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The effect of a special adjuvant with FSH on blood FSH level, superstimulation and embryo quality in dairy cattle

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Research Article

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Abstract

Follicle-stimulating hormone (FSH) must be applied at 12-h intervals over 4–5 days in the traditional cattle superovulation protocol, which still needs to be improved. This research paper evaluated the superovulation results obtained by a traditional protocol or by a single administration of FSH dissolved in Montanide™ ISA-206 VG (MonISA-206). Control cows were superovulated with 10 mL of FSH (500 µg pFSH + 100 µg pLH) from day 7 to day 10 (for 4 days, twice daily i.m. injections, decreasing doses). Cows in the EG10 and EG7.5 groups were injected i.m. with 20 mL (100%, 10 mL + 10 mL) or 15 mL (75%, 7.5 mL + 7.5 mL) of the FSH and MonISA-206 mixture at once on day 7. All cows were inseminated 12 and 24 h after oestrus onset. The cows presented no pathology at the injection sites. Plasma FSH levels differed between the groups, but the interaction between hour and group × time was not different. Superstimulation and embryo quality results were similar between the groups. A single injection of FSH (both 100% and 75% doses) dissolved in MonISA-206 led to adequate plasma FSH levels and similar superovulation results to traditional FSH treatment, and caused no pathology at the injection sites.

Introduction

Embryo transfer (ET), defined as the transfer of *in vivo* or *in vitro* produced embryos into the *cornu uteri* of a recipient, has been widely used for approximately five decades to improve the quality of production in farm animals (Choudhary *et al.*, 2016; Ünay *et al.*, 2024). The ET process consists of several stages, including the very critical superovulation (Sevgi *et al.*, 2019; Deguettes *et al.*, 2020; Ongaratto *et al.*, 2020). Follicle-stimulating hormone (FSH) is the most commonly and efficiently used agent for follicle stimulation, as it controls the follicular wave in the ovary (Deguettes *et al.*, 2020; Jahnke and Youngs, 2021). However, a disadvantage of using FSH in traditional superovulation applications is that, due to its short half-life (5 h or less), it must be applied repeatedly at 12-h intervals over 4 or 5 days to induce superovulation successfully (Bülbül *et al.*, 2013; Bó *et al.*, 2018; Gutiérrez-Reinoso *et al.*, 2023). It is emphasised that stress caused by these repeated injections (Bó and Mapletoft, 2014) and during animal handling (Edwards *et al.*, 1987) in donor animals and limits superovulation success by altering the preovulatory LH surge (Stoebel and Moberg, 1982). Simple application protocols are needed to reduce the extra care and labour needed, minimize mistakes, reduce the stress in donor animals and make monitoring of animals easier in cattle (Tríbulo *et al.*, 2012; Bó *et al.*, 2018; Gutiérrez-Reinoso *et al.*, 2023). Kimura (2016) also reported that a single administration of FSH for superovulation was simpler and more user-friendly, minimised the damage to the injection site and reduced the stress in cattle. Studies on the use of FSH have widely focused on both the slow release of FSH and its different chemical structures, especially to lengthen its $t_{1/2}$ (Ben-Menahem, 2018; Deguettes *et al.*, 2020; Gutiérrez-Reinoso *et al.*, 2023). For this purpose, FSH has been used by many researchers in studies together with polyvinylpyrrolidone (PVP), hyaluronan or aluminium hydroxide (Takedomi *et al.*, 1995; Tríbulo *et al.*, 2011; Biancucci *et al.*, 2016; Kimura, 2016). However, the inability of FSH to mix sufficiently with hyaluronan and

distribute it homogeneously in PVP solutions due to the high viscosity of PVP and the very low therapeutic index of aluminium hydroxide gel (Al-gel) have limited the use of these substances (Lindblad, 2004; Tríbulo *et al.*, 2011; Biancucci *et al.*, 2016).

