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Abstract

The size and location of the corpus luteum and the presence of coexistent follicles are crucial factors in synchronizing recipients and determining the suitability for embryo transfer. However, there has been a recent decline in conception rates after embryo transfer, which is attributed to environmental pollution, uterine inflammation, ovarian cysts, and other factors. Therefore, we conducted experiments to establish a novel criterion for successful embryo transfer assessment. To assess the suitability for embryo transfer one day before transfer, we conducted ultrasound examinations equipped with a vaginal probe to evaluate the corpus luteum and coexistent follicle. We found that instances with corpus luteum and coexistent follicles (diameter: >10 mm) constituted the majority (69.7%) of cases. When comparing the fertility rates of cases in which the corpus luteum and coexistent follicle (diameter: >10 mm) were located on the same ovary and cases in which they were not, higher fertility rates were observed when the corpus luteum and coexistent follicle (diameter: >10mm) were on different ovaries. Our study revealed a high incidence of corpus luteum and coexistent follicles with a diameter exceeding 10 mm. Therefore, our findings suggest that the co-occurrence of the corpus luteum and a large follicle can serve as a new standard for the evaluation of embryo transfer suitability.

Keywords: corpus luteum, coexistent follicle, embryo transfer, conception rate, Hanwoo

Introduction

Embryo transfer has been a long-standing practice in both humans and livestock, including cows, pigs, and sheep. This technique is used in various fields, including biotechnology research, breeding improvement, preservation of genetic resources, and infertility resolution (1-3). In the case of cows, this technique is instrumental in producing offspring with exceptional genetic traits. Embryos are generated using superovulation methods, ovum pick-up (OPU), and ovaries obtained from slaughtered animals, which are then transplanted into recipient cows (3-5). Additionally, various synchronization methods, primarily centered around ovum synchronization, are employed to transfer embryos into multiple recipients simultaneously in cows (6-10).

To ensure the success of embryo transfer, synchronizing the recipients and determining the presence or absence of the corpus luteum is crucial, which is typically accomplished through rectal palpation or ultrasound examination (6, 8, 11, 12). Furthermore, even in instances of synchronized estrus, various factors can impede recipient ovulation, including physiological irregularities, ovarian cysts, and endocrine inflammation (11, 13, 14). Additionally, research indicates that the highest conception rates are achieved when embryos are transferred into the uterine angle where the corpus luteum is present (15-17). Therefore, evaluating the presence, location, and size of the corpus luteum prior to embryo transfer is closely related to the pregnancy rate (11, 12, 18).

Recent studies have demonstrated that the diameter of the corpus luteum and the coexistent follicle also play a significant role in affecting conception rates before embryo transfer (15, 18). Specifically, the size of the corpus luteum exhibits a positive correlation with fertility rates. Conversely, the size of coexistent follicles has a negative correlation with conception rates (12, 15, 18, 19).

Furthermore, research has indicated that conception rates are influenced by the content and ratio of the reproductive hormones progesterone and estrogen (12, 15, 18, 19). Progesterone is a hormone critical for maintaining pregnancy, whereas estrogen positively influences follicle development. Therefore, higher progesterone levels, lower estrogen levels, and a higher progesterone-to-estrogen ratio are associated with relatively higher fertility rates (12, 15, 18, 19).

Given that estrogen is derived from follicles, coexistent follicles must be absent or small in size to ensure successful embryo transfer (15, 18).

Nevertheless, to the best of our knowledge, no previous studies have comprehensively compared and analyzed conception rates, progesterone, and estrogen concentrations when the corpus luteum and coexistent follicles are present on the same ovary compared to when they are located differently. Therefore, our study sought to compare the presence, size, and location of the corpus luteum and coexistent follicles in the context of the embryo transfer synchronization method and their impact on pregnancy rates. Additionally, we analyzed conception rates when the corpus luteum and coexistent follicle are on the same ovary versus when they are not, and compared the influence of progesterone and estrogen levels on fertility rates.

