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The effect of PGF2α injection in different times on the pregnancy rate in progesterone-based synchronization in Holstein cows

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Abstract: This study investigated the effect of prostaglandin F2α (PGF2α) injection at different times on pregnancy rate (PR) in Holstein cows undergoing progesterone-based fixed-time artificial insemination (FTAI). Group 1 (G1, n=30): A progesterone releasing intravaginal device (PRID) was placed intravaginally with a gonadotropin-releasing hormone (GnRH) injection on day 0. Eight days later, PGF2α was injected. PRID was removed on the 9th day, and after 60 hours, FTAI was performed with a GnRH injection. Group 2 (G2, n=30): Unlike G1, PGF2α was injected twice, with an interval of 24 hours, 8 and 9 days after the intravaginal placement of PRID. Group 3 (G3, n=30): PGF2α was injected 9 days after the intravaginal placement of PRID. Pregnancy diagnoses were performed by transrectal ultrasonography 45 days after FTAI. Blood samples were taken on the 8th day (for Groups 1 and 2), the 9th day (for Groups 2 and 3, PRID removal day), and on the day of FTAI (for Groups 1, 2 and 3) and serum progesterone (P4) concentration was determined. The pregnancy rate (PR) was 36.67%, 53.33%, and 43.33% in Groups 1, 2, and 3, respectively (P=0.194). In cows with a body condition score (BCS) <3, repeated PGF2α injections within a 24-hour interval increased the PR (G2: 70%) compared to the other two groups (G1: 20%, G3: 25%) (P=0.045). P4 concentrations decreased after PGF2α injections (P<0.001). In cows experiencing luteolysis, a significant difference was found in PR between G1 and G3 (P=0.04). In conclusion, PGF2α injection at 24-hour intervals in P4-based FTAI protocols in cows quantitatively increased both pregnancy and luteolysis rates and may improve fertility.

Keywords: *Cow, Luteolysis, PGF2α, Pregnancy, Progesterone*.

Holstein ineklerinde progesteron bazlı senkronizasyonda farklı zamanlarda PGF2α enjeksiyonunun gebelik oranına etkisi

Özet: Bu çalışmada, progesteron bazlı sabit zamanlı suni tohumlama (FTAI) uygulanan Holştayn ineklerde farklı zamanlarda prostaglandin F2α (PGF2α) enjeksiyonunun gebelik oranı üzerine etkisi araştırıldı. Grup 1 (G1, n=30): Progesteron salgılayan intravajinal cihaz (PRID) 0. günde vajinaya yerleştirildi ve gonadotropin salgılatıcı hormon (GnRH) enjeksiyonu yapıldı. Sekiz gün sonra PGF2α enjekte edildi. PRID, 9. günde çıkarıldı ve 60 saat sonra GnRH enjeksiyonu ile sabit zamanlı suni tohumlama (FTAI) gerçekleştirildi. Grup 2 (G2, n=30): Grup 1'den farklı olarak PGF2α, sabit zamanlı suni tohumlama (FTAI) intravajinal yerleştirilmesinden 8 ve 9 gün sonra, aralarında 24 saatlik bir süre bulunarak iki kez enjekte edildi. Grup 3 (G3, n=30): PGF2α, PRID'in intravajinal yerleştirilmesinden 9 gün sonra enjekte edildi. Gebelik teşhisleri FTAI'den 45 gün sonra transrektal ultrasonografi ile yapıldı. Kan örnekleri, 8. gün (G1 ve G2 için), 9. gün (PRID çıkarılma günü) ve FTAI gününde alındı ve serum progesteron (P4) konsantrasyonu belirlendi. Gebelik oranları Grup 1, 2 ve 3'te sırasıyla %36.67, %53.33 ve %43.33 olarak bulundu (P=0.194). Vücut kondisyon skoru (BCS) <3 olan ineklerde, 24 saat arayla tekrarlanan PGF2α enjeksiyonları gebelik oranını (G2: %70) diğer iki grupla (G1: %20, G3: %25) karşılaştırıldığında artırdı (P=0.045). P4 konsantrasyonları PGF2α enjeksiyonlarından sonra azaldı (P<0.001). Luteolizis uygulanan ineklerde Grup 1 ve Grup 3 arasında gebelik oranlarında anlamlı fark bulundu (P=0.04). Sonuç olarak, ineklerde P4 bazlı FTAI protokollerinde 24 saatlik aralıklarla PGF2α enjeksiyonunun gebelik ve lüteoliz oranlarını nicel olarak artırdığı ve fertiliteyi iyileştirebileceği sonucuna varıldı.