Montanide™ ISA-206 VG (MonISA-206) emulsions, which have been used as adjuvants for several years, especially in vaccines and some biological fluids that are expected to be released for a long time, consist of two liquids that do not mix (Waghmare *et al.*, 2014; Ibrahim *et al.*, 2015). It is a physical water-in-oil-in-water (w/o/w) emulsion that does not contain substances of animal origin (Balcaen *et al.*, 2016). A water particle dispersed in the oil globule is the internal phase in a second liquid particle, which is the continuous phase. While release from the aqueous phase in the outer part of the emulsion is rapid, the release from the aqueous phase in the inner part is slow (Dar *et al.*, 2013). Owing to this feature, it has been used for decades as a carrier or slow release agent, especially for double emulsions, vaccines, anticancer drugs, hormones and steroids (Cole and Whateley, 1997; Vlaia *et al.*, 2009; Dar *et al.*, 2013; Balcaen *et al.*, 2016). In addition, MonISA-206 is well tolerated by the body, has a low viscosity, can be stored at refrigerator temperature for more than a year and has no toxicity (Cofrades *et al.*, 2013; Waghmare *et al.*, 2014; Balcaen *et al.*, 2016).

There are few studies using MonISA-206 as an adjuvant for superstimulation in cattle. In a recent ovum pick-up study, Ciftci and Dinc (2023) reported similar oocyte responses in Holstein heifers, both of which were superstimulated with a single dose of FSH dissolved in MonISA-206 or repeated doses of FSH (in four equal doses 12 h apart). In another study (Cizmeci *et al.*, 2022), which examines the number of corpus luteum (CL) but not including the number/quality of embryos, an s.c. single dose of FSH dissolved in MonISA-206 administered as an adjuvant yielded CL results similar to those of the repeated FSH injections (in eight decreasing doses 12 h apart). Although there are few superstimulation studies (Ciftci and Dinc, 2023), this is the first report using Montanide™ ISA-206 VG, determining embryo quality and including FSH and MonISA-206 dose optimisation. In this study, we aimed to evaluate plasma FSH levels and the superovulatory effects of traditional FSH treatment (two daily injections, eight decreasing doses) or with a single administration of FSH dissolved in a specific adjuvant (Montanide™ ISA-206 VG). A single application of FSH+adjuvant was carried out at two different doses (100% vs 75%).

Materials and methods

Ethical statements

All procedures and protocols in the present study were approved by the International Center for Livestock Research and Training (Lalahan, Ankara) and the Animal Experiments Local Ethics Committee (Protocol Number: 27.12.2017/147).

Materials and animal management

This study was carried out at the International Livestock Research and Training Center Farm (39°58′14.16″ N latitude and 33°6′31.35″ E longitude, and an altitude of 1080 m above sea level) on 21 lactating Holstein cows with body condition scores (BCSs) between 3 and 3.5 (1–5 scale). All the cows were clinically healthy, normally cyclic (having at least two oestrous cycles of normal length, 20–22 days) and had no retention of placenta, metritis, mastitis, artificial insemination or mating after parturition. Animals were randomly allocated to three similar groups according to their age (month), daily milk yield (L), live weight (kg) and days in milk (days) data (Table 1).

Table 1. The characteristics of cows in the CG, EG10 and EG7.5 groups (±SEM)

Parameter	CG	EG10	EG7.5	*P*
n	7	7	7	
Age (month)	42.14 ± 3.28	42.14 ± 1.81	42.71 ± 2.65	0.985
Daily milk yield (L)	29.96 ± 1.43	29.79 ± 1.36	29.04 ± 1.45	0.889
Live weight (kg)	648.24 ± 2.49	651.60 ± 4.69	650.61 ± 3.28	0.797
Days in milk (days)	87.71 ± 6.19	85.00 ± 8.11	90.86 ± 7.85	0.857

There was no significant difference in age, daily milk yield, live weight and days in milk data of the cows in the CG, EG10 and EG7.5 groups ($P > 0.05$).

Table 2. Diet composition of the cows in the study

Ingredients	Amount (kg)
Corn silage	20
Wheat straw	3
Meadow grass	5
Alfalfa hay	3
Concentrate feed[a]	12
Bypass-oil[b]	0.5

The ratio was adjusted according to the National Research Council (2001) to provide nutrients for the cows. The animals were fed twice daily at 07:00 and 19:00 at a 60/40 forage/concentrate level according to the TMR (whole ration: complete feed).
[a]Concentrate feed includes 14.8% crude protein and 2.20 mcal/kg DM energy.
[b]Bypass-oil (Megalac, Volac International Ltd).

All animals were maintained and fed under a similar management system. They were housed in free stalls with self-locking head gates on the feed line. Animals were fed twice daily at 07:00 and 19:00 at a 60/40 forage/concentrate ratio according to Total Ration: Complete Feed (TMR) with the dietary composition adjusted according to the National Research Council (2021) (Table 2). They were supplemented with vitamins and minerals to meet their nutritional requirements and had *ad libitum* access to fresh water throughout the experiment.

