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Retrospective evaluation of factors affecting superovulatory response in embryo production in Simmental cattle

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Abstract: In the present study, it was aimed to investigate the effect of lactation status, days in milk (DIM), follicle stimulating hormone (FSH) dose, and repeated superstimulations on superovulation response and embryo yield in Simmental cattle. In the present study, 193 Simmental breed cattle (lactating, nonlactating, and heifer) were used as donors, of which 149 were superovulated only once and the other 44 were superovulated for 3 times. Progesterone-based estrus synchronization protocol was applied to the donors. Starting from day 7, FSH was given intramuscularly to donors in decreasing doses twice a day for 4 days. The donors were injected with prostaglandin $F_{2\alpha}$ at the 5th FSH treatment and progesterone source was removed 12 h later. Artificial insemination was performed twice after the removal of progesterone source. In the present study, 134 of the 149 donors who underwent a superovulation protocol for the first time responded to superovulation treatment (≥3 corpus luteum (CL); 89.93%) and the mean count of transferable embryos per donor was 5.58. No statistical difference was observed for the counts of CL, total oocyte/embryo, and transferable embryo among heifers, lactating cows, and nonlactating cows. A positive correlation was noted between DIM and embryo yield ($P < 0.05$). Superovulation response and embryo yield were increased depending on the dose of FSH in cows undergoing superstimulation treatment ($P < 0.05$). Although no statistical difference was detected between the embryo yields obtained from the first two applications in repeated superovulation treatments, the embryo yield decreased after the 3rd superstimulation. In conclusion, in Simmental breed cows, the embryo yield in superovulation applications was unaffected by lactation status, but was affected by the applied FSH dose and DIM. It was also concluded that the embryo yield decreased after the 3rd application in repeated superovulation treatments.

Key words: Days in milk, follicle stimulating hormone dose, lactation, repeated superstimulation, superovulation

1. Introduction

Embryo transfer is an assisted reproduction technique that involves the transfer of embryos from one donor to the recipients [1]. This biotechnology method allows the count of generations obtained from donors of high genetic value to be increased in a short time and the spread of the desired genetics quickly [2,3]. The most important factor affecting success in embryo production is the differences in the response of animals to superovulation treatments [4–6]. The number of transferable embryos collected at each uterine flushing also affects the success of embryo transfer program, that is, the superovulation response that affects the number of transferable embryos [7]. Differences in the superovulation response are mostly due to different gonadotropin hormone types (follicle stimulating hormone (FSH) or pregnant mare's serum gonadotropin (PMSG)) and doses, gonadotropin hormone application time, repeated superstimulation, age of donor, ovarian status at the time of treatment, lactation status, breed, and days in milk (DIM) [2, 4, 5, 8, 9].

According to the International Embryo Technology Society (IETS), the count of transferable in vivo-derived embryos per donor in the world is 6.74 [3]; the American Embryo Transfer Association reported this number as 6.6 in beef and 5.7 in dairy breeds [10]. Differences in the count of transferable embryos obtained after superovulation treatment have been reported in the dual-purpose breed Simmental cattle (beef and dairy) [11–13]. Breuel et al. [14] determined that Simmental cattle donors could achieve a greater ovarian sensitivity (the counts of oocyte/embryo and transferable embryo) to gonadotropins than beef cattle, such as Angus, Charolais, or Hereford donor. Karaşahin et al. [15] reported that transferable embryo rate in Simmental cattle was higher than that of Holstein and Brown Swiss. In previous studies on Simmental cattle, the average count of oocyte/embryo and transferable embryos

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were in the range of 9.2–20.5 and 4.0–12.8, respectively [11-17]. However, in most of these studies, factors affecting superovulation response in Simmental cattle have not been evaluated. Therefore, the effects of lactation status, DIM, FSH dose, and repeated superstimulation treatments on the response to superovulation in Simmental breed cattle were evaluated in this study.

2. Materials and methods

The experimental procedures were approved by the Ethics Committee of Selçuk University Faculty of Veterinary Medicine, Experimental Animals Production and Research Center (2018/99).

2.1. Location

This study was conducted between September and January at the Gözlü Agriculture Enterprise in the province of Konya, Turkey. The animals were periodically vaccinated for infectious bovine rhinotracheitis, foot-and-mouth disease, pox, bovine virus diarrhea, brucella, enterotoxemia, and fungal infections.

