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Effects of GnRH and hCG administration on conception rate and the subsequent estrous cycle on day 12 following artificial insemination in Holstein cows

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Abstract: The present study investigates the effects of gonadotropin-releasing hormone (GnRH) and human chorionic gonadotropin administration on the conception rate of cows 12 days after artificial insemination (AI). Included in the study were 150 high milk-yielding Holstein cows housed under the same husbandry and feeding conditions. All had calved at least once between the ages of 2.5 and 6 years, and were between 90 and 220 days in milk. In accordance with the farm's reproductive management approach, a controlled internal drug release (CIDR) device was inserted into the vagina concurrent with a GnRH injection (day 0), and the device was removed on day 7. Two prostaglandin F_{2α} (PGF_{2α}) injections were made 24 h apart, and a timed artificial insemination (TAI) procedure was performed in conjunction with a GnRH injection. The animals were randomly assigned to one of three groups 36 h after receiving the second dose of PGF_{2α}. On day 12 following the AI procedure, the first group (GnRH, n = 50) was given GnRH, the second group (hCG, n = 50) was given hCG, and the third group (Control, n = 50) received no additional treatment. Blood samples were collected for progesterone analysis on days 12, 16 and 20 following the AI procedure, and pregnancy testing was initiated on day 30. Pregnancy rates of 46%, 48%, and 42% were recorded in the GnRH, hCG, and Control groups, respectively ($p > 0.05$), with no significant differences noted between the experimental groups. The animals determined to be pregnant underwent an ultrasound scan on day 60, during which one embryonic death was noted in the Control group (4.7%). The progesterone levels observed in the GnRH and hCG groups on day 16 following AI were considerably greater than the 12th day progesterone levels ($p < 0.05$; $p < 0.01$). It was concluded from the study that GnRH and hCG injections on day 12 following AI increase progesterone levels, but have no influence on conception rates.

Key words: Cow, fertility, GnRH, hCG, plasma progesterone

1. Introduction

Continuous reproduction and fertility are critical for successful animal breeding. Calving intervals of around one year, as well as successful conception following the first artificial insemination (AI) after birth, are all factors affecting profitability on dairy farms. The additional yields associated with fertility are vital to the production economy [1].

While advances in biotechnology, selection, and animal breeding have led to significant increases in milk yield in cows, fertility studies have been less rewarding. Decreases in fertility can be attributed to undiagnosed estrus, failure to ovulate on time, luteal insufficiency, early embryonic death, high milk yield, energy metabolism imbalances, and various disorders. Since the estrus detection rate in dairy cows is reported to be less than 50%, various timed insemination programs have been developed and widely adopted [1-3].

The main hormone responsible for embryonic development and growth following insemination is

progesterone [4]. Insufficient secretion of progesterone during the early embryonic period leads to poor embryonic development, and such embryos fail to produce adequate levels of the interferon tau (IFN- τ) necessary for the inhibition of prostaglandin F_{2α} (PGF_{2α}) secretion. Consequently, maternal recognition of pregnancy does not occur in the subsequent stages, resulting in embryonic loss [5,6]. Hormonal treatments are administered postinsemination to increase conception rates or reduce embryonic losses in cows. Such treatments aim to increase progesterone levels [7] through the administration of gonadotropin-releasing hormone (GnRH), human chorionic gonadotropin (hCG), controlled internal drug release (CIDR), or progesterone-releasing intravaginal devices (PRID) following insemination [8,9].

High milk production has been linked to increased embryonic loss in previous research [10]. Toledo-Alvarado et al. [11] reported lower reproductive performance in Holstein cows compared to other breeds that they

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attributed to their higher milk yield. Cows with high milk production consume more feed, which increases hepatic blood flow and accelerates steroid catabolism, leading to reduced circulation levels of progesterone and estrogen [12]. It has thus been reported that progesterone supplementation can improve conception rates in high-yielding cows [13,14].

In recent years, infertility in cows has been treated with GnRH and hCG, and these treatments are now commonly used to synchronize estrus and ovulation, control follicular waves, and support luteal tissues. For such purposes, they can be administered 1–15 days before, during, or after AI [15].

Numerous studies have identified the first 3 weeks of pregnancy as critical for embryo viability. It has been reported that the level and consistency of progesterone secreted by the corpus luteum (CL) are critical for preventing embryonic loss during this procedure. For this reason, attempts are made to keep progesterone at an appropriate level in early-stage pregnancy through applications such as hCG and GnRH on different days of the estrus cycle.