Materials and Methods

Animals and Management

A total of 145 cows were employed in this experiment. The cows were reared at the Gyeongsangbuk-do Livestock Research Institute in accordance with the Hanwoo Korean Feeding Standard, and they were housed in a well-equipped space that provided ample room (300 m^2 for 15 cows) and stanchions. All experimental procedures involved in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Gyeongsangbuk-do Livestock Research Institute.

Experimental Design

Cows were synchronized using the E2/P4 (7), 2FTET (7), and J-synch (9) methods for embryo transfer. Detailed methods can be found in the related literature, as well as in Figure 1. The experiment included 40 cows subjected to the E2/P4 method, 73 cows in the 2FTET method, and 32 cows in the J-synch method (Fig. 1).

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1. E2/P4 method

For the E2/P4 synchronization method, a 2 mg intramuscular (i.m.) injection of estradiol benzoate (EB) (Samyang Anipharm Co., South Korea) was administered on day 0, along with the insertion of a 1.56 g progesterone-releasing device (Cue-Mate, Bioniche Animal Health, Australia) into the vagina at a random stage. On day 7, a 25 mg intramuscular injection of prostaglandin F2α (PGF2α) (Lutalyse, Zoetis, USA) was administered, and the progesterone-releasing device was removed. On day 8, a 2 mg i.m. injection of EB was administered. Estrus was confirmed on day 9, and on day 15, the corpus luteum was assessed through rectal palpation via ultrasound examination. On day 16, one embryo was transferred (Fig. 1).

2. 2FTET method

For the 2FTET synchronization method, a 2 mg i.m. injection of EB was administered on day 0, along with the insertion of a 1.56 g progesterone-releasing device into the vagina at a random stage. On day 6, a 25 mg intramuscular injection of prostaglandin F2α (PGF2α) is given, and the progesterone-releasing device is removed. Estrus is confirmed on day 8. On day 9, 250 µg of gonadotropin-releasing hormone (GnRH) (Gonadon, gonadorelin acetate, Dong Bang Co., South Korea) was administered via i.m. injection. On day 15, the corpus luteum was assessed through rectal palpation via ultrasound examination. On day 16, one embryo was transferred (Fig. 1). Prior to conducting the pregnancy test, a 2 mg i.m. injection of EB was administered, and a 1.56 g progesterone-releasing device was inserted into the vagina on day 33. The progesterone-releasing device was then removed on day 39, and pregnancy was confirmed through rectal palpation via ultrasound examination. If the cow was found to be pregnant, the pregnancy was recorded without any further treatment. In the case of a non-pregnant cow, a 25 mg i.m. injection of PGF2α was administered on day 33, and estrus was confirmed on day 41. On day 42, 250 µg of GnRH was i.m. injected. On day 48, the corpus luteum was examined via rectal palpation using ultrasound examination, and on day 49, a second embryo was transferred (Fig. 1).

3. J-synch method

For the J-synch synchronization method, a 2 mg i.m. injection of EB was administered on day 0, along with the insertion of a 1.56 g progesterone-releasing device into the vagina at a random stage. On day 6, a 25 mg i.m. injection of prostaglandin F2α (PGF2α) was administered, and the progesterone-releasing device was removed. Estrus was then confirmed, and 250 µg of GnRH was administered via i.m. injection on day 9. On day 15, the corpus luteum was assessed through rectal palpation using ultrasound examination, after which one embryo was transferred on day 16 (Fig. 1).