Anahtar Kelimeler: *Gebelik, İnek, Luteolizis, PGF2α, Progesteron*.

Introduction

In the last 30 years, challenges have arisen in detecting estrus signs in cows, paralleling the increase in milk yield. Consequently, pregnancy rate (PR) on first insemination has declined to 40%. The accurate determination of estrus symptoms and timely artificial insemination (AI) play a crucial role in improving fertility. Timed AI protocols have been developed to alleviate difficulties in detecting estrus symptoms. These protocols are widely adopted in both dairy and beef cattle worldwide. Using highly productive bulls has led to increased profitability both genetically and economically. Furthermore, it allows for pre-determination and planning of calf production (Kaçar, 2019).

The goal of a successful estrus synchronization program in dairy cows aims to achieve high fertility using fixed-time artificial insemination (FTAI) without the need for estrus detection. Strategies for ovulation control involve manipulating the corpus luteum (CL) lifespan with prostaglandin F2α (PGF2α), inducing follicle development, synchronizing ovulation, or inhibiting estrus through progesterone (P4) treatment (Thatcher et al., 2006). Depending on the presence of the CL in different periods of the estrus cycle in cows, PGF2α acts at different rates 78% at 5-9th days, 87.9% at 10-13th days, and %100 at 14-19th days of the estrus cycle (Freitas et al., 2021). It was determined that cows with a preovulatory dominant follicle showed signs of estrus 72 hours after PGF2α injection when PGF2α injection was performed showed signs of estrus 72 h later (Martins et al., 2011). Optimizing the size of the dominant follicle is important for timed AI protocols and synchronization of ovulation (Wiltbank et al., 2014). Pregnancy rates were found to be similar between cows with estrous and cows with timed AI (Santos et al., 2010; Wiltbank et al., 2014). Timed synchronization protocols reduced the time between birth and first delivery and increased the proportion of cows conceiving after the voluntary waiting period (Kim et al., 2020). In this study, the aim was to investigate the effect of PGF2α injection at different times on PR in Holstein cows undergoing P4-based FTAI.

Materials and Methods

The research was approved by the Kafkas University Local Ethics Committee for Animal Experiments (HADYEK numbered 2019/129) and permission was obtained from the Ministry of Agriculture and Forestry of Türkiye (dated 19.06.2019 and numbered 64445328-020-E1820373). The experiments were performed at private corporations located in Denizli, Türkiye.

Animals: The study was carried out between January and May, in Holstein cows, housed in a semi-open barn, fed with mixed ration and had access to *ad libitum* water. In the study, 90 clinically healthy Holstein cows, aged between 2-6 years old, weighing 450-550 kg, with a body condition score (BCS) of 2.5-3.5 (according to a 5-point scale with 0.25-point increments), producing 14-25 liters of milk, and at least 60 days postpartum, were used. Internal and external antiparasitic treatments and vaccination of cows were performed at least 25 days prior to synchronization protocols. The cows were fed twice daily, and drinkable water was supplied *ad libitum* from fixed water drinkers. Cows were fed with alfalfa hay, barley straw, wheat straw, corn silage, sugar beet pulp, crushed barley, and concentrated feed (2750 metabolic energy; crude protein 21%, crude cellulose 10%, crude fat 4%, crude ash 8%, calcium 1%, phosphorus 0.6% and sodium 0.45%). Cows were then randomized into 3 groups:

Group 1 (G1, *n*=30): The cows in this group were administered a progesterone releasing intravaginal device (PRID) intravaginally on day 0 together with 2 mL injection of gonadotropin-releasing hormone (GnRH, 50 µg Gonadorelin diacetate tetrahydrate, Ovarelin®, Ceva, Türkiye). Eight days after the application, a 5 mL injection of PGF2α (5 mg/mL Dinoprost, Enzaprost-T®, Ceva, Türkiye) was administered. The PRID was removed on day 9, and 60 hours later, FTAI was performed together with a 2 mL injection of GnRH. **Group 2 (G2, *n*=30):** The cows in this group were administered a PRID intravaginally on day 0 together with 2 mL injection of GnRH, similar to G1. On days 8 and 9 after the application, a 5 mL injection of PGF2α was administered. The PRID was removed on day 9, and 60 hours later, a 2 mL injection of GnRH was made together with FTAI. **Group 3 (G3, *n=30*):** The cows in this group were administered a PRID intravaginally on day 0, together with a 2 mL injection of GnRH similar to G1 and G2. Nine days after the application, a 5 mL injection of PGF2α was administered, and the PRID was removed. Sixty hours after PRID was removed, a 2 mL injection of GnRH was made with FTAI. Holstein sperma (Medivet®, Türkiye) was used for AI. Sperma was defrosted in a hot water bath at 37°C for 30 seconds just before the AI, and then placed recto-vaginally to the cornu uteri on which the Graafian follicle was detected. Single AI was performed for all groups in the study. To eliminate application differences, all AI procedures were performed by the same veterinary physician. Pregnancies were determined by transrectal ultrasonographic examination (5-7.5 MHz linear probe, Draminski iScan®, Draminski, Poland) which was performed 45 days after the AI procedure (Fig. 1).

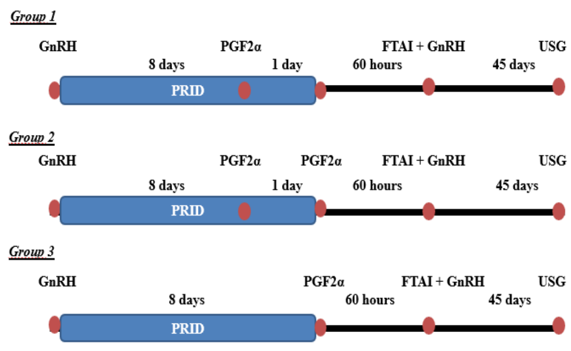

Figure 1. Procedures that were applied to all groups. GnRH: Gonadotrophin Releasing Hormone, PRID: Progesterone-releasing intravaginal device, PGF2α: ProstaglandinF2α, FTAI: Fixed-time artificial insemination, USG: Ultrasonography.

Analyzes of Blood Samples: On day 8 (Groups 1 and 2), day 9 (PRID removal days), and the day of AI, blood samples were collected from the *vena coccygea* into 8.5 ml vacutainer gel tubes (BD Vacutainer, BD, Türkiye). For the determination of P4 concentrations, blood samples were subjected to centrifugation at 1,300 g (NF400R®, Nüve,Türkiye) for 10 minutes to obtain serum samples. The serum samples were then stored at -18°C until the experiments.

Hormone analyzes were performed at the Department of Biochemistry of Kafkas of Science and Literature, Kars, Türkiye.

The P4 concentration in serum samples of cows obtained on different days was determined using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits (Catalogue no: 20112-1008, SunRed Biotechnology Company, Shanghai, China) according to the manufacturer's instructions. The assays were then analyzed with an ELISA reader (Epoch®, Biotek, USA). The assays were analyzed using an ELISA reader (Epoch®, Biotek, USA). The sensitivity of the kit was 0.029 ng/mL, with a measurement range of 0.05-40 ng/mL.

In our study, the changes in serum P4 on the 8th and 9th days of the experiment were determined. Additionally, serum P4 levels and active CL were determined on the day of AI, and the rate of luteolysis was determined. Cows with a serum P4 concentration of ≥1 ng/mL on day -3 and <1 ng/mL on day 0 are considered to have CL regression (Ribeiro et al., 2012). In this study, luteolysis of the CL P4 was accepted in cows with a serum P4 concentration of 1 ng/mL during the first PGF2α injection and <1 ng/mL at AI.

