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RESEARCH ARTICLE

The Effect of Different Follicular Waves and Serum Anti-Müllerian Hormone Levels on Superovulation Response in Dairy Heifers

Uğur Kara[1*], Davut Koca[2], Hakan Sağirkaya[3], Ali Osman Turgut[4], Abdil Burhaneddin Akkaya[5], Hasan Mutlu[6], Fatih Orhan[7] and Çiğdem Bolaç[2]

[1]Çukurova University, Faculty of Ceyhan Veterinary Medicine, Department of Obstetrics and Gynecology, Adana, Türkiye; [2]Yuzuncu Yil University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Van, Türkiye; [3]Uludağ University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial İnsemination, Bursa, Türkiye; [4]Siirt University, Faculty of Veterinary Medicine, Department of Animal Science, Siirt, Türkiye; [5]İdeal Animal Health Technologies Company, Afyonkarahisar, Türkiye; [6]Eastern Mediterranean Agricultural Research Institute, Adana, Türkiye; [7]Trakya Cattle Breeding Industry and Trade Inc., Kırklareli, Türkiye
*Corresponding author: ukara@cu.edu.tr

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ABSTRACT

Currently, there is a need for simplified protocols in *in vivo* embryo production and transfer programs, that can be simultaneously applied to a large number of donors, as well as for reliable methods to predict superovulatory responses of donors. The aim of this study was to investigate the effects of the first and second follicular wave, serum progesterone and anti-Müllerian hormone (AMH) concentrations on the superovulation response in dairy heifers. A total of 56 Holstein Friesian heifers were divided into two groups, and subjected to superovulation protocol at the first (FFD, n=28) or the second follicular wave (SFD, n=28), induced by GnRH treatment. Animal age, BCS and serum AMH levels before start of superovulation treatment differed non-significantly between the two groups, while serum progesterone was higher in SFD than FFD group ((4.41±0.36 v 2.03±0.41ng/mL; P<0.05). For superovulation in both groups, 450mg FSH was administered at 12h intervals, for a total of 8 decreasing doses. Donors were inseminated with sexed semen. Uterine flushings were performed on the 7th day of the following cycle. Non-significant differences were observed between FFD and SFD groups for total numbers of follicles, corpora lutea (CLs), total embryos, transferable embryos, Grade-1 embryos, degenerated embryos and unfertilized oocytes. Superovulation response in terms of total follicles, CLs, transferable embryos and Grade-1 embryos was higher (P<0.05) in cows with high serum AMH levels (480-920pg/mL) than cows with low serum AMH levels (160-280pg/mL) before start of superovulation treatment. In conclusion, progesterone levels at the beginning of superovulation and different follicular waves had no effect on superovulation response, while serum AMH level before the start of superovulation protocol could be used as a biomarker for donor selection.

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INTRODUCTION

In vivo embryo production (IVEP) has become an important biotechnical application in animal breeding programs aimed to enhance progress in genetic improvement and success of embryo transfer. The efficiency of this technology is largely dependent on the magnitude of the superovulatory response and the number of high quality embryos recovered from donor animals (Bó *et al.*, 2008; Bó and Mapletoft, 2014). Nevertheless, one of the principal constraints limiting the consistency of IVEP efficiency is the wide inter individual variations in ovarian responsiveness among donors (Bó *et al.*, 2008).

The evolution of superovulation protocols reflects a gradual shift from single dose equine chorionic gonadotropin (eCG/PMSG) treatments toward multiple dose follicle stimulating hormone (FSH) treatments. Studies on follicular wave dynamics revealed that synchronization of gonadotropin administration with the emergence of a follicular wave is a critical determinant of

superovulatory success (Nasser *et al.*, 1993; Mapletoft *et al.*, 2002). However, timing of follicular wave emergence can not be clearly detected in conventional protocols, resulting in mismatches between treatment initiation and ovarian physiological status, which negatively affect embryo yield and quality ((Nasser *et al.*, 1993).

To overcome these limitations, progress in real time ultrasonography has enabled the scientists to develop superovulation strategies based on follicular wave synchronization (Mapletoft *et al.*, 2002; Bó and Mapletoft, 2014). At present, three principal approaches have been developed to synchronize follicular wave in cattle. These are: the use of estradiol and progesterone combinations, administration of gonadotropin releasing hormone (GnRH) or luteinizing hormone (LH), and follicular ablation. Despite their effectiveness, each approach presents some limitations. The use of estradiol is prohibited in several countries. Follicular ablation requires specialized equipment and skilled personnel, while GnRH based protocols depend on the successful ovulation of the dominant follicle, which cannot be consistently ensured (Bó and Mapletoft, 2014; Mikkola *et al.*, 2019).