Administration of hCG during insemination or in the luteal phase can support ovulation of the Graafian follicle, prevent corpus luteum dysfunction, promote effective endogenous progesterone production, and increase pregnancy rates [16,17]. GnRH injections, on the other hand, induce follicular atresia, luteinization, or ovulation in the ovaries, leading to the formation of an accessory corpus luteum and a subsequent increase in progesterone levels. Elevating progesterone concentrations and reducing estrogen levels during the late luteal phase inhibits the regulation of oxytocin receptors, allowing PGF₂α secretion to be suppressed. Consequently, GnRH supports maternal recognition of the developing embryo and enhances conception rates [18]. It can thus be understood that fertility rates in cows may be increased by the administration of GnRH or hCG during the luteal phase of the estrous cycle [19]. Studies have demonstrated that GnRH/hCG treatment, when applied on day 12 post-AI, stimulates the formation of accessory corpora lutea, resulting in elevated progesterone concentrations and, consequently, improved pregnancy rates [20].

The present study investigates the effects of GnRH and hCG application on day 12 following AI on pregnancy rates, progesterone levels, and the subsequent estrous cycle of high milk-yielding multiparous and primiparous Holstein cows.

2. Materials and methods

This study was carried out with the approval of the Balıkesir University Animal Experiments Local Ethics Committee (Approval no: 2020/2-11).

2.1. Material

The study was conducted from February to June 2020 on a farm in Şanlı, Balıkesir province (39°48'54.0"N, 27°57'34.3"E), during which 1800 Holstein cattle, 700 of which were in lactation, were assessed. The enterprise's cows produced 9000–11,500 kg of milk throughout the 305-day lactation period. During the study period, the cows produced an average of 33 kg of milk. The enterprise implements a voluntary postpartum waiting period of 90 days for cows in the first lactation and 60 days for those in the second and subsequent lactations.

The study sample included 150 Holstein cows with regular estrous cycles between 60 and 90 days postpartum with no complications during parturition or the postpartum period.

2.2. Method

2.2.1. Synchronization application

In accordance with the enterprise's standard insemination program, a synchronization approach was applied to the cows following the voluntary waiting period, as indicated in Figure 1. On day 0, a CIDR device was inserted into the vagina of the cows, concurrent with a GnRH injection. On day 7, the CIDR device was withdrawn and two PGF₂α intramuscular injections were given at 24-h intervals. Fixed-time insemination was conducted along with a GnRH injection 36 h after the second PGF₂α dose.

2.2.2. Establishing study groups

The inseminated cows were randomly separated into three equally sized groups, as indicated in Figure 2.

- GnRH group (GnRH group), (n = 50): GnRH (10 µg Buserelin acetate, Receptal, Intervet, İstanbul) was administered intramuscularly to the cows in this group on day 12 following AI.
- hCG group (hCG group), (n = 50): 1500 IU hCG (1500 IU, Chorulon, Intervet, İstanbul) was administered intramuscularly to the cows in this group on day 12 following AI.
- Control group (C group), (n = 50): The cows in this group received no treatment.

2.2.3. Blood collection procedure

For progesterone analysis, half of the cows in the experimental and C groups (n = 25/group) were tested on days 12, 16, and 20 following AI. To this end, 10 mL blood samples were collected into serum tubes from the coccygeal vein, and the serum was separated by centrifugation at 3000 rpm for 15 min. The separated sera were stored at -20 °C until the time of analysis.

2.2.4. Progesterone analyses

Progesterone analyses were conducted using the electrochemiluminescence Immunoassay (ECLIA) approach (Architect i2000SR, Abbott, USA). The minimum progesterone measurement value of the method was 0.1 ng/mL.

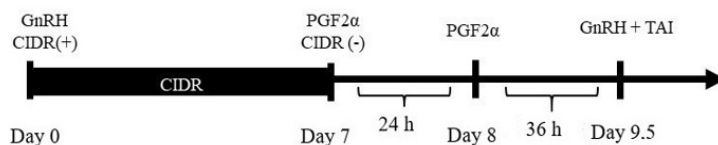


Figure 1. Routine synchronization protocol followed by the farm.