Distinguish experimental groups by measurement of corpus luteum and coexistent follicle

The presence and diameter of the corpus luteum and coexistent follicle were measured using ultrasonic equipment equipped with vaginal probe ultrasonography (4Vet Slim, DRAMINKI, Poland). As illustrated in Supplementary Figure 1, the subjects were divided into four experimental groups. The “Only CL” group comprises cases where only the corpus luteum is present in the left or right ovary. The "CL+MF" group represents cases in which both the corpus luteum and a middle-sized (5–10 mm) coexistent follicle are observed. The “CL+LF” group consists of cases with the corpus luteum and a large-sized (>10 mm) coexistent follicle. The “LF” group includes cases where there is no corpus luteum, only a large-sized coexistent follicle. The “X” group pertains to cases in which neither a corpus luteum nor a follicle is detected. For further analysis within the “CL+LF” groups, the combination of corpus luteum and the coexisting large follicle is designated as “Same side_CL/LF” when they are within the same ovary and “Other side_CL/LF” when they are found in different ovaries in Supplementary Figure 2.

Embryo production, embryo transfer, and pregnancy test

The embryos utilized in this experiment were previously described in detail in a paper published by our research team (5). Cumulus-oocyte complexes were collected and cultured through the OPU method, and fresh embryos were subsequently transferred to the recipient. To enhance the accuracy of the experiment and eliminate potential confounding factors that could impact conception rates, a single expert conducted both the measurement of the corpus luteum and coexistent follicle and embryo transfer. Pregnancy testing was carried out via rectal palpation and ultrasound equipment (HS-101V; Honda, Japan) at least 23 days after embryo transfer.

Plasma collection and concentration of progesterone and estrogen ELISA kit

Blood was drawn from the cow’s jugular vein one day prior to the embryo transfer, followed by centrifugation to separate the plasma. Using the isolated plasma, the levels of progesterone and estrogen in the blood were analyzed. The Bovine Progesterone ELISA kit (CSB-E08172b, CUSABIO Co., USA) and the Bovine Estradiol ELISA kit (CSB-E08173b, CUSABIO Co., USA) were employed for this analysis.

Statistical Analysis

The chi-square test was used to analyze the conception rate according to the size and location of the corpus luteum and coexistent follicle. Additionally, the correlation between conception rates and the levels of progesterone and estrogen was statistically examined through a 2-way ANOVA, followed by Tukey’s multiple comparisons test for *post hoc* analysis (GraphPad Prism, version 8.0.1, GraphPad Software Inc., USA).

Results

Table 1 summarizes the results related to the distribution of corpus luteum and coexistent follicle one day before embryo transfer, categorized by the synchronization method. The “Only CL” group accounted for 7.6%, the “CL+MF” group represented 8.3%, the “CL+LF” group comprised 69.7%, the “LF” group was at 11.7%, and the “X” group constituted 2.8% (Table 1). Notably, the “CL+LF” group exhibited a significantly higher percentage compared to the other groups, with a significant difference observed among the experimental groups ($p<0.001$). No significant differences were observed between the presence of corpus luteum and coexistent follicle based on the synchronization methods E2/P4, 2FTET, and J-synch. In instances where the “LF” group (n=17) and the “X” group (n=4) were urgently vaccinated against FMD to prevent disease transmission (n=28), the vaccinated cows were subsequently excluded from the embryo transfer procedure (Table 2).

A total of 96 cows out of the 145 synchronized cows underwent fresh embryo transfer using the OPU method (Table 2). The conception rates for embryo transfer according to the synchronization methods were determined to be 57.1% for the E2/P4 method, 37.1% for the 2FTET method, and 48.1% for the J-synch method. Importantly, no significant differences in conception rates were observed among the synchronization methods examined herein (Table 2). Among the experimental groups categorized based on the presence of corpus luteum and coexistent follicle, the "Only CL" group had a conception rate of 28.6%, the "CL+MF" group achieved 33.3% conception rate, and the "CL+LF" group yielded 43.0% conception rate. Notably, there were no significant differences in conception rates between these experimental groups.

Our study confirmed that a larger corpus luteum size is associated with a higher conception rate. As illustrated in Fig. 2, there was a significant difference in corpus luteum size according to pregnancy status ($p<0.001$). However, no significant difference was observed when analyzing the relationship between the size of the coexistent follicle and pregnancy (Fig. 2).