Numerous alternative approaches, such as modifications of FSH dosing schedule (Guerra *et al.*, 2012; Çizmeci *et al.*, 2023; Lalchhanhima *et al.*, 2025), controlled-release gonadotropin formulations (Deguettes *et al.*, 2020), use of recombinant FSH (Gutiérrez-Reinoso *et al.*, 2022), and adjunctive pre-superovulatory treatments (Bó and Mapletoft, 2014) have been investigated to enhance embryo yield and quality. However, donor related variations indicate that ovarian characteristics play an important role in the embryo production (Ireland *et al.*, 2007; Hashimoto *et al.*, 2023). Previous studies have also revealed that these variations may be related to the size of the follicular population present in the ovaries at the initiation of superovulation treatment (Ireland *et al.*, 2007).

Accordingly, the identification of reliable biomarkers for donor selection and prediction of superovulatory response has emerged as a priority area in reproductive research. Anti-Müllerian hormone (AMH), a glycoprotein member of the transforming growth factor-β family, is secreted by granulosa cells of growing antral follicles and is widely accepted as an indirect indicator of ovarian follicular reserve (Monniaux *et al.*, 2012). Several studies conducted in beef and dairy cattle have reported relationship between circulating AMH concentrations and both superovulatory response and embryo yield (Rico *et al.*, 2009; Souza *et al.*, 2015; Koca *et al.*, 2024a). However, studies that evaulated predictive value of AMH prior to superovulation in dairy heifers are still limited.

Fertility challenges associated with high milk yield, heat stress, and metabolic demands have increased the importance of IVEP and embryo transfer in dairy production systems (Miles *et al.*, 2023). On the other hand, the biological and economic sustainability of these techniques depends on optimizing superovulatory responses and accurately identifying donors with high embryo producing potential (Okawa *et al.*, 2025). However, the influence of follicular wave origin on follicular development, oocyte competence, and transferable embryo yield in dairy breeds has not been fully described. In the light of this situation, the objectives of the present study were: (a) to compare the superovulatory responses of the first and second follicular waves in dairy heifers; and (b) to evaluate the effect of serum AMH concentrations before the start of superovulation protocol on the superovulatory response in dairy heifers and to assess their potential utility as a biomarker for donors selection.

MATERIALS AND METHODS

Animals: The current study was conducted on a commercial dairy farm located in the Lüleburgaz district of Kırklareli province, Türkiye (41°24′1″ N, 27°24′38″ E) during the spring and the autumn of 2025. A total of 56 clinically healthy Holstein Friesian heifers aged between 13 and 15 months and weighing between 370 and 400kg were enrolled. All animals exhibited regular estrous cycles and showed no history or clinical evidence of any reproductive disorders. Body condition scores (BCS) were assessed using a 5-point scale as described by Ferguson *et al.* (1994); the values of BCS ranged between 3.00 and 3.50.

Experimental heifers were managed under similar barn and housing conditions. The diet consisted of cereal straw, alfalfa hay, wheat silage, corn silage, and a commercial heifer concentrate containing 20% crude protein, 3.80% ether extract, and 2634 kcal/kg metabolizable energy. The total mixed ration provided approximately 11.69kg of dry matter per animal per day.

Three weeks prior to the superovulation protocol, all 56 donor heifers received a single oral bolus containing omega-3 and omega-6 fatty acids and β-carotene (110g; Eicosapentaenoic acid 4.475%, Docosahexaenoic acid 13.424%, β-carotene 55mg; Fertibol®, Mazzoleni SPA, Italy). Fresh water and mineral blocks were available ad libitum. All animals were vaccinated routinely against Infectious Bovine Rhinotracheitis (IBR), Bovine Viral Diarrhea (BVD), Bovine Respiratory Syncytial Virus (BRSV), Bovine Parainfluenza-3 (PI3), enterotoxemia, mastitis, foot-and-mouth disease, and pasteurellosis according to the herd health program. Antiparasitic treatments for internal and external parasites were administered biannually. Before application of experimental treatments, transrectal ultrasonographic examinations of the uterus for any infection, and ovaries for ovarian structures and cyclic ovarian activity (based on the presence or absence of functional corpus luteum) were performed using a B-mode, real time linear array transducer of 7.5MHz frequency (Ibex® Pro, EI Medical Imaging, USA).

Experimental design: The experimental donor heifers (n=56) were randomly allocated into two experimental groups (n=28 per group). In the first group, superovulation treatment was initiated during the first follicular wave following GnRH-induced ovulation under progesterone support (FFW). In the second group, superovulation was performed during the second follicular wave following GnRH-induced ovulation of the dominant follicle from the first wave (SFW).