Preparation of the FSH and MonISA-206 mixture

The adjuvant used in the study was purchased from a commercial firm (Montanide™ ISA-206 VG, Seppic, France), filtered through an injector filter (0.22 µm, Merck, Millex, Germany) and then sterilised. Lyophilised FSH powder (Stimufol, total: 500 µg pFSH + 100 µg pLH, Reprobiol®, Belgium) was diluted in 10 mL of diluent from the kit. Diluted FSH and MonISA-206 in a 50:50 (v/v; 10 mL + 10 mL) ratio were homogeneously mixed by vortexing for 10 min via an automatic mixer (Stuart SA8 VORTEX, UK) at a low shear rate (300 rpm) at 30 °C to form a w/o/w emulsion (Dar *et al.*, 2013).

Superovulatory treatments and artificial inseminations

The experimental design is illustrated in Fig. 1. All cows received a controlled intravaginal drug release (CIDR) containing 1.38 g

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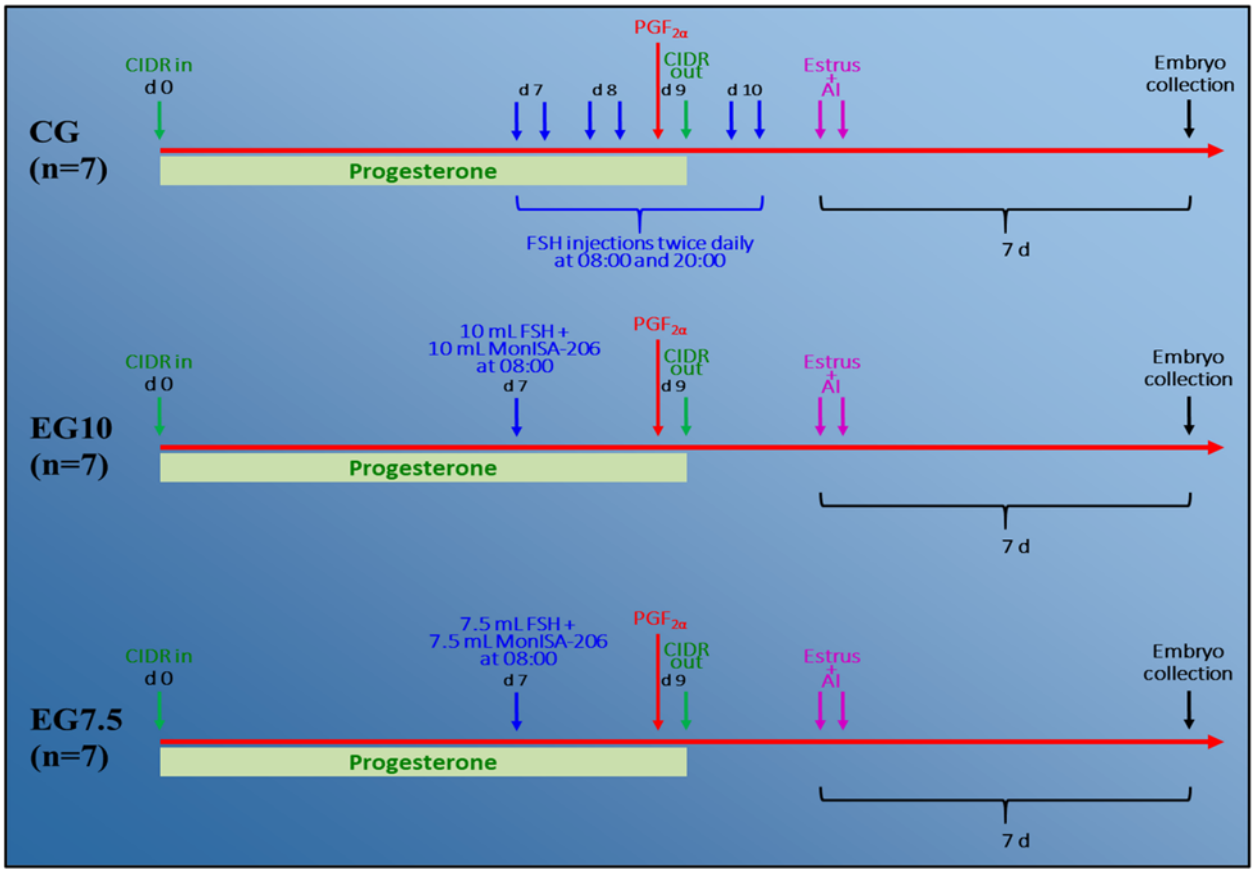