Statistical analysis: SPSS 18 (SPSS®, Chicago, IL, USA) package was used for the statistical analysis of the data obtained from the experiments. The PR was calculated using the Chi-square test. Age, milk yield, BCS, and P4 levels of the groups were evaluated using One-Way ANOVA, and multiple comparisons were made with the Tukey HSD test. A paired-t test was used for paired comparison in the intra-group analyzes, multiple dependent groups were evaluated with Repeated Measures ANOVA, and multiple comparisons were calculated using Bonferroni correction. The data in the study were presented as mean ± standard error of the mean (SEM). Values of P <0.05 were considered statistically significant in the evaluation of results.

Results

The age, BCS, and milk yields of the cows in the groups showed no significant difference (P>0.05). The average age of all groups was 4.38-4.5 years (P=0.894), BCS ranged between 2.90 and 2.95 (P=0.843), and milk yield ranged between 20.8 and 22.1 L (P=0.175).

Although PR did not differ statistically among the groups, the PR was found to be higher in G2 compared to the other groups. The PR of G2 was approximately 16% higher than in G1, and there was a trend towards statistical significance (Table 1).

Although the highest luteolysis rate was 70.0% (21/30) in G2, a significant difference (P=0.183) was not observed. However, there was a statistical trend towards luteolysis formation in G2 and G3 (P=0.067, Table 1).

For pregnancy rates in cows with luteolysis, there was no significant difference between G1 and G2 (P=0.309). Similarly, no significant difference in PR was determined between G2 and G3 (P=0.2). However, a significant difference was found between G1 and G3 (P=0.04, Table 1).

Investigations were conducted based on the BCS, and it was observed that PGF2α injections at 24-hour intervals did not significantly affect the PR in cows with BCS≥3 (P=0.931). However, in cows with BCS<3, it was determined that PGF2α applications significantly increased their PR (G2: 70%) (P<0.05) compared to the G1 (G1: 20%) (Table 2).

Progesterone concentrations in cows at PGF2α injections and at the time of AI are presented in Table 3. It was found that serum P4 levels did not significantly differ on different days between groups (P>0.05). However, it was determined that the concentration of P4 significantly decreased within groups after PGF2α injections (P<0.001).

Progesterone concentrations were 0.82 ± 0.05 ng/mL in G1, 0.84 ± 0.05 ng/mL in G2, and 0.75 ± 0.07 ng/mL in G3. There was no significant difference in P4 concentrations between groups (P=0.51; Table 4).

Table 1. Luteolysis rate, pregnancy rate and pregnancy rates with luteolysis of groups.

Groups	Luteolysis, % (n/total)	Pregnancy rate of cows in all groups, % (n/total)	Pregnancy rates of cows with luteolysis, % (n/total)
Group 1	60.0 (18/30)	36.67 (11/30)	61.1^a (11/18)
Group 2	70.0 (21/30)	53.33 (16/30)	76.2 (16/21)
Group 3	46.7 (14/30)	43.33 (13/30)	92.9^b (13/14)
P value	0.183	0.431	0.040

$^{a-b}$: Shows statistically significant differences between groups in the same line (P<0.05).

Table 2. Pregnancy rates (%) according to body condition score of cows.

Groups	BCS <3		BCS ≥3		*P* value
	%	n/Total	%	n/Total	
Group 1	20[a]	2/10	45	9/20	0.180
Group 2	70[b]	7/10	45	9/20	0.196
Group 3	25[ab]	2/8	50	11/22	0.222
P Value	0.045		0.931		-
Total	39.29	11/28	46.77	29/62	0.704

For BCS<3; *P* levels were determined as *P*=0.025 between Group 1 and Group 2, *P*=0.80 between Group 1 and Group 3, and *P*=0.058 between Group 2 and Group 3 (BCS=body condition score). [a-b]: Shows statistically significant differences between groups in the same line.

Table 3. Progesterone concentrations (ng/mL) between groups and different days.