2.2. Selection of donors

In the present study, 193 Simmental breed cattle were used as donors. A total of 149 cattle were superovulated once, and the other 44 were superovulated for 3 times. The donors used once in the study were divided into three groups: heifers (n = 19), non-lactating cows (n = 29, cows gave birth at least once, milk yield decreased/dried off due to various diseases such as mastitis, foot diseases and not milked), and lactating cows (n = 101). The cows used in the repeated superovulation treatments were divided into two groups: nonlactating (n = 15) and lactating (n = 29). First, donors were selected using a herd management software, and rectal and ultrasonographic examinations were performed. Superovulation was performed on donors with no problems detected on genital organs (adhesions, cysts, and metritis etc.), with CL present on the ovary, with an ovarium size ≥6 cm, and the number of follicles ≥5 during examinations. In addition, cows were selected on the basis of the following parameters: age between 2.5 and 4 years, body weight of 600–800 kg, and body condition score of 3–3.5, and heifers were selected between 16 and 18 months.

2.3. Synchronization protocol

Estrus synchronization was performed with a progesterone-based protocol. The progesterone source (Eazi-Breed CIDR, Zoetis, USA) was placed intravaginally and the GnRH analog (buserelin acetate, Receptal, MSD, USA) was administered intramuscularly (Day 0). On day 9, $PGF_{2\alpha}$ (dinoprost tromethamine, Dinolytic, Zoetis, USA) was injected intramuscularly in the morning, and CIDR was removed in the evening. Donors were inseminated artificially on fixed time at 48 and 60 h (day 11) following the removal of progesterone source. Two frozen/thawed straws were used in each insemination, and each straw contained $>7 \times 10^6$ motile spermatozoa. All artificial inseminations (AI) were conducted by an experienced technician.

2.4. Superovulation protocol

Within the synchronization protocol, the donors were treated with 8 decreasing doses of FSH intramuscularly starting from day 7.

2.4.1. Donors superovulated once

A total of 400 μg FSH (Stimufol, Reprobiol, Belgium) was applied to the heifers (n = 19) for superovulation. Cows were randomized (using random number tables) and divided into two subgroups according to FSH dose: the FSH 400 μg group (80–80, 60–60, 40–40, and 20–20 μg) (n = 34), and FSH 500 μg group (100–100, 75–75, 50–50, and 25–25 μg) (n = 96). The cows were then divided into two subgroups (lactating and nonlactating cows) to determine the effect of lactation status on their groups.

2.4.2. Repeatedly superovulated donors

A total of 44 cows were superovulated with 500 μg FSH at three different times (with intervals of at least 45–60 days) to determine the effect of repeated superovulation treatments. These cows were divided into lactating and nonlactating subgroups to determine the effect of lactation status on repeated superovulation treatment.

2.5. Collection of embryos

The ovaries of donors were examined rectally and ultrasonographically on day 7 following AI, and the CL number was determined by ultrasonography. Uterine flushing was performed in donors with at least three CLs on both ovaries.

The uterine flushing application was performed during epidural anesthesia (5–8 mL, lidokain HCl, Adokain, Sanovel, Turkey). First, a balloon catheter (2-way foley catheter, silicone, 16–20 inches) was inserted to the uterus horn, and the uterine lumen was washed several times (3–4 times, 300–400 mL in total) with Ringer's lactate solution (calf serum + kanamycin). The embryos were collected in a filter (EmCon filter, 75 μm). After the uterine flushing process, the filter was taken to the laboratory.

2.6. Evaluation and classification of embryos

The developmental stages and quality of the obtained embryos were evaluated under a stereomicroscope according to the IETS criteria [18]. The assessment of embryo quality was made according to morphological integrity. Code I (excellent or good) corresponded to very low levels of irregularity between the cells, a ratio of >85% viable embryonic cells, and a round and unfolded zona pellucida. Code II (fair) is characterized by a medium level of irregularity between the cells and a viable cell ratio of 50%. Code III (poor) is characterized by irregularities

in the form of the embryo and a viable cell ratio of 25%. Code IV (dead or degenerated) are embryos with oocytes or dead cells with uncompleted division.