Figure 1. Experimental design for the groups. AI: artificial insemination; CIDR: controlled intravaginal drug release; d: day; FSH: follicle-stimulating hormone; MonISA-206: Montanide™ ISA-206 VG; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$.

of progesterone (Eazi Breed™ CIDR®, Pfizer, Australia) at a random stage of the oestrous cycle (day 0) (Sevgi *et al.*, 2019; Ünay *et al.*, 2024). In the control group (CG) ($n = 7$), cows were injected with 10 mL of FSH (Stimufol, total: 500 µg pFSH + 100 µg pLH, Reprobiol®, Belgium) from day 7 to day 10 (4 days in total) twice daily (at 08:00 and 20:00) i.m. injections with decreasing doses (2, 2, 1.5, 1.5, 1, 1, 0.5 and 0.5) (traditional protocol) (Biancucci *et al.*, 2016; Gutiérrez-Reinoso *et al.*, 2023; Ratsiri *et al.*, 2021). Cows in the second group (EG10) ($n = 7$) were injected i.m. with 20 mL (into the neck area on the right and left sides as 10 + 10 mL) of Stimufol and MonISA-206 mixture (10 mL Stimufol [500 µg pFSH + 100 µg pLH] + 10 mL MonISA-206) at once on day 7 at 08:00. In the third group (EG7.5) ($n = 7$), the cows were superstimulated as in the EG10 group with 15 mL (in the neck area on the right and left sides as 7.5 + 7.5 mL) of Stimufol and MonISA-206 mixture (7.5 mL Stimufol [375 µg pFSH + 75 µg pLH] + 7.5 mL MonISA-206) at once injected i.m. on day 7 at 08:00. All cows in all groups were injected i.m. with 25 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (dinoprost, Dinolytic, Pfizer, Turkey) at 08:00 h and the CIDR was removed from the vagina at 20:00 h on day 9.

Following CIDR removal, all cows in the groups were visually checked for oestrus (standing heat, vaginal discharge) three times a day (30 min each). Rectal palpation also verified oestrus (fluctuant dominant follicles and uterine tonus). All cows showed oestrus 30–36 h after CIDR removal and were inseminated artificially (AI) twice, 12 and 24 h after the onset of oestrus by a skilled technician using frozen-thawed semen (20×10^6 spermatozoa/straw with approximately 60% progressive motility after thawing) from the same bull.

Superovulation response assessment, embryo collection and evaluation

Superovulation response assessment, embryo collection and evaluation were performed 7 days after the first AI. Superovulation response was evaluated by rectal palpation and ultrasonographical examination (7.5 MHz linear-array transrectal probe, HASVET, Mindray® DP50, China) of the ovaries according to the number of corpora lutea.

Embryo collection was carried out as described previously (Sevgi *et al.*, 2019). Briefly, each cornu uterus was flushed separately via a non-surgical method after epidural anaesthesia (Adokain, Sanovel) with a disposable two-way Foley catheter (Bioniche, Belleville, CANADA) inserted into the uterine horn through the cervix. Ringer's lactate (500 mL for each cornu uterus, Ringer VIP, Polifarma, Turkey) containing 1% foetal calf serum (FCS) (N-4267, Sigma-Aldrich, St. Louis, MI, USA) and 0.125% kanamycin sulphate (Kanovet, Vetaş, Turkey) was used for flushing medium. After the recovered lavage fluid was filtered (72 µm Emcon filter, Agtech, USA), the embryos were transferred to a 90-mm Petri dishes containing phosphate buffer solution supplemented with 20% FCS + 0.4% bovine serum albumin, and evaluated under a stereomicroscope (Eclipse TE300, Nikon, Japan) at 20× magnification. The embryos were evaluated morphologically according to the quality and viability criteria determined by the International