Superovulation treatment: All donor heifers of both groups received two intramuscular injections of cloprostenol sodium (500µg; Esrumate®, Vet Pharma Friesoythe GmbH, Germany) 14 days apart. In the FFW group, a progesterone-releasing intravaginal device (PRID Delta®, 1.55g

progesterone; Ceva Santé Animale, France) was inserted concurrently with the second $PGF_2\alpha$ injection (Day 0). On the evening of Day 6, (10µg) of buserelin acetate, an analogue of GnRH (Receptal®, Intervet International GmbH, Germany) was administered intramuscularly, and the intravaginal device was replaced with the new one. In the SFW group, estrus was assumed to have occurred three days (Day 0) after the second $PGF_2\alpha$ injection. On the evening of Day 6, (20µg) of buserelin acetate was administered intramuscularly. Double dose of GnRH was used in SFW group (20µg) compared to FFW group (10µg) keeping in view the report of Melo *et al.* (2024) that increasing the GnRH dose at high P4 concentrations in Holstein heifers positively affected the ovulation response of the dominant follicle. In both groups, ovulation of the dominant follicles was monitored by ultrasonographic examination (5–8 MHz; Ibex® Pro, EI Medical Imaging, USA) on day 8 (48h after GnRH injection). There were 28 heifers in each group, and each showed one dominant follicle present on their ovaries at the time of the initial GnRH treatment. Ultrasonography performed 48 hours after the first GnRH treatment in all donor heifers revealed that ovulation had occurred in the dominant follicles. Superovulation was induced in both groups using a total dose of 450µg of FSH (Stimufol®, Reprobiol SRL, Belgium), administered intramuscularly in eight decreasing doses at 12h intervals, starting on the evening of Day 8 (Mapletoft *et al.*, 2002; Nasser *et al.*, 2011). Beginning with the seventh FSH injection, three doses of cloprostenol sodium (500µg) were administered intramuscularly at 12h intervals. In the FFW group, the intravaginal device was removed at the time of the final FSH administration. Twenty-four hours after the last FSH dose, 20µg of buserelin acetate (GnRH analogue) was administered intramuscularly in both groups (Fig. 1). Artificial insemination was performed three times at 6h intervals beginning 16h after the final GnRH injection, using frozen-thawed sex-sorted semen (Ultraplus High Purity). At the time of the first insemination, trans-rectal ovarian ultrasonography was performed to determine the total number of follicles (diameter >10mm) present on the ovaries.

Embryo recovery and evaluation: On Day 7 of the subsequent estrous cycle, uterine flushing was performed for embryo recovery. Prior to embryo recovery, transrectal ultrasonography was conducted to record the number of corpora lutea (CLs) and remaining follicles. Epidural anesthesia was achieved using 4mL of a local anesthetic (Adokain®, Vem İlaç, Türkiye). Uterine flushing was carried out using 1000mL of lactated Ringer's solution supplemented with 1% fetal bovine serum and 0.1% gentamicin (Kara *et al.*, 2020). Following embryo recovery, each donor received an intramuscular injection of cloprostenol sodium (500µg) and intrauterine administration of cephapirin benzathine (500mg; Metrinew®, Alke Health Products, Türkiye).

Embryos were evaluated under a stereomicroscope and classified according to the morphological criteria established by the International Embryo Transfer Society (IETS; Robertson and Nelson, 2010). Embryos of grades 1, 2, or 3 were considered transferable (Fig. 2). Furthermore, to evaluate the effect of superovulation protocols on the developmental stage, embryos were divided into two separate categories: those in early developmental stages and those in advanced developmental stages. Embryos of morula, compact morula and early blastocyst stages were designated as Morphology-1 (M1) embryos, while those of blastocyst and expanded blastocyst stages were designated as Morphology-2 (M2) embryos (Bó and Mapletoft, 2013).

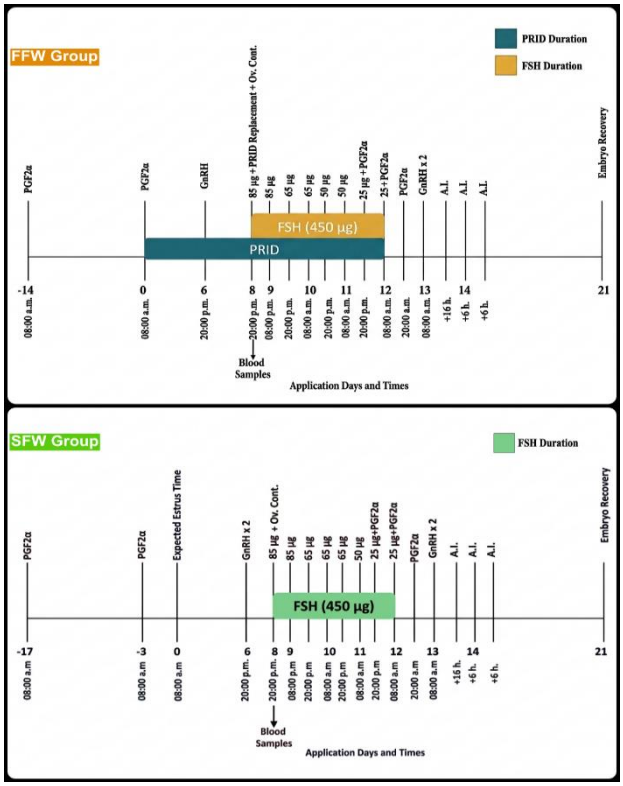

Fig. 1: Superovulation protocols applied in cows of FFW and SFW groups.