Figure 3 illustrates the analysis of progesterone and estrogen levels in the blood for the experimental groups, categorized based on the presence of corpus luteum and coexistent follicle. In terms of progesterone content, the “Only CL” group exhibited the highest levels compared to the other groups. Furthermore, there was a tendency for progesterone levels to decrease as the size of the follicle increased, with a significant difference observed between the groups (Fig. 3). Regarding estrogen content, the “Only CL” group had the lowest levels compared to the other groups. Notably, significant differences were detected only between the “Only CL” and “CL+MF” groups and between the “CL+MF” and “LF” groups ($p<0.05$).

Furthermore, as indicated in Table 3, the conception rates were compared by distinguishing between cases in which the corpus luteum and coexistent follicle (>10 mm) were present in the same ovary (“Same side CL/LF” group) and cases where they were located in different ovaries (“Other side CL/LF” group). Upon comparing the fertility rates, we found that the “Other side CL/LF” group tended to have a higher fertility rate than the “Same side CL/LF” group, although this difference did not reach statistical significance (Table 3).

Although the results were not statistically significant, a comparison of the progesterone levels in the blood showed that the “Other side CL/LF” group had higher progesterone content than the “Same side CL/LF” group. Conversely, the blood estrogen levels were higher in the “Same side CL/LF” group compared to the “Other side CL/LF” group (Fig. 4).

Discussion

Studies are actively underway to investigate synchronization methods aimed at enhancing the fertility rate of embryo transfer in cows (6, 7, 10, 11, 17, 20). Given that cows are domestic animals, the primary objective of embryo transfer tends to be profit-driven rather than genetic resource preservation. Furthermore, embryo transfer can result in the production of offspring with outstanding genetic traits, making it a potentially more lucrative option compared to artificial insemination (2, 3, 17). However, it is important to note that embryo transfer complex preparation procedures, advanced technology, and additional expenses related to purchasing embryos. Therefore, its utilization rate is lower when compared to artificial insemination (9, 15, 21).

Additional research efforts are thus needed to address issues such as reducing the acquisition costs and improving the low conception rates associated with embryo transfer. The outcomes of these efforts could be highly promising, as they would establish a basis for the generation of substantial profits through transplantation, in addition to significantly expediting the genetic improvement process. Traditionally, rather than conducting a detailed confirmation of the size of the corpus luteum and coexistent follicle prior to embryo transfer through ovarian ultrasound, a rectal test relying on palpation is commonly performed (11, 19). Technology based on ultrasonic equipment with rectal or vaginal probes has recently gained widespread popularity, albeit with the drawback of requiring specialized expertise. Our research team focuses on oocyte collection using OPU methods, embryo production, and embryo transfer, and therefore our team members are highly skilled in handling ultrasound equipment equipped with a vaginal probe (5). By leveraging this expertise, our study confirmed that the ratio of both corpus luteum and coexistent follicle (>10 mm) was notably high, reaching approximately 69.7% (101 out of 145 cows), thus exceeding previous findings. For instance, Msahiko et al. (15) reported that the ratio of both the corpus luteum and coexistent follicle (>10mm) was 32.8% (24/73 cows).

Numerous studies have demonstrated that the corpus luteum secretes progesterone, a hormone crucial for maintaining pregnancy, whereas the follicle secretes estrogen, a hormone necessary for follicle development (1, 3, 16, 19, 22).Therefore, we inferred that the presence of only the corpus luteum during embryo transfer positively impacts conception rates. However, the presence of both the corpus luteum and coexistent follicle has an adverse effect on pregnancy maintenance, thereby negatively affecting conception rates.Although it is physiologically ideal for only the corpus luteum to be present during embryo transfer, the exact mechanism underlying the simultaneous presence of the coexistent follicle remains to be fully understood. Hypotheses have been proposed, suggesting that cow-related diseases and environmental factors, such as environmental pollution, uterine inflammation, and ovarian cysts, may be primary contributing factors (1, 3, 13, 22).