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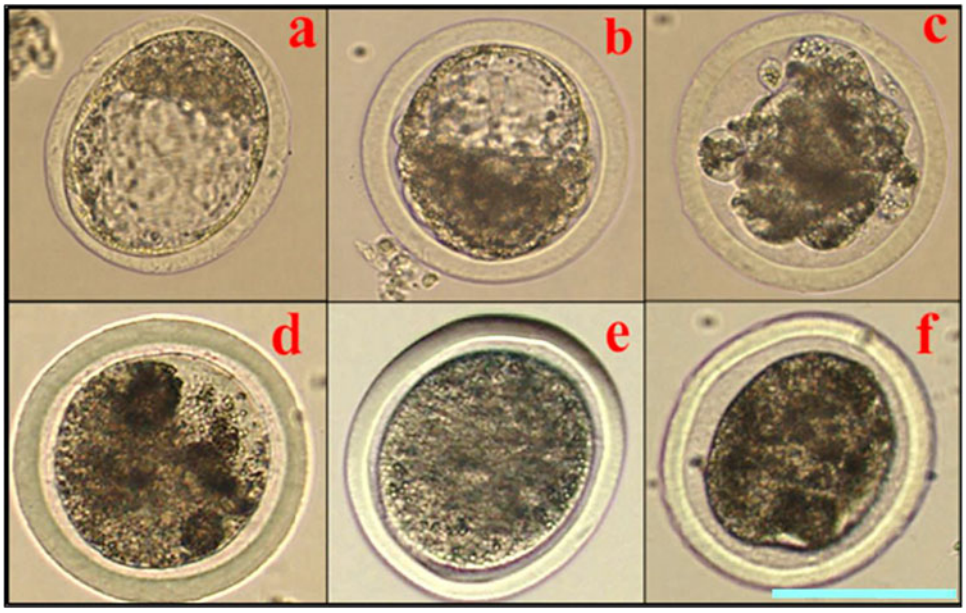

Figure 2. Morphological evaluation of embryos. (a) Grade 1 (excellent/good), (b) grade 2 (fair), (c) grade 3 (poor), (d) degenerated embryos and (e,f) unfertilized ova. Scale bar 100 µm.

Embryo Transfer Society (Wright 1998). Grade 1 (excellent/good), grade 2 (fair) and grade 3 (poor) embryos were defined as transferable (Fig. 2) (Biancucci *et al.*, 2016; Bülbül *et al.*, 2013).

Plasma FSH level analysis

Blood was collected from all the cows at 0, 1, 2, 4, 8, 12, 24, 36, 48, 60, 72, 84 and 96 h after the start of FSH treatment (just before the FSH injections, which coincided with the FSH application hours in all cows in all groups) from the jugular vein into vacuum tubes containing EDTA. Blood samples were centrifuged within 2 h after collection at 3000 × *g* for 10 min to separate the plasma. Plasma samples were stored at −20 °C until they were analysed. Plasma FSH concentration was measured using a competitive enzyme immunoassay kit (pFSH Elisa Kit, Cusabio Biotech Co. Ltd., Houston, TX 77054, USA, Catalogue Number: CSB-E15856B) specified for the determination of bovine FSH with intra- and inter-assay coefficient variations of <15% and <15%, respectively (Ratsiri *et al.*, 2021).

Statistical analyses

The homogeneity of variance test was used to determine the distribution of the data. Normality of the data distribution was assessed with the Shapiro–Wilk test, and the homogeneity of variance was assessed with Levene's test. The average age, daily milk yield, live weight and days in milk of cows in the CG, EG10 and EG7.5 groups were compared via analysis of variance (one-way ANOVA). The Mann–Whitney non-parametric test was used to compare the mean numbers of corpora lutea, total ova/embryos, transferable (grades 1, 2 and 3) and degenerated embryos, and unfertilised ova between the groups, as the data did not have a normal distribution. Data were square root transformed before statistical analysis to normalise the variance for plasma FSH levels. Repeated measures analysis of variance was used to determine whether there was a difference between groups and times (h) for plasma FSH levels. Following the analysis of variance, Tukey's test with a significance level of $P < 0.05$ was used to identify different groups. Least square means and standard errors are expressed as $\bar{X} \pm$ SEM. The SPSS package (IBM® SPSS Statistics for Windows, version 21) was used for all statistical analyses. Differences were considered significant at $P < 0.05$.

Results

All animal injection sites were checked for hard masses, cysts, sacs, scar tissue, oedema, painful lesions, fistulas, fibrous tissue and abscess formation until 30 days after injection. There were no pathological findings at the injection sites in the cows.

The cows' plasma FSH levels (ng/mL) by group and hour (±SEM) are presented in Table 3 and Fig. 3. The difference in FSH levels between groups was significant ($P = 0.014$), whereas the interactions of hour ($P = 0.087$) and group × time ($P = 0.987$) were not significant.