Transfer of embryos to recipients: Transferable embryos were washed in TCM-199 medium supplemented with gentamicin and 20% fetal calf serum at 38.5°C and transferred to recipient cows within 6h of collection. Embryo recipients were 253 lactating Holstein cows aged 2-6 years, calved normally around 67 days before. The embryos were transferred into the upper 1/3 of the uterine horn on the side of the corpus luteum of suitable recipients having a mature corpus luteum (≥2cm in diameter) on day-7 of estrus cycle (Kara *et al.*, 2020), determined through ultrasonography. Pregnancy examination in recipients was performed on day 28 after embryo transfer using transrectal ultrasonography with B-mode, real-time linear array transducer of 7.5-MHz frequency (Ibex® Pro, EI Medical Imaging, USA).

Blood collection and analysis: Blood samples for AMH determination were collected from the coccygeal vein on Day 5 following the first administration of prostaglandin $F_2\alpha$ ($PGF_2\alpha$). Samples were drawn into 9-mL serum tubes containing clot activator (Ayset®, Türkiye). A second set of blood samples was collected at the onset of FSH treatment to determine progesterone concentrations. Blood samples were centrifuged at 2000×g for 15min, serum was harvested and stored at −80°C until analysis for AMH or progesterone concentrations.

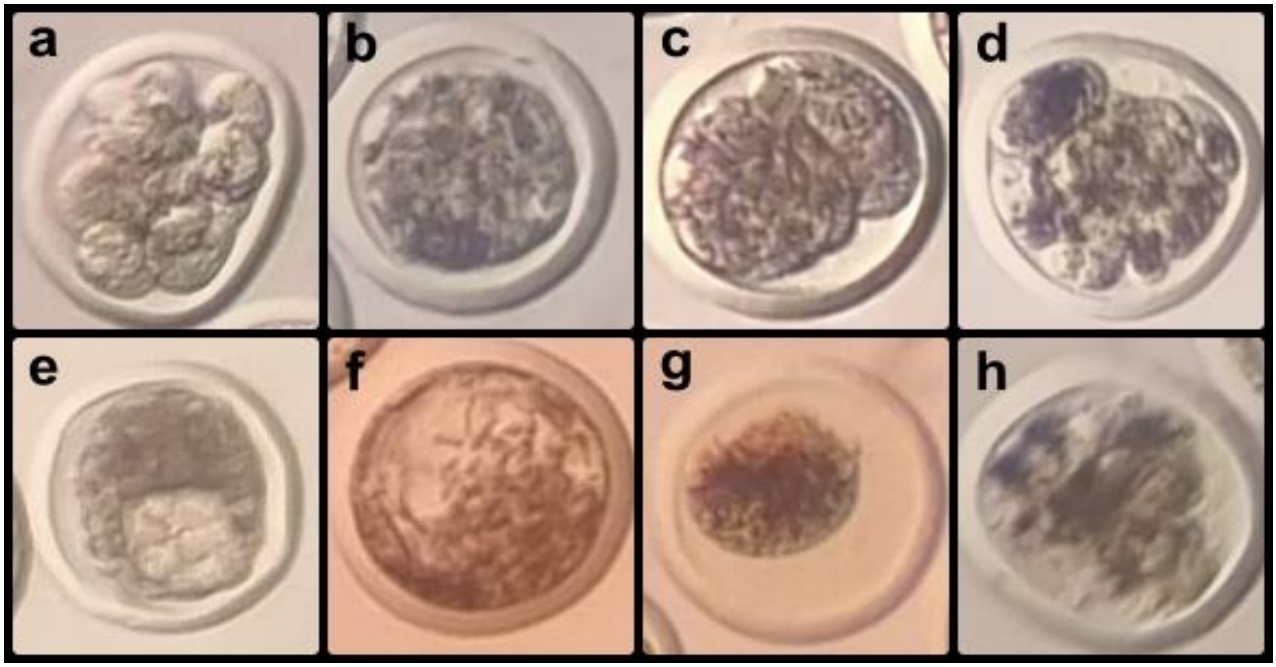

Fig. 2: Bovine embryos of different developmental stages and qualities obtained during the present study. a): morula; b): grade 1 compact morula; c): grade 2 compact morula; d): grade 3 compact morula; e): grade 1 early blastocytst; f): grade 1 blastocyst; g): unfertilized oocyte; h): degenerated embryo. The procedure was performed using a stereomicroscope with 80X magnification.