2.7. Statistical analysis

SPSS 25 statistical package program was used for the data analysis. The data were presented as mean ± standard deviation, and minimum–maximum values. The suitability of the data for repeated-measures analysis of variance (comparison of the results obtained from repeated superovulation treatments) was evaluated with Mauchy's sphericity test and Box-M variance homogeneity test. For the comparison of means, repeated measures variance analysis was used. When the parametric tests (repeated measurements in factorial order; fixed effect) failed to meet the prerequisites of variance analysis, Greenhouse and Geisser [19] or Huynh and Feldt [20] tests with a degree of freedom correction were used. Multiple comparisons among groups (comparison of the findings obtained from heifers, lactating, and nonlactating donors with superovulation treatment) were performed using the adjusted Bonferroni test. Variables were evaluated after checking the normality and homogeneity of variance prerequisites (the Shapiro–Wilk and Levene tests). When performing data analysis, independent 2 groups *t*-test (Student's *t*-test) was used for comparison of two groups (for example, comparing FSH doses); when prerequisites were not met, the Mann–Whitney U test was used. For the comparisons of three and more groups (comparison of the findings obtained from heifers, lactating, and nonlactating donors with superovulation treatment), one-way analysis of variance, the Tukey honestly significant difference test, the Kruskal–Wallis, or the Bonferroni–Dunn tests were used. The relationship between the two continuous variables was evaluated using the Pearson correlation coefficient and the Spearman correlation coefficient when the parametric test did not meet the prerequisites. $P < 0.05$ was accepted for the significance level of the tests.

3. Results

In the present study, 134 of the 149 donors who underwent the superovulation protocol responded to the superstimulation (≥3 CL, 89.93%) and 15 donors (11 lactating and 4 nonlactating) showed no response. The mean counts for total CL, total oocyte/embryo, transferable embryo, Code I, II, and III embryos, and degenerated and unfertilized oocytes are given in Table 1.

Table 1. Descriptive statistics for the day of uterine flushing in donors that responded to superovulation treatments.

Parameters	n	Mean	SD	Min	Max
Right ovary (Counts of CL)	134	5.85	3.47	0	14
Left ovary (Counts of CL)	134	5.18	3.66	0	15
Total CL	134	11.01	6.81	3	29
Total oocyte/embryo	134	10.01	6.66	0	28
Transferable embryos	134	5.58	3.78	0	23
Code I embryos (Excellent or good)	134	2.53	1.34	0	19
Code II embryos (Fair)	134	1.95	1.43	0	13
Code III embryos (Poor)	134	1.13	0.71	0	7
Degenerated embryos	134	3.13	2.27	0	16
UFO	134	1.29	1.21	0	13
Excellent compact morula	134	1.08	1.03	0	9
Excellent early blastocyt	134	0.66	0.25	0	6
Excellent blastocyt	134	0.78	0.78	0	11
Fair compact morula	134	1.54	1.11	0	13
Fair early blastocyt	134	0.24	0.59	0	7
Fair blastocyt	134	0.07	0.15	0	3
Poor compact morula	134	1.07	0.87	0	1
Poor early blastocyt	134	0.01	0.08	0	1
Poor blastocyt	134	0.01	0.08	0	1

CL: corpus luteum, UFO: unfertilized oocyte, SD: standard deviation.

Of the 134 donors, 31 (23.13%) had <5 oocytes/embryos, 52 (38.80%) had 6–10 oocytes/embryos, 41 (30.59%) had 11–20 oocytes/embryos, and 10 (7.46%) had >20 oocytes/embryos. When the count of transferable embryos was evaluated, 51 donors (38.05%) had 1–5 transferable embryos, 41 donors had (30.59%) 6–10 embryos, and 16 (11.94%) donors had >10 embryos. A total of 26 donors (19.40%) responded to superovulation without yielding any transferable embryos.

Table 2 presents the results of superovulation of heifers, lactating cows, and nonlactating cows. The mean of total CL counts reached 11.79 ± 7.17 in nonlactating cows, 11.13 ± 7.20 in lactating cows, and 9.21 ± 3.04 in heifers ($P > 0.05$). No statistical difference was observed in the total oocyte/embryo and transferable embryo counts ($P > 0.05$). Figure shows the development period and quality classification of embryos according to lactation status of donors.

The relationship between DIM and superovulation results is given in Table 3. The mean DIM of cows used in this study was 126 days. Correlation analysis revealed a positive correlation between DIM and the counts of transferable embryo and Code I quality embryo ($P < 0.01$).

Table 4 presents the data on the counts of total CL, total oocyte/embryos, transferable embryos, Code I, II, and III embryos, and degenerated/unfertilized oocytes after the effect of FSH dose was evaluated regardless of the lactation status. Although FSH dose showed no effect on total CL count, the embryo yields in donor cows treated with 500 µg of FSH was higher than that of the donors treated with 400 µg of FSH ($P < 0.05$). Table 5 shows the results for different doses of FSH in accordance with lactation status.