Groups	8th day	9th day	FTAI	*P* value
Group 1	1.85±0.13[a]	-	1.12±0.09[b]	<0.001
Group 2	2.06±0.05[a]	1.73±0.22[a]	1.01±0.05[b]	<0.001
Group 3	-	2.01±0.14[a]	1.17±0.08[b]	<0.001
P value	0.372	0.268	0.292	-

[a-b]: Shows statistically significant differences between groups in the same line (*P*<0.001). FTAI: Fixed Time artificial insemination. The data in the table were presented as mean ± standard error of the mean (SEM).

Table 4. Progesterone concentration (ng/mL) in cows with luteolysis.

Groups	Mean	Standard error of the mean (SEM)
Group 1	0.82	0.05
Group 2	0.84	0.05
Group 3	0.75	0.07
P value	0.510	

Discussion

The absence of complete luteolysis in dairy cows with FTAI practices is reported to reduce fertility (Ribeiro et al., 2012; Giordona et al., 2013). In dairy cows, two PGF2α injections at 8-hour intervals have been suggested to be more effective than a single PGF2α injection in inducing luteolysis (Hölper et al., 2023). It was determined that PR increased as a result of FTAI with PGF2α injected at intervals of 7-8 hours in beef cattle (Alnimer et al., 2019). In Simmental cattle, on the 7th and 8th days of OvSynch protocol, 29.5% PR was obtained in the classical OvSynch group, while it was 36.5% in the OvSynch group where a double dose of PGF2α was injected. In the beef cows that received the five-day CoSynch protocol, fertility rates were reported as 48%, 51%, and 55%, respectively, following a single dose of PGF2α on day 5, a double dose on day 5, and two doses on the 5th day at 8-hour intervals (Giordona et al., 2012). In Simmental cows, when synchronized with the seven-day OvSynch+CIDR protocol, a 9% increase in PR was observed, and a positive statistical trend was detected as a result of a second injection of PGF2α 24 hours after the first PGF2α injection (Kaçar et al., 2018). It was reported that PGF2α injections did not increase the PR in the cows administered the PRID-Synch protocol for seven and five days, with an interval of 24 hours. However, performing a second injection of PGF2α reduced the percentage of cows whose luteal regression was not completed, and a tendency to increase PR was determined by timed AI (Santos et al., 2016). In contrast to these studies, a study with the OvSynch protocol found no significant difference in ovulation and PR after the second GnRH application in cows with or without an additional PGF2α treatment one day after (Brusveen et al., 2009). In the presented study, the rate of luteolysis in the groups was not different. It was determined that PGF2α applied twice at twenty-four-hour intervals did not increase the rate of luteolysis compared to the other groups. The inconsistency in luteolysis rates and pregnancy outcomes across studies could stem from several factors. For instance, differences in breed-specific responses to hormonal treatments may play a significant role, as breeds exhibit distinct reproductive physiology and sensitivity to PGF2α. Additionally, the physiological status of the cows, such as parity, lactation stage, or the functional status of the CL at the time of treatment, can significantly influence treatment outcomes. Study-specific conditions, including variations in protocol implementation, timing of injections, or environmental stressors, may also contribute to the observed differences (Nascimento et al., 2014). Therefore, further research is needed to elucidate these differences and to optimize synchronization protocols by considering breed-specific responses, physiological conditions, and environmental influences. Such efforts could lead to a better understanding of how PGF2α injections can be utilized more effectively in improving luteolysis rates and overall reproductive performance.