Previous literature has discouraged embryo transfer when both the corpus luteum and coexistent follicle are simultaneously present (12, 15, 23). Excluding the "CL+LF" (69.7%), "LF" (11.7%), and "X" (2.8%) groups, our findings confirmed that the aforementioned strategy is rather inefficient, as only 15.9% of cases allowed for embryo transfer (7.6% for "Only CL" and 8.3% for "CL+MF"). To address these limitations, we divided the CL+LF group into the "Same side CL/LF" and "Other side CL/LF" groups and compared the levels of progesterone and estrogen in the blood.Existing literature has already reported that successful embryo transfer is associated with high progesterone concentration and low estrogen concentration (12, 15, 19). In this study, we confirmed that the "Other side CL/LF" group exhibited higher progesterone levels and lower estrogen levels compared to the "Same side CL/LF" group, although this difference was not statistically significant. Therefore, our findings suggest that embryo transfer can be considered when the corpus luteum and coexistent follicle are present in different ovaries.

Our findings highlighted the importance of meticulously assessing the presence and size of both the corpus luteum and coexistent follicle through ultrasound equipment to ensure the successful embryo transfer. Moreover, our findings provide foundational insights to study the mechanisms underlying the simultaneous presence of the corpus luteum and coexistent follicle. Therefore, the results of this study offer a valuable theoretical basis to guide the decision-making process regarding embryo transfer in cows, thus contributing to the improvement of farmers' income, as well as conception rates.

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Tables and Figures

Table 1. Changes in corpus luteum and coexistent follicle size before embryo transfer (n=145).
(a-b: Values with different letters, a and b, are significantly different at P<0.001)

Group	E2/P4		2FTET		J-synch		Total	
	No. of Cow	%	No. of Cow	%	No. of Cow	%	No. of Cow	%
Only CL	4	10.0%	5	6.8%	2	6.3%	11	7.6% [a]
CL+MF$	9	22.5%	1	1.4%	2	6.3%	12	8.3% [a]
CL+LF$	22	55.0%	56	76.7%	23	71.9%	101	69.7% [b]
LF[$]	5	12.5%	9	12.3%	3	9.4%	17	11.7% [a]
X	0	0.0%	2	2.7%	2	6.3%	4	2.8% [a]
Total	40	100.0%	73	100.0%	32	100.0%	145	100.0%

[$] Medium (5–10 mm) and large (>10 mm) coexistent follicle with corpus luteum, [a,b] Corpus luteum and follicle size was analyzed using the chi-square test. Statistical significance was set at $p < 0.001$.

Table 2. Conception rate according to corpus luteum size, coexistent follicle size, and embryo transfer method (n=96).

Group	E2/P4		2FTET		J-synch		No. of pregnant cow/Total	Pregnancy rates (%)
	No. of Cow	%	No. of Cow	%	No. of Cow	%		
Only CL	-*	-	1/5	20.0%	1/2	50.0%	2/7	28.6%
CL+MF[$]	-*	-	1/1	100.0%	0/2	0.0%	1/3	33.3%
CL+LF[$]	4/7*	57.1%	21/56	37.5%	12/23	52.2%	37/86	43.0%
Total	4/7*	57.1%	23/62	37.1%	13/27	48.1%	40/96	41.7%

[$] Medium (5–10 mm) and large (>10 mm) coexistent follicle with corpus luteum. A total of 21 cows belonging to the large follicle and X groups did not undergo embryo transfer. * A total of 28 cows were vaccinated against foot-and-mouth disease three days after the embryo transfer and were therefore excluded from the experiment.

Table 3. Conception rate according to the position of the coexistent follicle (>10mm) prior to embryo transfer (n=86).