The numbers of CLs, total ova/embryos, transferable (grades 1, 2 and 3) and degenerated embryos, and unfertilized ova in the groups (±SEM) are presented in Table 4. There was no significant difference between the groups in any criteria ($P > 0.05$). The number of CL in the cows in the study was two in one cow in the CG group, three in one cow in the EG10 group and ≥4 in all other cows. That is, the superovulatory responses of the cows in all the groups were similar.

Discussion

Although few studies have evaluated superstimulation with a single dose of FSH dissolved in MonISA-206 or traditional protocol (Cizmeci *et al.*, 2022; Ciftci and Dinc, 2023), this is the first report to determine embryo quality and include FSH and MonISA-206 dose optimisation. In the study, there were no pathological situations at the injection sites in any of the cows. All cows were in oestrus 30–36 h after CIDR removal and were AI twice. Although

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Table 3. Least square means ($\bar{X}$) of plasma FSH levels (ng/mL) according to group and hour (±SEM)

Factors	$\bar{X}$ ± SEM
Group	$P = 0.014$
CG	14.49 ± 0.987[a]
EG10	9.39 ± 0.987[b]
EG7.5	10.47 ± 0.987[b]
Hour	P = 0.087
0	4.52 ± 2.055
1	12.95 ± 2.055
2	15.85 ± 2.055
4	12.74 ± 2.055
8	11.32 ± 2.055
12	11.88 ± 2.055
24	10.70 ± 2.055
36	12.55 ± 2.055
48	11.14 ± 2.055
60	11.64 ± 2.055
72	11.07 ± 2.055
84	11.59 ± 2.055
96	10.90 ± 2.055
Group × hour	$P = 0.987$

[a,b]: there is a significant difference between means with different superscripts ($P < 0.05$). FSH: follicle stimulating hormone.

plasma FSH levels differed between groups, there was no significant difference in hour or interaction effect between group × time. The number of CLs and embryo qualities obtained with a single injection of FSH dissolved in Montanide™ ISA-206 VG were similar to those obtained with traditional FSH treatment. Furthermore, similar results were obtained in both the EG10 and EG7.5 groups using two different doses of FSH (100% vs 75%).

The commercial Montanide adjuvant has been reported to be safe with fewer side effects (Waghmare *et al.*, 2009). However, some side effects (oedema at the injection site reabsorbed in the next 3–4 weeks or abscesses that eventually ulcerate and/or open the fistula and also develop scar tissue and heal in 4–8 weeks) at the injection site have been reported for another Montanide category (Montanide ISA 50V2) following s.c. application (Arguedas *et al.*, 2022). Previous studies in which cows were superstimulated by injection of FSH+MonISA-206 neither examined nor stated any pathological situation at the injection sites (Cizmeci *et al.*, 2022; Ciftci and Dinc, 2023). In our study, there was no pathological situation at the injection sites in any cow, which is compatible with previous studies reporting that MonISA-206 was well tolerated and had no toxicity (Cofrades *et al.*, 2013; Waghmare *et al.*, 2014; Balcaen *et al.*, 2016). However, as our study was not designed to evaluate the safety, detailed studies using different materials and methods (possibly with more animals) need to be carried out to assess the safety of FSH+MonISA-206 treatment.

Similar or different plasma FSH levels have been reported following traditional FSH treatment or the application of a single FSH dissolved in PVP (Takedomi *et al.*, 1995), Al-gel (Kimura *et al.*, 2007) or saline (Hiraizumi *et al.*, 2015) for superovulation. Although the plasma FSH levels were similar between the groups that received a single s.c. injection of 20 AU pFSH dissolved in 10 or 50 mL of saline and the control (traditional 12-h interval protocol), there were differences when 30 AU pFSH was used (Hiraizumi *et al.*, 2015). The absorption rate and plasma levels of FSH in donors are affected by the injection route (Gabriela Farías-Delgado *et al.*, 2023), the type and amount of adjuvant used (Hiraizumi *et al.*, 2015) and the BCS of the cow (Bó *et al.*, 1994). Bó *et al.* (2018) reported similar plasma FSH levels in beef cows with medium to high BCSs after a single s.c. injection of FSH in the neck region (into adipose tissue) or behind the shoulder. However, the plasma FSH profiles were similar following i.m. or s.c. injection in the neck region (with little adipose tissue), which differed from those following s.c. injection behind the shoulder in Holstein cows (Bó *et al.*, 2018). In our study, as in previous studies that used traditional protocols, plasma FSH levels in the CG increased, peaked at approximately 2 h and decreased to the level of the EG10 and EG7.5 groups until the next injection (12 h later), probably because of its half-life (Bó *et al.*, 2018; Gutiérrez-Reinoso *et al.*, 2023). However, plasma FSH remained at a certain level until 96 h in the EG10 and EG7.5 groups, likely because of the slow release agent MonISA-206 (Cole and Whateley, 1997; Vlaia *et al.*, 2009; Dar *et al.*, 2013; Balcaen *et al.*, 2017). In this study, the difference in plasma FSH levels between groups was significant, whereas the interaction between time and group × time was insignificant. Although few studies have evaluated plasma FSH levels after the use of MonISA-206 as an adjuvant, the results of this study are compatible with those published recently by Cizmeci *et al.* (2022). They also reported similar plasma FSH levels at all sampling times in Holstein cows superstimulated using a traditional protocol with a single s.c. injection of FSH dissolved in MonISA-206 (Cizmeci *et al.*, 2022). In our study, the reduced FSH dose used in the EG7.5 group led to an FSH profile similar to that of the EG10 group.