To increase the regression of a newly created CL resulting from GnRH injection and optimize fertility, two doses of PGF2α administration were required. High P4 concentrations were found to suppress LH release and prevent ovulation when GnRH is administered (Lima et al., 2013). Increased circulating P4 concentrations around AI time create a suboptimal environment during the transport of sperm and ovum in the genital canal, resulting in decreased fertility (Brusveen et al., 2009). The circulating P4 levels above 0.5 ng/mL during timed AI were reported to reduce fertility by more than 50% (Souza et al., 2007). This was significantly effective in the regression of CL due to the large percentage of cows with high levels of P4 during the second GnRH treatment of the OvSynch protocol. Surprisingly, despite the significant increase in the percentage of cows with low P4 during the second GnRH (63.2% and 91.0%), it was observed that the pregnancy rate did not increase in the cows with high P4 concentrations at the second PGF2α treatment. It is possible that giving an additional dose of PGF2α earlier (approximately 12 hours following the normal PGF2α treatment) would lower the circulating P4 earlier and yield a positive effect on the PR (Brusveen et al., 2009). The ratio of cows with a low P4 concentration 48 hours after the first PGF2α treatment of the Ovsynch protocol was reported as 94% (Souza et al., 2007). In cows that received a second injection of PGF2α during the Ovsynch protocol, the proportion of cows with a P4 value lower than 0.4 ng/mL during the final GnRH administration was higher compared to the control group (95.6% and 84.6%, respectively). However, it has been demonstrated that the fertility of cows with complete luteolysis did not reflect that observation (Brusveen et al., 2009). In the present study, the number of cows with P4 levels <1 ng/mL during AI was higher, at 70%, in cows that received a PGF2α injection with a 24 hour-interval. However, this luteolysis rate was not reflected in the pregnancy rate. The lowest luteolysis rate was observed in cows that received PGF2α on the 9th day, at 46.7%. Interestingly, these cows were found to have a higher PR compared to other groups. According to these results, it may be suggested that luteolysis in this group was complete and sufficient, making the uterus more suitable for pregnancy. 24 hours after the first PGF2α application using the ReSynch protocol, a second injection of PGF2α has been shown to induce luteal regression fully and tends to increase the PR. However, it was found that a double dose of PGF2α injection administered at one time produced no effect (Barletta et al., 2018). On the 5th day of the sexual cycle, 41% of heifers responded to a single PGF2α treatment, whereas none of the lactating or non-lactating cows responded. On the seventh day, 88% of heifers and 90% of non-lactating cows responded to a single PGF2α treatment, whereas only 66% of lactating cows responded (Nascimento et al., 2014). Therefore, in this study, we found no differences in PR between the groups. However, PGF2α injections at 24-hour intervals showed a statistical trend towards increasing PR compared to the cows administered PGF2α on day 8 (G1). Despite the numerical difference in PR, the absence of a statistically significant difference can be attributed to the limited number of cows used in each group. This indicates that the sample size may be insufficient to detect significant differences. A larger sample size is needed in future studies to obtain more robust results and to understand better the effects of PGF2α injections on luteolysis and pregnancy rates. On the eighth day, PGF2α injection has been shown to reduce PR, suggesting that early and inadequate luteolysis may negatively affect fertility rates.

In Nelore breed cattle with progesterone-based AI, the pregnancy rate was determined to be 69.75% in cows with BCS≥2.75, whereas it was only 32.98% in cows with BCS <2.75. For protocols with FTAI applications, it was recommended that BCS should be at least 2.5 to ensure a positive energy balance in cows and heifers (Pereira et al., 2018). On the other hand, animals with higher BCS are expected to have higher levels of nutritional reserves, which can be observed from the distribution of body fat on the animal's body surface. The PR in cows with BCS≤2.5 was found to be lower at 28.7%, compared to the groups with BCSs of 2.75-3 (37%) and > 3.25 (40.9%) (Ribeiro et al., 2012). PGF2α injections at 24-hour intervals did not change the PR of the cows with BCS≥3 compared to other groups. Surprisingly, in cows with BCS<3, PGF2α injections at 24-hour intervals increased the PR compared to different groups. This finding has been interpreted as meaning that two PGF2α injections can provide complete luteolysis in cows with low BCS.

Conclusion

In conclusion, in P4-based FTAI applications, PGF2α injections with a 24-hour interval in increased PR in Holstein cows. In addition, the rate of luteolysis was higher in the cows in this group. In light of these findings, PGF2α injections at 24-hour intervals may be effective in improving fertility. However, these results should be further verified by additional research using larger sample sizes.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (19.09.2019, 2019/129 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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