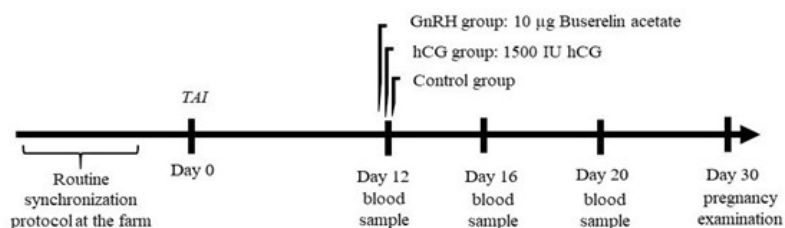


Figure 2. Study groups and blood collection procedure.

2.2.5. Pregnancy examinations

Three cows from each of the GnRH and hCG groups and seven cows from the C group were reinseminated after exhibiting estrus on the expected estrus days following insemination (between days 19 and 24). Pregnancy examinations of cows that did not exhibit estrus at the expected time were performed transrectally on day 30 by an experienced veterinarian using real-time ultrasonography (Falco Esaote, Pie Medical, Netherlands). Cows determined not to be pregnant during the experiment were monitored without treatment for 30 days for signs of estrus. Those that showed estrus were inseminated and pregnancy was assessed by ultrasound 30 days later. Cows determined to be pregnant on day 30 underwent a second ultrasound examination on day 60, at which time failure to detect pregnancy was considered indicative of embryonic death.

2.2.6. Statistical analysis

IBM SPSS Statistics, version 25.0 (IBM Corp., Armonk, NY, USA) was used for the statistical evaluation of the data. The pregnancy examination results and embryonic death rates of the groups on days 30 and 60 following AI were compared among groups with a chi-square (χ^2) test. Average progesterone levels on days 12, 16 and 20 following AI were analyzed with Kruskal–Wallis and Friedman tests. Average progesterone values of pregnant and nonpregnant cows on days 12, 16, and 20 following AI were determined by a *t* test. The pregnancy rates on day 30, based on the progesterone levels determined on the measurement days, and the pregnancy rates on day 30 according to the number of lactations were determined through a χ^2 analysis.

3. Results

The pregnancy and embryonic death rates of the GnRH, hCG and C groups on the days 30 and 60 are presented in Table 1. The pregnancy rates on day 30 following

AI were 46%, 48%, and 42% in the GnRH, hCG, and C groups, respectively ($p > 0.05$). The pregnancy rates recorded during the second pregnancy examinations on day 60 were 46%, 48%, and 40% in the GnRH, hCG, and C groups, respectively ($p > 0.05$). No embryonic deaths were recorded in the GnRH and hCG groups in the two pregnancy examinations, while one embryonic death (4.7%) was recorded in the C group between days 30 and 60.

The mean plasma progesterone concentrations of the groups on days 12, 16, and 20 post-AI are presented in Table 2. A statistically significant difference was observed between the plasma progesterone levels of the treatment groups on days 12 and 16 ($p < 0.05$). In the GnRH group, no significant difference was observed between days 16 and 20, while a statistically significant difference was detected in the hCG group ($p < 0.01$). No significant differences were noted in the plasma progesterone levels across the three time points in the C group ($p > 0.05$).

The mean progesterone levels of the pregnant and nonpregnant cows on days 12, 16, and 20 postinsemination are presented in Table 3 and Figures 3, 4, and 5. While the measurements taken on days 12 and 16 were not statistically significant across all study groups, higher progesterone levels were observed in the pregnant cows than in the nonpregnant cows other than in the C group ($p > 0.05$). The postinsemination progesterone levels measured on day 20 were significantly higher in the pregnant cows than in nonpregnant cows in all three groups (GnRH: $p < 0.01$, hCG: $p < 0.01$, C: $p < 0.01$). A proportional increase in blood progesterone level was observed in the GnRH and C groups as the pregnancy progressed, while in the hCG group, a proportional decrease was determined only between days 16 and 20. Blood progesterone levels in the nonpregnant cows in the C group showed a proportional decrease on days 12, 16, and 20, while in the experimental

Table 1. Pregnancy and embryonic loss rates of experimental and control groups on days 30 and 60 postinsemination ($p > 0.05$)

Group	n	Day 30 (n/50)	Day 60 (n/50)	Embryonic death (n/n, %)
GnRH	50	23/50 (46%)	23/50 (46%)	0/23 (0.0%)
hCG	50	24/50 (48%)	24/50 (48%)	0/24 (0.0%)
Control	50	21/50 (42%)	20/50 (40%)	1/21 (4.7%)

Table 2. Mean progesterone levels of the experimental and control groups measured on days 12, 16, and 20 postinsemination.