Group	No. of pregnant cow	Total	Pregnancy rates (%)
Same side CL/LF	13	42	31.0%
Other side CL/LF	24	44	54.5%
Total	37	86	43.0%

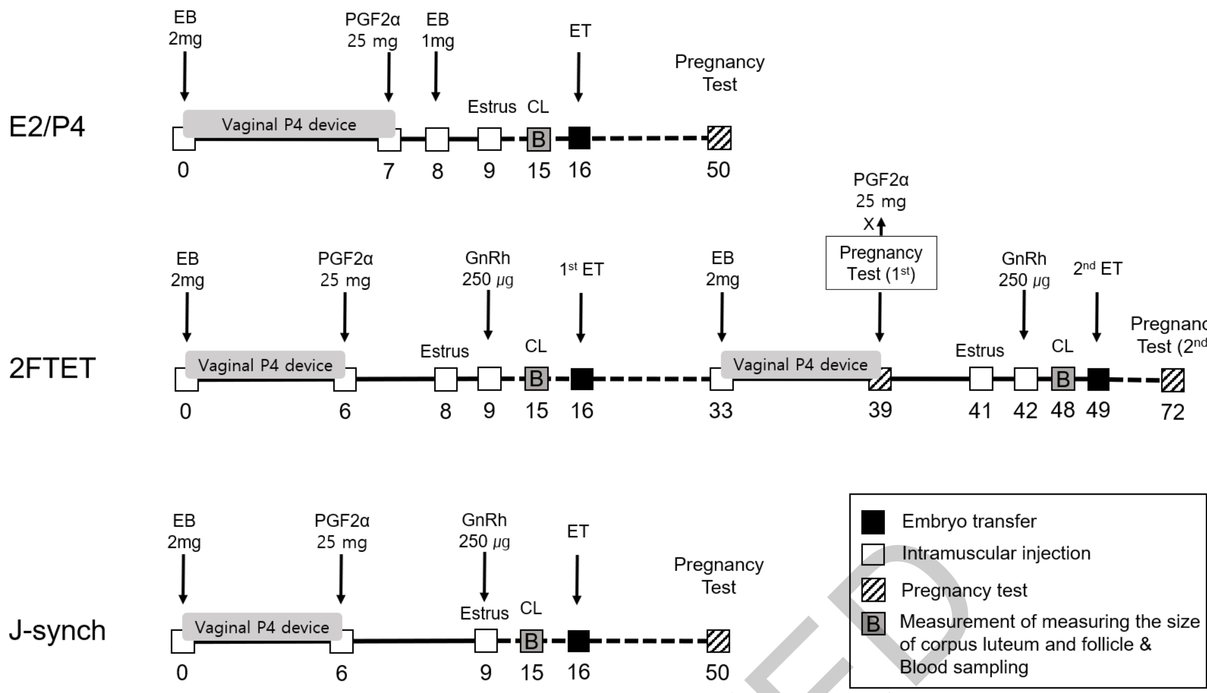

Figure 1. Synchronization method utilized in the experiment. Black square boxes represent embryo transfers, white square boxes denote intramuscular injections, shaded square boxes indicate pregnancy tests, and "B" within a square box indicates measurement of corpus luteum and coexistent follicle to determine embryo transfer, with blood collection for further analysis.