The donors were subgrouped based on the total CL count. Tables 6 and 7 present the relationship between total CL counts and embryo yield. As the count of CL increased, the counts of degenerated embryos and oocytes also increased ($P < 0.05$).

Table 8 presents the results from the repeated superovulation treatments. According to these findings, the repeated superovulation treatments in Simmental cattle caused a decrease in superovulation response and embryo yield after the 3rd superovulation treatment.

Table 2. Embryo yields based on lactation status (heifer, lactating, and nonlactating cows) of donors that responded to superovulation treatments.

Items	Heifer	Lactating cows	Nonlactating cows
n	19	90	25
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD
Right ovary (Counts of CL)	5,00 ± 2.0	5.86 ± 3.61	6.34 ± 3.73
Left ovary (Counts of CL)	4.21 ± 1.65	5.29 ± 3.92	5.45 ± 3.67
Total CL	9.21 ± 3.04	11.13 ± 7.20	11.79 ± 7.17
Total oocyte/embryo	7.21 ± 3.17	10.29 ± 6.99	11.16 ± 7.25
Transferable embryos	3.79 ± 2.11	5.88 ± 4.04	5.88 ± 4.73
Code I embryos (Excellent or good)	1.74 ± 1.19	2.89 ± 2.69	1.84 ± 1.59
Code II embryos (Fair)	1.21 ± 0.75	1.88 ± 1.27	2.76 ± 2.19
Code III embryos (Poor)	0.84 ± 0.76	1.14 ± 1.08	1.28 ± 1.05
Degenerated embryos	2.95 ± 2.09	3.03 ± 2.75	3.60 ± 2.21
UFO	0.58 ± 0.53	1.32 ± 3.16	1.72 ± 2.20

CL: corpus luteum, UFO: unfertilized oocyte, SD: standard deviation.

Table 3. Correlation coefficient (r)[a] between days in milk (DIM) and superovulation results in lactating cows.

	Total CL	Total oocyte/embryo	Transferable embryos	Code I embryo	Code II embryo	Code III embryo	Degenerated embryo	UFO
DIM	0.218*	0.209*	0.326**	0.299**	0.036	0.164	0.108	–0.156

[a] Pearson coefficient; *, $P < 0.05$; **, $P < 0.01$. CL: corpus luteum, UFO: unfertilized oocyte, DIM: days in milk.

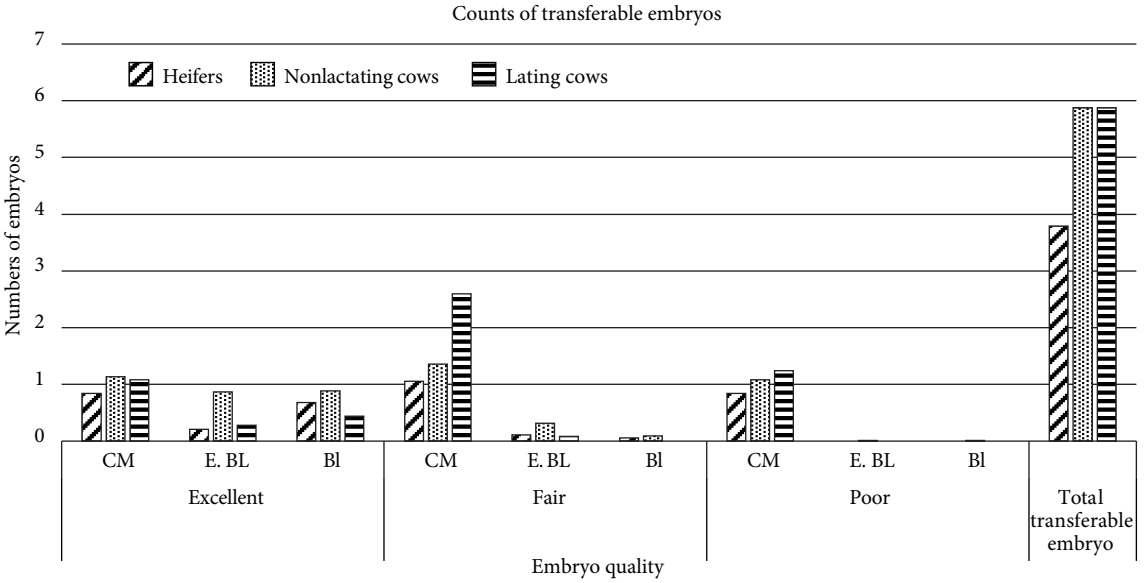

Figure. Developmental stage and quality classification of embryos based on the lactation status in donors (superovulated once) that responded to superovulation treatments. (CM: compact morula, E. BL: early blastocyst, Bl: blastocyst; Excellent: Code I quality embryos, Fair: Code II quality embryos, Poor: Code III quality embryos).