Group	n	Day 12 (ng/mL)	Day 16 (ng/mL)	Day 20 (ng/mL)	p
GnRH	25	2.36 ± 0.35 ^a	3.19 ± 0.23 ^b	2.48 ± 0.38 ^{ab}	0.021
hCG	25	2.03 ± 0.25 ^a	3.17 ± 0.39 ^b	1.90 ± 0.37 ^a	0.008
Control	25	2.68 ± 0.26	2.73 ± 0.23	2.54 ± 0.40	0.672

a, b: Values within a row with different superscript letters differ significantly at $p < 0.05$

Table 3. Mean progesterone levels of pregnant and nonpregnant cows in experimental and control groups on days 12, 16, and 20 postinsemination.

Group	Day 30 of pregnancy (P)	Day 12 (ng/mL)	p	Day 16 (ng/mL)	p	20 th day (ng/mL)	p
GnRH (n = 25)	P: + (n=13)	2.99 ± 0.57	0.061	3.20 ± 0.33	0.960	3.50 ± 0.47	0.004
	P: - (n=12)	1.68 ± 0.29		3.18 ± 0.34		1.39 ± 0.44	
hCG (n = 25)	P: + (n=15)	2.1 ± 0.28	0.700	3.43 ± 0.52	0.428	2.84 ± 0.46	0.001
	P: - (n=10)	1.91 ± 0.47		2.78 ± 0.60		0.51 ± 0.25	
Control (n = 25)	P: + (n=12)	2.67 ± 0.37	0.986	3.02 ± 0.30	0.237	3.88 ± 0.53	0.001
	P: - (n=13)	2.68 ± 0.39		2.46 ± 0.34		1.31 ± 0.36	

P(+): Pregnant, P(-): Nonpregnant

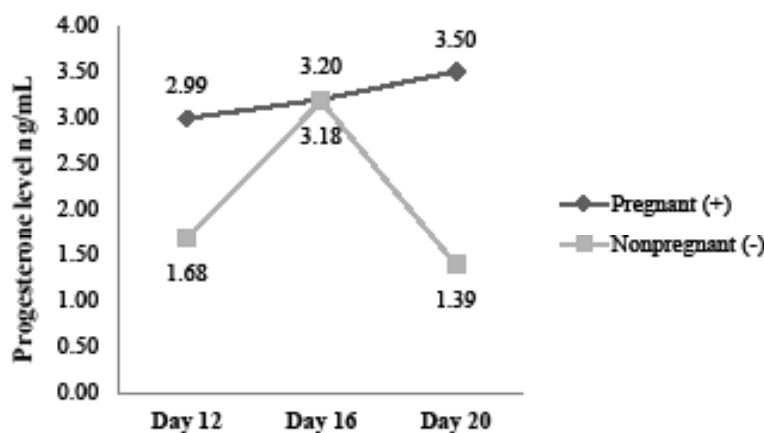


Figure 3. Progesterone levels in pregnant and nonpregnant cows in the GnRH group on days 12, 16, and 20.

groups, a proportional increase in progesterone levels was noted on day 16. This increase was considered to be influenced by the treatments administered on day 12. A significant decrease in progesterone was observed on day 20 in all of the groups.

The effects of the different progesterone levels measured on days 12, 16, and 20 in the C and experimental groups on day 30 are presented in Table 4. Significant differences in pregnancy rates were observed among the groups based on the progesterone levels measured on day 20 ($p < 0.01$).

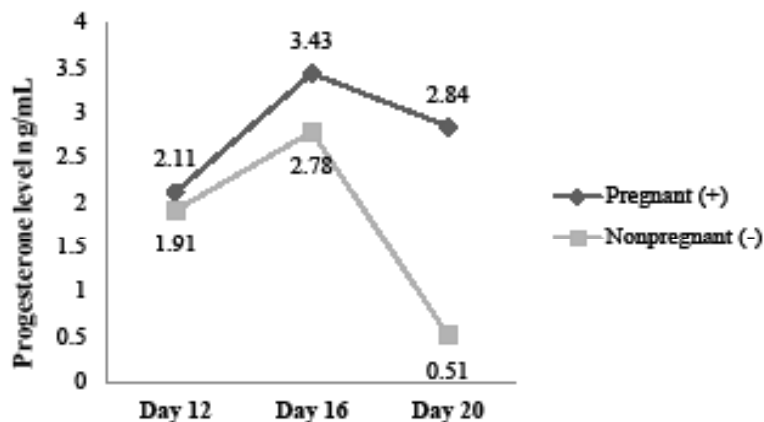


Figure 4. Progesterone levels in pregnant and nonpregnant cows in the hCG group on days 12, 16, and 20.