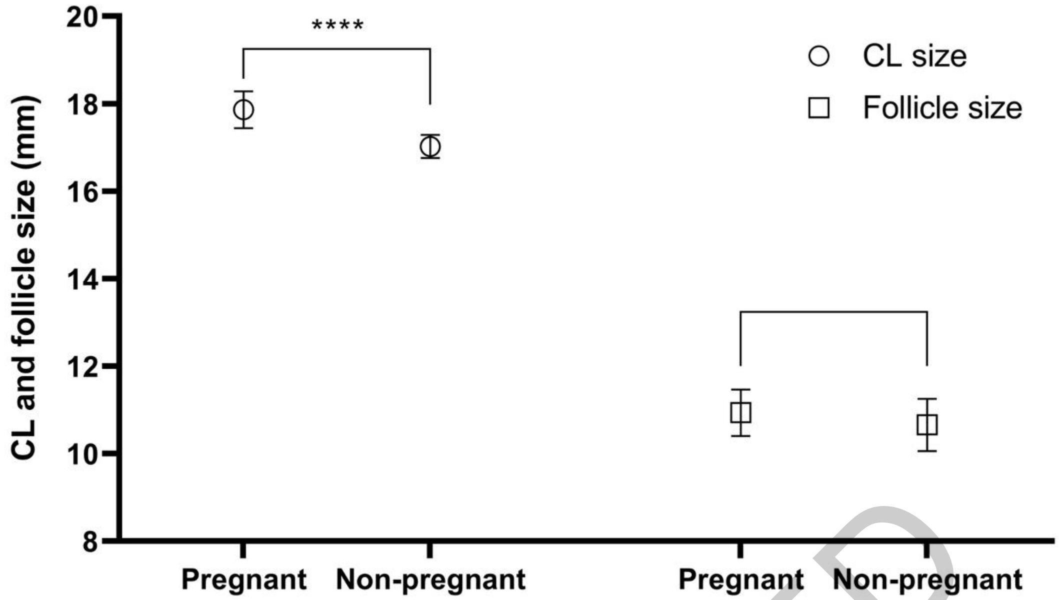

Figure 2. Size of corpus luteum and coexistent follicle according to pregnancy (n=96). Differences in the size (mean ± SEM) of the corpus luteum and follicle were analyzed via two-way analysis of variance (ANOVA) (Tukey's multiple comparisons test). **** Significance level p<0.001.