In addition to the traditional superovulation protocol, successful superovulatory responses have also been reported in cattle using a single injection of FSH combined with adjuvants (Takedomi *et al.*, 1995; Kimura *et al.*, 2007; Tríbulo *et al.*, 2011; Biancucci *et al.*, 2016). Tríbulo *et al.* (2011) reported comparable results in superstimulated beef cows with a single i.m. injection of FSH diluted in a hyaluronan-based slow-release formulation (SRF) compared to traditional treatment. Although they obtained similar CL numbers in groups injected with different concentrations of SRF, embryo quality was better in cows treated with FSH at 100% SRF than in those treated with FSH at 50% SRF. In another study, Biancucci *et al.* (2016) compared the traditional superovulation protocol with the one using FSH dissolved in 0.5% hyaluronan. The total dose in the hyaluronan group was split in two (67% and 33%) and injected i.m. 48 h apart. The researchers reported a better superovulation response and embryo quality, and attributed this result to the adequate plasma FSH concentration in hyaluronan-treated heifers (Biancucci *et al.*, 2016). Al-gel has also been used to dissolve FSH and to superovulate cattle via a single i.m. injection (Kimura *et al.*, 2007). Although plasma FSH levels were higher up to 4 h post-treatment in the traditional protocol and from 8 to 36 h in the Al-gel protocol (with 30 mg FSH), similar superovulation results were reported. The amount of Al-gel (5 mL vs 10 mL) also did not affect superstimulation results (Kimura *et al.*, 2007). Some studies have also used FSH dissolved in PVP to compare traditional superovulation protocols. Takedomi *et al.* (1995) reported that a single s.c. injection of FSH dissolved in PVP led to a plasma

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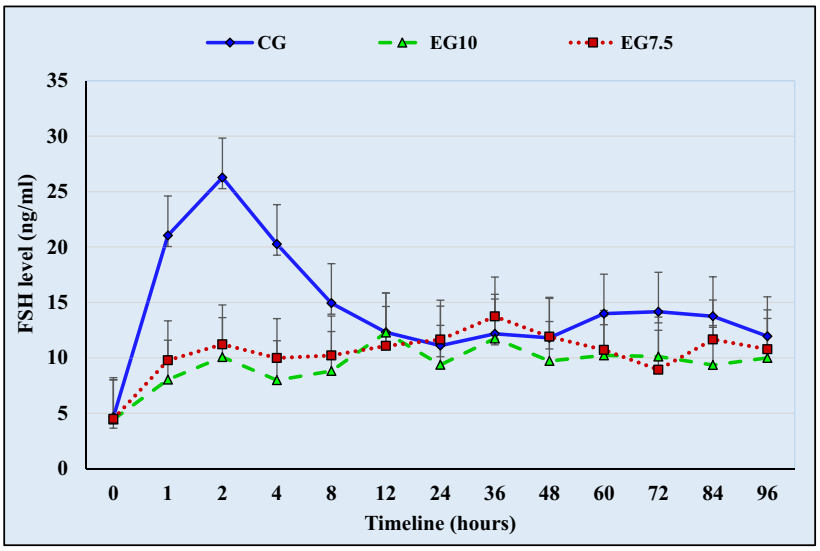

Figure 3. Plasma follicle stimulating hormone (FSH) levels (ng/mL) by group and time in hours.