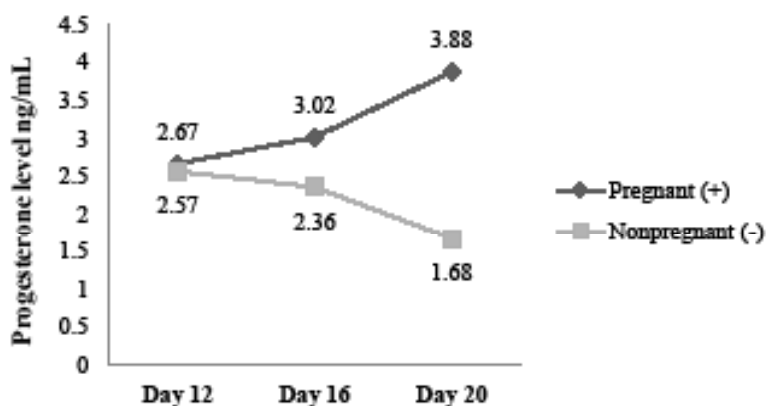


Figure 5. Progesterone levels in pregnant and nonpregnant cows in the Control group on days 12, 16, and 20.

The cows with progesterone levels of ≥ 2 ng/mL in the experimental and C groups recorded higher pregnancy rates than those with levels between 1 ng/mL and 2 ng/mL, as well as those with levels below 1 ng/mL.

The number of cows with serum progesterone levels of ≥ 1 ng/mL measured on days 12, 16, and 20 in the C and experimental groups were shown in Table 5. For the GnRH group, the number of cows with serum progesterone levels ≥ 1 ng/mL were 22, 24 and 18 on days 12, 16, and 20, respectively, while values of 20, 22 and 13 were recorded for the hCG group and 23, 24, and 19 for the C group. In the hCG group, the number of cows with serum progesterone levels ≥ 1 ng/mL measured on day 20 was significantly lower than the number determined on day 16 ($p < 0.05$).

Pregnancy rates on day 30 according to lactation numbers in the C and experimental groups are presented in Table 6. In the experimental groups, a decrease in pregnancy rates was detected in primiparous and multiparous cows as the number of lactations increased,

with no statistical difference between the groups ($p > 0.05$). In the C group, pregnancy rates were significantly higher in multiparous cows than in the primiparous cows ($p < 0.05$).

In the study, the number of cows that showed estrus symptoms were reinseminated before the pregnancy examination on day 30 after fixed-time AI, as well as between days 30 and 60 of the second pregnancy examination. The pregnancy rates obtained from these cows after the second insemination are presented in Table 7. Prior to the day 30 pregnancy examination, three cows in each of the GnRH and hCG groups and seven in the C group exhibited estrus and were inseminated. Between days 30 and 60 postinsemination, three cows in each of the GnRH and hCG groups and two cows in the C group were inseminated after exhibiting estrus. The pregnancy rates of the cows that were reinseminated after exhibiting estrus during the 60-day follow-up were 66.66% in the GnRH and hCG groups, and 44.44% in the C group.

Table 4. The effect of different progesterone (P₄) levels measured on days 12, 16, and 20 post-insemination in the experimental and control groups on day 30.

Group	Day 12			p	Day 16			p	Day 20			p
	P ₄ level (ng/mL)	N	Day 30 of pregnancy n/n (%)		P ₄ level (ng/mL)	n	Day 30 of pregnancy n/n (%)		P ₄ level (ng/mL)	n	Day 30 of pregnancy n/n (%)	
GnRH	< 1	3	0/3 (0.0%)	0.116	< 1	1	1/1 (100.0%)	0.618	< 1	7	0/7 (0.0%)	0.005
	1 ≤ 2	10	5/10 (50.0%)		1 ≤ 2	4	2/4 (50.0%)		1 ≤ 2	3	2/3 (66.6%)	
	≥ 2	12	8/12 (66.7%)		≥ 2	20	10/20 (50.0%)		≥ 2	15	10/15 (73.3%)	
hCG	< 1	5	2/5 (40.0%)	0.588	< 1	3	1/3 (33.3%)	0.530	< 1	12	3/12 (25.0%)	0.003
	1 ≤ 2	9	6/9 (66.7%)		1 ≤ 2	7	5/7 (71.4%)		1 ≤ 2	1	1/1 (100%)	
	≥ 2	11	7/11 (63.6%)		≥ 2	15	9/15 (60.0%)		≥ 2	12	11/12 (91.7%)	
Control	< 1	2	1/2 (50.0%)	0.602	< 1	1	0/1 (0.0%)	0.104	< 1	6	0/6 (0.0%)	0.006
	1 ≤ 2	4	1/4 (25.0%)		1 ≤ 2	6	1/6 (16.7%)		1 ≤ 2	4	1/4 (25.0%)	
	≥ 2	19	10 / 19 (52.6%)		≥ 2	18	11/18 (61.1%)		≥ 2	15	11/15 (73.3%)	