Serum concentrations of AMH and progesterone were quantified using an electrochemiluminescence immunoassay (ECLIA) method, as previously validated (Koca *et al.*, 2023; Koca *et al.*, 2024b; Turgut and Koca, 2024a,b; Cetin and Koca, 2025). Analyses were performed using Elecsys® AMH (Human) and Elecsys® Progesterone assays on a Cobas e601 analyzer (Roche Diagnostics, Germany). Assay validation, calibration procedures, and quality controls were conducted in accordance with the manufacturer's guidelines. The analytical sensitivities were 10.0pg/mL for AMH and 0.05ng/mL for progesterone. The coefficients of variation for intra-assay and inter-assay ranged from 0.6 to 1.5% for AMH and 0.8 to 1.8% for progesterone.

Statistical analysis: Statistical analyses of the data were performed using the Minitab 21 software. The Anderson–Darling normality test was used to evaluate the normal distribution of the data. Analysis of descriptive parameters (Age, BCS, AMH) and comparison of progesterone levels, numbers of total follicles, total CLs, total embryos, transferable embryos and first quality embryos between protocols were performed using Independent Samples t-test. Comparisons of donor superovulation responses between groups, including anovulatory follicles, morphology-1 (M1) and morphology-2 (M2) embryos, degenerated embryos, and unfertilized oocyte counts, were performed using the Mann–Whitney U test. To evaluate the effect of AMH regardless of superovulation protocols, all donors were divided into three groups: low (160-280pg/mL, n=19), medium (290-440pg/mL, n=19), and high (480-920pg/mL, n=18). Regardless of the protocol, possibe effects of donors' AMH levels on superovulation response, anovulatory follicle, M1 and M2 embryo, degenerated embryo and unfertilized oocyte numbers were analyzed using the Kruskal–Wallis test. Comparison of the numbers of total follicles, total corpora lutea, total embryos, transferable embryos and Grade-I embryos between G1, G2, G3 were performed using one-way ANOVA. Tukey's multiple comparison test was used as post-hoc analysis to compare all possible pairwise differences between groups. Analyses of pregnancy success rates of embryos obtained were performed using Chi-Square test. Statistical significance levels were determined as $P<0.05$.

RESULTS

In this study, 56 Holstein Friesian heifers were used as donors. Non-significant differences were recorded between experimental groups with respect to age, BCS, or serum anti-Müllerian hormone (AMH) concentrations at the start of the protocol (Table 1). Ultrasonography showed that in both groups, before starting FSH applications, ovulation of the dominant follicles and new follicular wave synchronization were achieved with GnRH in all donors. At the beginning of FSH administration, serum progesterone concentrations were significantly lower ($P<0.05$) in the FFW group than in the SFW group (2.03±0.41 vs. 4.41±0.36ng/mL, respectively (Table 1).

Table 1: Descriptive statistics about serum AMH levels, age, BCS and serum progesterone levels for cows of FFW and SFW groups

Variable	Groups	Mean±SEM	Minimum	Maximum
AMH (pg/mL)	FFW	417±33.1[a]	180	920
	SFW	365±30.7[a]	160	840
Age (months)	FFW	14.83±0.30[a]	12.400	17.300
	SFW	15.89 ± 0.33[a]	12.200	19.600
BCS	FFW	3.26±0.04[a]	3.0000	3.5000
	SFW	3.39±0.04[a]	3.0000	4.0000
P4 (ng/ml)	FFW	2.03 ± 0.41[b]	0.050	7.060
	SFW	4.41 ± 0.36[a]	0.070	6.920

Note: Differences in mean values between the two groups for each parameter are statistically non-significant.

Superovulation response: There were 28 heifers in each group, and, each showed one dominant follicle present on

their ultrasonographic examinations at the time of the initial GnRH treatment. Ultrasonography performed 48 hours after the first GnRH treatment in all donor heifers revealed that ovulation had occurred in the dominant follicles. As shown in Table 2, there were non-significant differences in total number of follicles, corpora lutea, non-ovulated follicles and unfertilized oocytes between FFW and SFW groups. Similarly, the differences in numbers of transferable embryos, grade-1 embryos and degenerated embryos, obtained in the FFW and SFW groups were non-significant. When evaluated according to embryo morphology and developmental stages, no difference was found between M1 (morula, compact morula and early blastocyst) and M2 (blastocyst and expanded blastocyst) embryos between the two groups. The developmental stages and quality of embryos obtained from developing follicles of different follicular waves also did not differ. Thus, no differences were observed between the FFW and SFW groups in terms of donor superovulation response and embryo yield. Moreover, differences in serum progesterone levels recorded at the beginning of FSH administration also had no effect on the superovulation response and embryo yield.