Table 4. Results of embryo yield based on FSH dose in cows that responded to superovulation treatments.

Items	FSH 400 mg (n = 30)	FSH 500 mg (n = 85)	P
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	
Right ovary (Counts of CL)	5.08 ± 2.59	6.27 ± 3.82	> 0.05
Left ovary (Counts of CL)	4.55 ± 2.75	5.53 ± 3.05	> 0.05
Total CL	9.62 ± 4.99	11.78 ± 7.55	> 0.05
Total oocyte/embryo	7.76 ± 4.41	11.32 ± 7.38	< 0.05
Transferable embryos	4.20 ± 3.36	6.38 ± 4.30	< 0.05
Code I embryos (Excellent or good)	2.12 ± 1.39	2.76 ± 1.77	< 0.05
Code II embryos (Fair)	1.41 ± 1.18	2.26 ± 1.65	< 0.05
Code III embryos (Poor)	0.69 ± 0.31	1.38 ± 1.07	< 0.05
Degenerated embryos	2.80 ± 2.09	3.32 ± 2.83	> 0.05
UFO	0.71 ± 0.26	1.62 ± 0.61	> 0.05

CL: corpus luteum, UFO: unfertilized oocyte, FSH: follicle stimulating hormone, SD: standard deviation.

However, the lactation status had no effect on the outcome of superovulation response and embryo yield.

4. Discussion

The success of embryo production in cattle is closely related to the response to superovulation protocol, which varies from animal to animal [21,22]. This difference in response to superovulation protocol is the most important factor determining the profitable and efficient application of embryo technology [4,7,23].

The counts of total CL, total oocyte/embryo, and transferable embryos obtained after the superovulation treatment were similar to the results of previously reported studies [1,3,7,24]. In this study, the ratio of donors that responded to superovulation treatment (≥3 CL) was 89.93%, and the mean count of transferable embryos

Table 5. Results of embryo yield based on FSH dose in lactating and nonlactating cows that responded to superovulation treatments.

Items	Lactating cows		Nonlactating cows	
	FSH 400 mg (n = 21)	FSH 500 mg (n = 69)	FSH 400 mg (n = 9)	FSH 500 mg (n = 16)
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD
Right ovary (Counts of CL)	4.95 ± 2.27	6.11 ± 3.87	5.42 ± 3.89	7.00 ± 3.58
Left ovary (Counts of CL)	4.64 ± 2.93	5.47 ± 3.16	4.92 ± 3.82	5.82 ± 3.62
Total CL	9.59 ± 4.85	11.56 ± 7.70	10.33 ± 7.53	12.82 ± 6.94
Total oocyte/embryo	7.81 ± 3.99	11.04 ± 7.53	8.78 ± 5.25	12.50 ± 6.79
Transferable embryos	4.29 ± 2.56	6.36 ± 3.34	4.89 ± 2.62	6.44 ± 5.29
Code I embryos (Excellent or good)	2.67 ± 1.76	2.96 ± 1.95	1.67 ± 1.23	1.94 ± 1.83
Code II embryos (Fair)	1.29 ± .084	2.06 ± 1.36	2.11 ± 1.31	3.13 ± 1.61
Code III embryos (Poor)	0.38 ± 0.32	1.38 ± 0.95	1.11 ± 0.53	1.38 ± 0.54
Degenerated embryos	2.38 ± 1.91	3.23 ± 2.17	3.44 ± 1.74	3.69 ± 2.05
UFO	0.95 ± 1.12	1.43 ± 1.18	0.44 ± 0.33	2.44 ± 1.13

CL: corpus luteum, UFO: unfertilized oocyte, FSH: follicle stimulating hormone, SD: standard deviation.

Table 6. Embryo yields based on total CL counts determined in all donors (heifer, lactating, and nonlactating cows) on day 7 of uterine flushing.