Table 5. Number of cows with serum progesterone levels (P₄) ≥ 1 ng/mL measured on days 12, 16, and 20 postinsemination in the control and experimental groups.

	Day 12 P ₄ ≥ 1 ng/mL (n / n, %)	Day 16 P ₄ ≥ 1 ng/mL (n / n, %)	Day 20 P ₄ ≥ 1 ng/mL (n / n, %)	p
GnRH	22/25 (88%)	24/25 (96%)	18/25 (72%)	0.051
hCG	20/25 ^{ab} (80%)	22/25 ^b (88%)	13/25 (52%) ^a	0.010
Control	23/25 (92%)	24/25 (96%)	19/25 (76%)	0.070
P	0.444	0.424	0.156	

a, b: Values within a row with different superscript letters differ significantly at p < 0.05

Table 6. Pregnancy rates on day 30 postinsemination according to lactation numbers in the control and experimental groups.

Group	Lactation number	n	Pregnancy rate on day 30 n / n (%)	p	χ ²
GnRH	1	23	11/23(47.8%)	0.811	0.057
	≥ 2	27	12/27 (44.4%)		
hCG	1	22	11/22 (50.0%)	0.802	0.063
	≥ 2	28	13/28 (44.4%)		
Control	1	23	6/23 (26.1%)	0.035	4.428
	≥ 2	27	15/ 27 (55.6%)		

The number of inseminations per pregnancy within the first 60 days, the number of inseminations that led to pregnancy, and the total pregnancies achieved in each group are presented in Table 8. A total of 23 cows in the

GnRH group, 24 cows in the hCG group, and 21 cows in the C group became pregnant following the initial insemination, and 12 cows subsequently became pregnant. The number of inseminations per pregnancy in each group

Table 7. Number of cows with estrus symptoms reinseminated before or after the day 30 pregnancy examination, and pregnancy rates of cows after second insemination (%)

Group	Number of cows showed estrus symptoms before pregnancy examination (n)	Number of cows showed estrus symptoms after pregnancy examination (n)	Number and pregnancy rates of cows after second insemination n/n (%)
GnRH	3	3	4/6 (66.66%)
hCG	3	3	4/6 (66.66%)
Control	7	2	4/9 (44.44%)

Table 8. Number of pregnancies achieved according to the number of inseminations, and number of inseminations per pregnancy in the first 60 days.

Group	Pregnant in the first insemination (n/50)	Pregnant in the second insemination (n/n)	Number and rate of pregnancy obtained total inseminations n/n (%)	Number of inseminations per pregnancy (n/n)
GnRH	23/50	4/6	27/50 (54%)	2.07 (27/56)
hCG	24/50	4/6	28/50 (56%)	2.00 (28/56)
Control	21/50	4/9	25/50 (50%)	2.36 (25/59)

was 2.07 for the GnRH group, 2.00 for the hCG group, and 2.36 for the C group. The overall pregnancy rates resulting from all inseminations conducted within the first 60 days postinsemination were 54% for the GnRH group, 56% for the hCG group, and 50% for the C group.

4. Discussion

Fertility performance in dairy farming is a crucial factor for sustainable animal husbandry. Parameters such as preservation of breeding value, milk yield, and reproductive performance have been assessed based on calving-pregnancy interval data and the number of inseminations per pregnancy. Significant improvements in milk yield have been achieved in recent years as a result of feeding, animal breeding, and genetic studies, while problems such as embryonic death, infertility, delayed ovulation, subestrus, anovulation have also arisen [21].