Table 2: Comparison of superovulation response parameters between cows of FFW and SFW groups

Variables (numbers)	Groups	Mean±SEM	Minimum	Maximum
Total follicles	FFW	15.82 ± 0.91[a]	5.000	25.000
	SFW	15.36 ± 1.11[a]	4.00	27.00
Total corpora lutea	FFW	12.96 ± 0.99[a]	4.000	22.000
	SFW	11.68 ± 1.12[a]	1.00	26.00
Total embryos	FFW	8.21 ± 0.88[a]	0.000	21.000
	SFW	7.75 ± 0,99[a]	0.00	25.00
Transferable embryos	FFW	7.57 ± 0.84[a]	0.000	19.000
	SFW	7.03 ± 0.97[a]	0.000	23.000
Grade I embryos	FFW	6.67 ± 0.82[a]	0.000	18.000
	SFW	5.64 ± 0.98[a]	0.000	23.000
Degenerated embryos	FFW	0.64 ± 0.24[a]	0.000	6.000
	SFW	0.71 ± 0.16[a]	0.000	3.000
Unfertilized oocytes	FFW	0.28 ± 0.12[a]	0.000	3.000
	SFW	0.60 ± 0.19[a]	0.000	4.000
Unovulated follicles	FFW	1.75 ± 0.33[a]	0.000	6.000
	SFW	1.53 ± 0.26[a]	0.000	4.000
M1 embryos*	FFW	2.28 ± 0.65[a]	0.000	15.000
	SFW	2.75 ± 0.41[a]	0.000	8.000
M2 embryos*	FFW	5.17 ± 0.61[a]	0.000	12.000
	SFW	4.25 ± 0.81[a]	0.000	15.000

Values with different superscript letters within a column for each parameter show significant differences between groups (P<0.05). *M1 embryos: morula, compact morula and early blastocyst stages embryos. M2 embryos: blastocyst and expanded blastocyst stages embryos.

Effect of serum AMH levels on superovulation response and embryo yield: In the present study, superovulation response was significantly higher (P<0.05) in the high-AMH group (G3) than in the low-AMH group (G1). Moreover, significant differences were observed in different ovarian superovulation response and embryo yield parameters among the groups based on serum AMH levels (Table 3). The mean values for total numbers of follicles, corpora lutea, total embryos, transferable embryos and grade-1 embryos were the lowest in the low AMH group (G1) and highest in the high AMH group (G3), the difference was significant (P<0.05). However, there were non-significant differences in these parameters between groups G1 and G2, as well as between groups G2 and G3. However, the effects of serum AMH level before initiation of superovulation protocol on the number of unovulated follicles, unfertilized oocytes, degenerated embryos, M1 and M2 embryos were non-significant, although the value was slightly higher in SFW than in FFW group (Table 3).

Table 3: Effects of different AMH levels on superovulation response parameters in donor cows

Variables (numbers)	Groups	n	Mean±SEM	Minimum	Maximum
Total follicles	G1	19	12.95 ± 1.14[b]	4.00	24.00
	G2	19	16.16 ± 1.09[ab]	9.00	25.00
	G3	18	17.7 ± 1.26[a]	5.00	27.00
Total corpora lutea	G1	19	9.37 ± 1.09[b]	1.00	18.00
	G2	19	12.58 ± 1.11[ab]	4.00	21.00
	G3	18	15.17 ± 1.36[a]	4.00	26.00
Total embryos	G1	19	2.44 ± 0.88[b]	0.00	11.00
	G2	19	5.61 ± 1.51[ab]	0.00	23.00
	G3	18	7.20 ± 0.87[a]	0.00	15.00
Transferable embryos	G1	19	5.05 ± 0.77[b]	0.00	11.00
	G2	19	7.89 ± 0.80[ab]	3.00	15.00
	G3	18	9.06 ± 1.48[a]	0.00	23.00
Grade I embryos	G1	19	3.84 ± 0.77[b]	0.00	10.00
	G2	19	6.84 ± 0.73[ab]	3.00	15.00
	G3	18	7.89 ± 1.51[a]	0.00	23.00
Degenerated embryos	G1	19	0.42 ± 0.19[a]	0.00	3.00
	G2	19	0.73 ± 0.15[a]	0.00	2.00
	G3	18	0.88 ± 0.36[a]	0.00	6.00
Unfertilized oocytes	G1	19	0.42 ± 0.19[a]	0.00	3.00
	G2	19	0.26 ± 0.10[a]	0.00	1.000
	G3	18	0.66 ± 0.28[a]	0.00	4.00
Unovulated follicles	G1	19	1.55 ± 0.32[a]	0.00	4.00
	G2	19	2.05 ± 0.44[a]	0.00	6.00
	G3	18	1.66 ± 0.33[a]	0.00	4.00
M1 embryos	G1	19	1.78 ± 0.49[a]	0.00	6.00
	G2	19	2.89 ± 0.82[a]	0.00	15.00
	G3	18	2.88 ± 0.64[a]	0.00	10.00
M2 embryos	G1	19	3.15 ± 0.73[a]	0.00	9.00
	G2	19	5.00 ± 0.79[a]	0.00	12.00
	G3	18	6.06 ± 1.01[a]	0.00	15.00

Mean values with different superscript letters in a column for each parameter show significant differences among groups (P<0.05). M1embryos: morula, compact morula and early blastocyst stages embryos. M2 embryos: blastocyst and expanded blastocyst stages embryos.