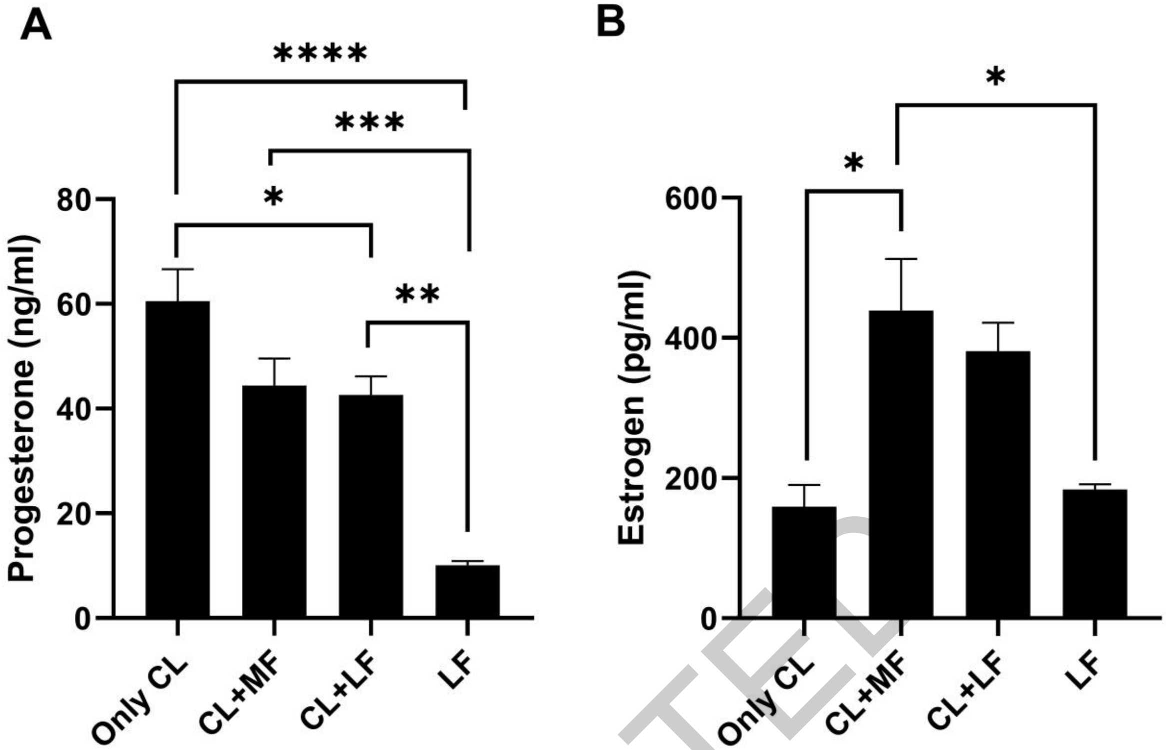

Figure 3. Plasma concentration of progesterone and estrogen 1 day before embryo transfer (n=96). The gray bar represents the plasma concentration of progesterone and estrogen categorized into four groups based on corpus luteum and follicle size. Differences in plasma concentration (mean ± SEM) of progesterone and estrogen were analyzed using two-way analysis of variance (ANOVA) (Tukey's multiple comparisons test). **** Significance level $p<0.001$. *** Significance level $p<0.005$. ** Significance level $p<0.01$. * Significance level $p<0.05$.

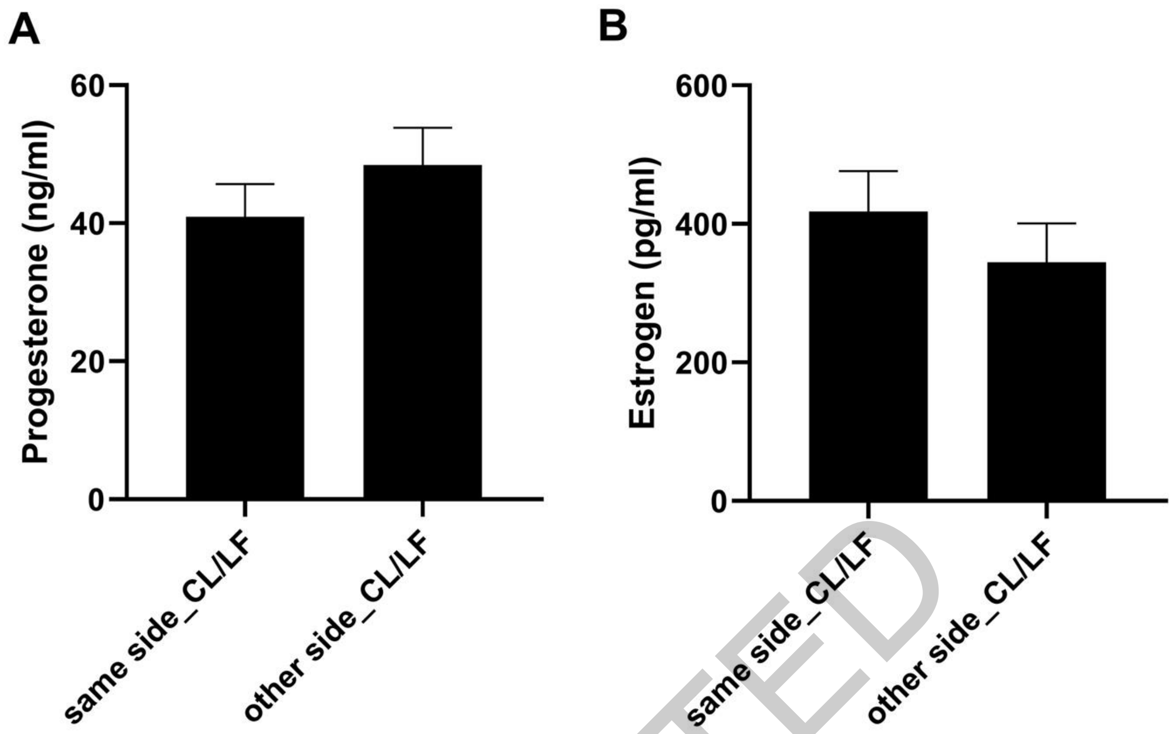

Figure 4. Plasma concentration of progesterone and estrogen a day before embryo transfer according to the location of corpus luteum and coexistent follicle (n=86). The gray bars represent plasma progesterone and estrogen concentrations categorized into two groups based on the location of the corpus luteum and coexistent large follicle.