Early embryonic loss is one of the main contributors to low pregnancy rates in dairy cows. Approximately 70%–80% of embryonic losses occur within the first 16 days following fertilization, and a significant proportion of these occur between days 8 and 16 [21–24]. Luteolysis prevention is vital for the maintenance of pregnancy, requiring sufficient secretion of IFN- τ by the embryo to suppress the release of PGF 2α . Embryos with slow development are unable to produce adequate levels of IFN- τ , and the resulting inability to prevent luteolysis leads to embryo loss. In this regard, progesterone supplementation prior to maternal acceptance of pregnancy is vital for its establishment and

maintenance [25]. Progesterone levels can be increased through such exogenous hormone applications as progesterone, GnRH and hCG. The present study aims to enhance the pregnancy rate in cows by reducing estrogen levels and induce the ovulation of the dominant follicle on day 12 post-AI, subsequently increasing progesterone levels.

In the present study, applications on day 12 reduced the number of cows with low progesterone levels (≤ 1 and 1–2 ng/mL) on day 16. Although the applications contributed positively to progesterone levels, no such positive effect was noted on the pregnancy rate, which we attributed to inadequate early embryonic development. Serum progesterone levels on day 12 in all groups suggested that ovulation occurred in 80% of the cows, confirming the presence of a corpus luteum (CL). This finding could be linked to the synchronization protocol implemented on the farm on which the study was conducted. The higher number of active CLs in the C group prior to the intervention would appear to be coincidental. Nonetheless, studies on hormonal support have highlighted the importance of ovarian function. Initiating the study after the co-synch + CIDR treatment likely mitigated potential deficiencies, suggesting that the administration of GnRH and hCG on day 12 may not have provided any additional benefit.

Previous studies in the literature report that applications of GnRH or hCG on or before day 12 postinsemination led to an increase in pregnancy rates. These findings suggest

that the applied hormonal treatments may have enhanced reproductive efficiency by improving conception rates and reducing the need for repeat inseminations [19,26–29]. The absence of any difference in pregnancy rates between the experimental and C groups in the present study may be attributable to the routine synchronization program operated by the enterprise on which the study was conducted. A co-synch+CIDR synchronization protocol was implemented, and it was thought that the GnRH applied at the beginning and end of the program synchronized the ovulations and supported luteal tissue formation after ovulation. As there were no embryos with weak development requiring support on day 12, the pregnancy rates did not differ between the groups. Only one case of embryonic death was noted between days 30 and 60, and it was thus concluded that GnRH or hCG applications on day 12 postinsemination are unnecessary on farms applying co-synch+CIDR protocols for reproductive herd management.

There have also been studies reporting that postinsemination GnRH or hCG applications have no effect on pregnancy rates, but rather only increase blood progesterone levels, as well as studies reporting a negative effect on pregnancy rates. The administration of GnRH and hCG concurrently with AI and on day 12 postinsemination had no effect on the pregnancy rate and did not lead to any statistically significant changes in progesterone levels [30]. Ataman et al. [20] observed that GnRH applied on day 12 postinsemination had no significant effects on pregnancy and embryonic death rates in Brown Swiss cattle, but reported that progesterone levels were higher on days 18 and 21. In another study in which GnRH was applied to Holstein heifers on days 0, 5, and 12 postinsemination, no significant difference was noted between the progesterone values measured on the application days. It has been reported that the progesterone levels measured on days 5 and 12 postinsemination were higher than in the control group may be due to the increased pregnancy rate and reduced embryonic loss attributable to the application [31]. In a study in which GnRH was administered to Holstein cows on day 12 post-AI, the number of accessory corpus lutea was higher in the experimental group (21.1%) compared to the control group (6.7%) [24]. According to results, the researchers stated that GnRH and hCG facilitated the formation of accessory corpus lutea, and the progesterone level increased accordingly. The application of GnRH and hCG increased progesterone levels in the present study; however, the absence of any corresponding increase in pregnancy rates indicated that progesterone levels at this stage (day 12) contributed little to the establishment of pregnancy. It was further concluded from the study that the increased progesterone concentration

on day 16 was associated indirectly with the formation of accessory corpus lutea; however, this positive situation was not reflected in the pregnancy rates.

Another possible explanation for the lack of variation in pregnancy rates among the groups in the present study may be the similarity in plasma progesterone concentrations at the time of treatment administration. However, progesterone levels in peripheral blood circulation were lower in cows with higher milk yields, which could be attributed to the increased blood flow in the liver due to intensive feed intake and changes in the steroid metabolism [32]. Although progesterone levels remained high in both the experimental and control groups after the applications in the study, this may not be reflected in pregnancy rates.