Pregnancy rates: In this study, a total of 140 embryos were transferred from donors in the FFW group, including 126 embryos of grade I, 12 of grade II, and 2 of grade III. In SWF group, 113 embryos were transferred, including 103 of grade I, 8 of grade II, and 2 of grade III. Results showed non-significant difference between the FFW and SFW groups in terms of pregnancy rates at day 28 after embryos were transferred to recipients (Fig. 3).

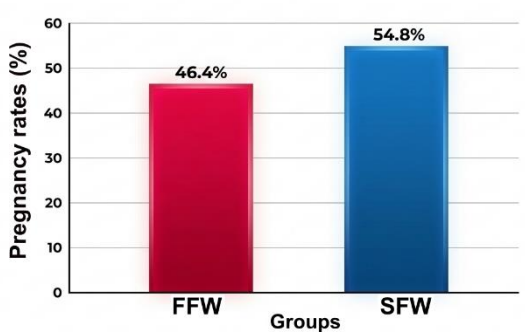

Fig. 3: Comparison of pregnancy rates between cows of FFW and SFW groups.

DISCUSSION

In recent years, research on superovulation protocols in cattle has primarily focused on developing strategies that eliminate the need for estrus detection, allow simultaneous application to large numbers of donors, and enable fixed-time interventions. In this context, protocols based on synchronization of a new follicular wave using GnRH offer a major practical advantage by removing the requirement for estrus monitoring in donor animals (Mapletoft and Bó, 2013). However, the success of these approaches depends on the successful induction of ovulation of the existing dominant follicle following GnRH administration (Martinez *et al.*, 1999).

The ovulatory response to GnRH is known to be influenced by multiple factors such as size and functional status of the dominant follicle, the stage of the estrous cycle, and the steroid hormone levels (Atkins *et al.*, 2008; Colazo *et al.*, 2008). Previous studies in lactating dairy cows have demonstrated that ovulation rates following GnRH treatment applied at random stages of the cycle are relatively low, and that increasing dose of GnRH may improve ovulation response (Colazo *et al.*, 2009; Sartori *et al.*, 2023). Similarly, increase in ovulation rates was reported in Holstein heifers following administration of higher GnRH doses (Melo *et al.*, 2024). These findings indicate the pivotal role of both GnRH dosage and timing in achieving effective follicular wave synchronization.

Following GnRH induced ovulation of the dominant follicle, initiation of FSH treatment for superovulation is generally recommended within 36-60h that coincide with the emergence of a new follicular wave (Bó and Mapletoft, 2014). In Holstein heifers, ovulation has been reported to occur between 23 and 54h after GnRH administration, with the subsequent follicular wave emerging approximately two days later (Picard-Hagen *et al.*, 2015). Consistent with these observations, ovulation of dominant follicles was confirmed in all donors in both groups in this study via ultrasonographic examination 48h after GnRH treatment.

Based on these physiological considerations, the present study evaluated the suitability of superovulation treatment during the first follicular wave by modifying a conventional protocol. Donors were presynchronized using two doses of cloprostenol sodium administered at 14 days interval to synchronize the first and second follicular waves. To enhance ovulation induction of the dominant follicle during diestrus in second follicular wave, 20µg GnRH was administered. These procedures are supported by those of a previous report by Melo *et al.* (2024). The results demonstrated successful ovulation following GnRH treatment in both experimental groups, indicating effectively synchronization of follicular dynamics in both groups.

Rivera *et al.* (2011), in their studies on lactating donor cows, reported that in the group that underwent superovulation after the first follicular wave synchronization without exogenous progesterone support, a P4 concentration of <1ng/ml during the growth of ovulation follicles reduced embryo quality by either lowering oocyte quality or slowing embryo development until day 7. According to these workers, exogenous P4 supplementation is necessary for optimal follicular development in superovulation treatments during the first follicular wave. In this study, serum P4 concentrations at the initiation of superovulation treatment were lower in the FFW group than in the SFW group. This situation may be related to the presence of an active corpus luteum acting as an endogenous P4 source during the second wave in SFW group (Rivera *et al.,* 2011).

