secretory capacity of the CL. Therefore, it was presumed that there was a significant difference between Group II and Groups I and III on day 5.

It has been reported that heifers that underwent removal of granulosa cells from pre-ovulatory follicles had lower concentrations of plasma progesterone than cows that had not undergone removal of granulosa cells [27]. In addition, confirmation of ovulation by rectal palpation might cause exfoliation of the granulosa cells of the follicle after ovulation, which might interfere with the development and formation of the CL and lead to the deterioration of CL function, resulting in infertility [23]. Therefore, in the practical field artificial inseminators and veterinarians tend to avoid confirmation of ovulation by rectal palpation. However, our results clearly indicate that the confirmation of ovulation by rectal palpation or transrectal ultrasonography did not negatively affect the subsequent development and formation of the CL or conception rate.

Additionally, it was also indicated that insemination at an optimal time is important for obtaining a high conception rate. At present, to determine whether the timing of AI is appropriate, there is no alternative to confirming ovulation 24 h after AI, especially for cows kept in tie-stall barns as standing estrus may not be detectable. In this study, the optimal time for AI was carefully determined in accordance with previously reported estrous signs scores [19, 20]. However, in 3, 4, and 4 cows in Groups I, II, and III, respectively, ovulation, development, or formation of the CL were not verified after AI. Therefore, it is important to check ovulation to obtain information on cows that have received AI with a normal estrous cycle.

Furthermore, there are concerns about artificial ovulation or rupture of follicles when ovulation is confirmed by rectal palpation. The follicle that approaches ovulation increases in volume and the apex of the follicle wall bulges outward to form an avascular area. The tissue in this part is very thin, and eventually the follicle ruptures owing to the stress of even a small intrafollicular pressure, leading to ovulation [28]. Therefore, palpation of the follicle immediately before ovulation may cause it to rupture and be artificially ovulated. There are no reports on the effect of this artificial ovulation or rupture of follicles on the development and formation of the CL and the conception rate. Immediately before ovulation, the oocyte is surrounded by cumulus cells released from the cumulus oophorus, and floats in follicular fluid [28]. Therefore, if the oocyte is captured by the ovarian fimbria transferred to the abdominal cavity of the oviduct, and taken up into the ampulla of the oviduct, there is a possibility of conception after artificial ovulation or rupture of follicles. However, if a follicle that takes a long time to ovulate is artificially ovulated or ruptured, the oocyte will not be released or transferred, which may lead to infertility. In addition, rough and careless palpation at such times may damage the oviduct and ampulla of the oviduct, causing infertility; therefore, it should be strictly avoided.

In clinical practice, artificial inseminators and veterinarians should be concerned about the time of AI is suitable or not. In such a case, it is recommended to confirm ovulation 24 h after AI and performed a subsequent second AI, as necessary. On the other hand, in this study, a subsequent second AI was also carried out as a result of confirmation of ovulation, but in a certain number of cows in which ovulation was not confirmed or was recognized, no development or formation of a CL was observed afterwards. If it is possible in clinical practice to identify in advance the cows that do not ovulate or do not form the CL even after ovulation before AI or before the subsequent second AI, it may help determine whether AI is not performed or ovulation synchronization procedures should be performed, and thus, the conception rate may be further improved. Further studies are required to determine whether additional AI should be performed if ovulation is not observed.

Conflict of interests: None of the authors have any conflict of interest to declare.

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