Table 4. Superovulatory responses of the cows in the groups (±SEM)

Parameters	CG	EG10	EG7.5
Corpora lutea	6.86 ± 0.99	6.00 ± 0.72	7.14 ± 1.28
Total ova/embryos	3.57 ± 0.97	2.29 ± 0.47	2.57 ± 0.97
Transferable embryos	2.14 ± 0.80	1.57 ± 0.43	1.71 ± 0.64
Grade 1	1.43 ± 0.61	1.29 ± 0.36	1.29 ± 0.52
Grade 2	0.57 ± 0.30	0.29 ± 0.18	0.29 ± 0.29
Grade 3	0.14 ± 0.14	0	0.14 ± 0.14
Unfertilized ova	0	0.14 ± 0.14	0.14 ± 0.14
Degenerated embryos	1.43 ± 0.37	0.57 ± 0.20	0.71 ± 0.36

FSH profile and a superovulatory response similar to the traditional protocol. A recent study (Cizmeci *et al.*, 2022) using a single s.c. administration of FSH dissolved in MonISA-206 reported CL results similar to those of the repeated FSH injections. In our study, the superstimulation results were similar in all the groups. The similar superstimulation results in the groups can be attributed to the adequate FSH profiles in the groups as stated in previous reports (Biancucci *et al.*, 2016). In this study, the reduced FSH dose used in the EG7.5 group led to an adequate FSH profile and similar superstimulation results to the other groups.

Some studies have reported different embryo quality outcomes using different agents and protocols. In a recent study, Gabriela Farías-Delgado *et al.* (2023) reported similar superstimulation success (number of antral follicles) in groups receiving a single epidural dose of FSH and a traditional protocol (eight decreasing doses). However, the number of transferable embryos was twice as high in the epidural group. They concluded that the plasma FSH levels in the epidural group were inadequate and lower after the 50th hour than those in the traditional protocol. Biancucci *et al.* (2016) reported better embryo quality with FSH + hyaluronan than with the traditional protocol because of the adequate plasma FSH concentration. Similar transferable embryo results following a single FSH + Al-gel application with the traditional protocol have also been reported (Kimura *et al.*, 2007). In another study (Takedomi *et al.*, 1995), a single s.c. FSH + PVP injection resulted in embryo quality similar to that of the traditional protocol. Embryo quality in our study was similar in all groups. Although no study has used MonISA-206 and compared embryo quality, we can still conclude that our superovulation protocol using a single injection of FSH dissolved in MonISA-206 resulted in embryo quality similar to the traditional protocol. In addition, the reduced FSH dose used in the EG7.5 group resulted in embryo quality similar to that of the other groups. As FSH controls the follicular wave in the ovary (Deguettes *et al.*, 2020; Jahnke and Youngs, 2021), our embryo quality results may be due to adequate plasma levels of FSH in all groups. However, the number of animals and embryos obtained may be a limitation of this study, and further research is recommended.

Conclusion

A single injection of FSH dissolved in Montanide^{TM} ISA-206 VG resulted in adequate plasma FSH levels and similar superstimulation and embryo quality results as traditional FSH treatment without causing any pathological changes at the injection sites. Furthermore, both the EG10 and EG7.5 groups (with 100% vs 75% FSH doses) showed similar results to each other and to the traditional protocol. In conclusion, the use of a single administration of the FSH+Montanide^{TM} ISA-206 diluent can be suggested because it is a simple and easy way to achieve superovulation in lactating cows. However, large-scale field studies should also be carried out.

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Data availability. The study data are available from the corresponding author upon reasonable request.

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Author contributions. Conceptualization and methodology were performed by M.A.Y., S.H.K. and B.B. Superovulatory treatment, ovary examination and embryo collection were performed by M.A.Y., T.Ç., R.S., A.O., M.S. and E.S. Blood sample collection and plasma analyses were performed by B.K., İ.Ü., B.A. and Y.E. Statistical analyses were performed by S.H.K. and B.B. Interpretation of the results was performed by M.A.Y. and B.B. M.A.Y., T.Ç., R.S., A.O., E.S., B.A., Y.E. and B.B. wrote the first drafts of the study. Revision of the article was performed by M.A.Y. and B.B. All authors read and approved the final manuscript.

Ethical approval. All procedures and protocols in the present study were approved by the International Center for Livestock Research and Training (Lalahan, Ankara) Animal Experiments Local Ethics Committee (Protocol Number: 27.12.2017/147).

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