Low P4 concentrations (<1ng/mL) during follicular development is associated with increased LH pulsatility, premature follicular and oocyte maturation, and decreased embryo quality (Inskeep, 2004). This issue is particularly important for follicles developing during the first follicular wave when P4 levels are low. Therefore, exogenous P4 supplementation has been proposed as an alternative strategy to improve parameters related to the superovulation during this period (Nasser *et al.*, 2011). In the present study, it was observed that exogenous P4 treatment through the use of PRID during the first follicular wave did not affect number of transferable embryos or embryo quality. Instead, number of transferable embryos and embryo quality were comparable to those obtained during the second follicular wave. Moreover, total follicles and CLs count are key indicators of superovulation response. In this study, no differences were observed between groups in terms of total follicle or CL numbers, indicating that an effective superovulatory response can be achieved during the first follicular wave by P4 supplementation and GnRH synchronization in dairy heifers. These findings are supported by those of Nasser *et al.* (2011).

In our study, the numbers of transferable embryos obtained in the FFW and SFW groups (7.57±0.84 and 7.03±0.97, respectively) were comparable. Additionally, we obtained first-quality embryo numbers of 6.67±0.82 and 5.67±0.98 in the FFW and SFW groups, respectively ($P>0.05$). Similarly, Rivera *et al.* (2011) reported the number of transferable embryos in the first and second follicular wave groups supplemented with exogenous P4 as 8.1±2.1 and 5.0±1.6, and the number of first-quality embryos as 7.0±2.0 and 4.7±1.6, respectively. These findings support the critical role of the P4 treatment on embryo quality during follicular development. Moreover, the absence of differences in the numbers of degenerated embryos, unfertilized oocytes, and anovulatory follicles between FFW and SFW groups indicates the reliability and safety of the applied protocol during the first follicular wave in this study. In our study, the results of the first follicular wave superovulation protocol supported by exogenous P4 were comparable to those obtained in superovulation treatments applied during the second follicular wave (with active corpus luteum present on the ovaries), which were designed similarly to traditional superovulation protocols. The fact that these results are consistent with those of Rivera *et al.* (2011) in lactating dairy donors and Nasser *et al.* (2011) in Nelore (*Bos indicus*) donors reveals two important suggestions; 1): Elimination of the need to wait until the middle of the estrus cycle for the the second follicular wave usually done in traditional superovulation protocols; and 2): Adoptation of fixed-time superovulation in dairy heifers by synchronizing the first follicular wave with exogenous P4 support.

Additionally, in the present study, developmental stages of embryos obtained from both groups were evaluated. The embryos were classified into two categories

according to their developmental stage: M1 embryos (morula, compact morula, and early blastocyst stages) and M2 embryos (blastocyst and expanded blastocyst stages). However, there were no differences in developmental stages of embryos derived from follicles that developed under different P4 conditions during distinct follicular waves. These findings support the idea that superovulation can be effectively achieved with external P4 supplementation during the first follicular wave.

Individual variability in superovulatory response remains one of the major limitations to the common application of this approach (Mikkola *et al.*, 2019). The success of superovulation protocols in cattle and the superovulatory response of donors is evaluated according to the number of transferable embryos obtained and the increase in embryo numbers (Bó *et al.*, 2010). In this study, it was observed that donors with higher serum anti-Müllerian hormone (AMH) concentrations had significantly higher superovulatory responses in terms of total follicle number, corpus luteum number, total embryo yield, number of transferable embryos and number of G-1 embryos. A strong correlation has been reported between serum AMH concentration and the gonadotropin-responsive antral follicle population in the ovaries of cows (Rico *et al.*, 2009). Studies in dairy cows (Souza *et al.*, 2015; Aziz *et al.*, 2017; Koca *et al.*, 2024a), Simmental cows (Sevgi *et al.*, 2019), and beef cows (Torres-Simental *et al.*, 2021) have also reported a strong correlation between serum AMH levels and superovulation response. These findings confirm the role of AMH as a reliable biomarker in predicting superovulation performance. Finally, pregnancy rates following transfer of embryos derived from different follicular waves were comparable between the two groups, indicating similar developmental ability of embryos.

Conclusions: In conclusion, fixed-time superovulation protocols applied following the synchronization of different follicular waves induced by GnRH had no effect on superovulation responses and embryo yields in Holstein heifers. Moreover, superovulation can be effectively achieved during the first follicular wave in dairy heifers with appropriate exogenous P4 support. AMH has emerged as a promising biomarker for the selection of donor heifers with superior ovarian reserves and higher embryo production potential, thus offering a practical tool to improve the efficiency of superovulation programs. Overall, these results highlight the need for further research to improve simplified, fixed-time, and practical superovulation strategies, validate reliable biomarkers for predicting ovarian reserve and embryo yield, and evaluate their integration into routine in vivo embryo production programs.

Ethical approval: The experimental procedures used in the present study were approved by the Research Ethics Committee of Tekirdağ Namık Kemal University, Local Ethics Committee for Animal Experiments (Ethics Approval Number: T2025-2799/01), Turkey.

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