One of the leading causes of embryonic loss in cows is luteal insufficiency, which was characterized by low levels of progesterone [5]. Recent studies have emphasized the crucial role of progesterone in embryo development in the early luteal period following insemination [33]. It has been reported that rapid increases in progesterone levels within the first 5 days following ovulation are associated with higher pregnancy rates [34]. Although data supporting this finding was lacking in the present study, it was thought that the higher progesterone levels recorded in the early and late luteal periods could have had a positive effect on the pregnancy rate. If there was no pregnancy, it might have a positive contribution to pregnancies in the following cycle to treat luteal insufficiency. It is worthy of note that the pregnancy rate following the subsequent insemination of cows that did not conceive during the first insemination was higher in the GnRH and hCG groups than in the C group. As such, GnRH and/or hCG administration on day 12 following insemination may be recommended due to their positive effects on the pregnancy rate in subsequent inseminations, even if it did not increase the pregnancy rate. This may be due to the high progesterone level before estrus, which may have a positive effect on oocyte quality, ovulation, and the early luteal period [35].

Progesterone is a useful biomarker for pregnancy in cows. Cows with progesterone levels of ≥ 2 ng/mL in blood/milk samples taken between 19 and 24 days following insemination were considered pregnant. In the present study, fewer cows were identified as pregnant during the day 30 pregnancy examinations than had plasma progesterone levels ≥ 2 ng/mL on day 20 postinsemination. This indicated a loss of 23.80% based on progesterone levels, one reason for which may be embryonic death. Erdem [22] reported a 27% embryonic death rate between days 24 and 27 days. In the present study, as pregnancy examinations were performed with real-time ultrasound on days 30 and 60, no embryonic death was detected

by ultrasound within the day 20–30 interval, and only one embryonic death was detected in the day 30–60 interval. This may be due to the fact that the study was conducted with cows housed under the same conditions, management, care, feeding conditions, and good animal welfare practices (quality feed, physical conditions, etc.) in the enterprise.

The number of lactations was an important fertility parameter affecting the pregnancy rate. As the number of lactations increased, so did milk yield, embryonic loss, and incidences of reproductive disease [34,36]. Besbacci et al. [37] reported that postinsemination GnRH and hCG applications improved the outcome in primiparous cows with low fertility, but provided no benefit to multiparous cows with good fertility. Similarly, Nascimento et al. [38] reported that hCG applications on day 5 postinsemination increased the pregnancy rates in primiparous cows more than in their control group. Furthermore, when the pregnancy rates were evaluated according to the number of lactations, better rates were achieved in the primiparous cows in the experimental groups than in the control group. In addition, while the pregnancy rates of primiparous and multiparous cows in the experimental groups were similar ($p > 0.05$), the pregnancy rate among primiparous cows was significantly lower than that of the multiparous cows ($p < 0.05$). Based on these findings, it can be concluded that GnRH and hCG administration at the time of first lactation in dairy enterprises may create a numerical difference, although the difference may not be statistically significant, mirroring the findings of the present study. This positive effect would reduce the rate of repeat breeder cows and may be important in cases where pregnancy is critical (such as inseminations with female (X-chromosome) sperm).

5. Conclusion

In the present study, GnRH and hCG application on day 12 postinsemination had a positive effect on progesterone levels, but no significant effect on pregnancy rate. The applied insemination protocol could be a determining

factor on pregnancy rates in postinsemination applications. Contrasting studies have reported both positive and negative effects of insemination protocols on conception rates and embryonic losses, and this should be taken into consideration when planning postinsemination interventions. In the present study, GnRH or hCG applications were deemed unnecessary on day 12 postinsemination when a co-synch+P4 protocol was adopted. Although there was no statistical difference in the pregnancy rates reported for the groups, such treatments may be recommended when achieving pregnancy is of critical importance. Endocrinological and ultrasonographic examinations involving a larger number of cows are required to evaluate the possible outcomes of the applied method on pregnancy rate and the subsequent cycle process in cows with high breeding value and milk yield.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethical approval

All procedures applied in the present study were approved by the Ethic Committee of Balıkesir University (2020-2-11).

Author contributions

Experimental design: HE and MHY. All authors contributed equally to the data collection, analysis, and writing of the final manuscript.

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