Items	Total CL counts			P
	≤ 10	11–19	≥20	
n	53	70	11	
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	
Right ovary (Counts of CL)	3.96 ± 1.42	7.34 ± 1.67	13.00 ± 3.13	< 0.05
Left ovary (Counts of CL)	3.06 ± 1.53	6.70 ± 1.79	12.55 ± 5.14	
Total CL	6.98 ± 2.45	14.04 ± 2.33	25.55 ± 8.01	
Total oocyte/embryo	5.53 ± 3.17	11.16 ± 3.85	24.36 ± 9.69	
Transferable embryos	3.26 ± 2.14	6.27 ± 3.87	12.36 ± 8.22	
Code I embryos (Excellent or good)	1.77 ± 1.54	2.74 ± 1.87	4.82 ± 2.98	
Code II embryos (Fair)	1.04 ± 0.74	2.03 ± 0.93	5.82 ± 3.55	
Code III embryos (Poor)	0.45 ± 0.33	1.49 ± 0.91	2.09 ± 1.16	
Degenerated embryos	1.81 ± 1.42	3.61 ± 2.48	6.36 ± 4.30	
UFO	0.38 ± 0.11	1.26 ± 0.95	5.91 ± 3.58	

CL: corpus luteum, UFO: unfertilized oocyte, SD: standard deviation.

per donor was 5.5. Chebel et al. [25] also observed that 92.9% of donors responded to superovulation, and the mean count of transferable embryos per donor was 4.7 ± 0.2. Mikkola and Taponen [26] included nontransferable embryos in calculating the success of superovulation in donors. In this study, the number of donors that did not yield transferable embryos was 26 (19.40%) despite their response to the superovulation treatment. Silva et al. [8] also reported that they could not obtain viable embryos from approximately 20% of donors after superovulation treatment. Superovulation administration is an unnatural process for cattle. Normally, in cyclic cattle, one ovum

Table 7. Counts of transferable embryos based on total CL counts determined in all donors (heifer, lactating, and nonlactating cows) on uterine flushing day.

Counts of total CL	Counts of transferable embryo		
	Heifer	Lactating cows	Nonlactating cows
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
≤10	3.00 ± 0.69^{aA}	3.33 ± 1.82^{aA}	3.37 ± 1.35^{aA}
11-19	5.14 ± 1.53^{aB}	6.60 ± 2.23^{aB}	5.61 ± 2.59^{aB}
≥20	-	12.71 ± 5.58^{aC}	11.75 ± 4.81^{aC}

[a-b]: differences in the rows, [A-C] differences in the columns. CL: corpus luteum, SD: standard deviation.

Table 8. Embryo yields obtained from cows with repeated superovulation treatments.

Items	1st superovulation treatment			2nd superovulation treatment			3rd superovulation treatment		
	Lact.	Nonlact.	Total	Lact.	Nonlact.	Total	Lact.	Nonlact.	Total
n	29	15	44	27	13	40	24	12	36
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
Total CL	11.56 ± 5.67^{A}	15.09 ± 5.00^{x}	12.64 ± 5.65^{a}	12.20 ± 3.87^{A}	12.00 ± 5.09^{x}	12.14 ± 4.21^{a}	8.48 ± 4.68^{B}	8.36 ± 4.31^{y}	8.44 ± 5.24^{b}
Total oocyte/embryo	9.18 ± 5.62^{A}	13.60 ± 5.33^{x}	10.56 ± 5.83^{a}	11.14 ± 4.37^{A}	11.00 ± 4.73^{x}	11.09 ± 4.41^{a}	8.05 ± 5.29^{B}	7.00 ± 3.12^{y}	7.72 ± 4.69^{b}
Transferable embryo	5.80 ± 3.49^{A}	9.45 ± 5.85^{x}	6.92 ± 4.77^{a}	6.16 ± 4.08^{A}	6.27 ± 3.52^{x}	6.19 ± 3.87^{a}	3.40 ± 1.69^{B}	3.64 ± 2.76^{y}	3.47 ± 2.67^{b}
Code I embryo	3.28 ± 2.46^{A}	4.55 ± 3.08^{x}	3.67 ± 2.62^{a}	2.80 ± 1.66^{A}	2.00 ± 1.36^{y}	2.56 ± 1.56^{ab}	1.48 ± 0.71^{B}	1.18 ± 0.76^{yz}	1.39 ± 0.83^{b}
Code II embryo	1.84 ± 1.04^{A}	2.64 ± 1.77^{x}	2.08 ± 1.64^{a}	2.28 ± 2.05^{A}	3.00 ± 2.4^{xy}	2.50 ± 2.15^{ab}	1.52 ± 1.18^{A}	1.64 ± 1.06^{yz}	1.56 ± 1.17^{ac}
Code III embryo	0.84 ± 0.51^{A}	2.18 ± 2.15^{x}	1.25 ± 1.08^{a}	1.08 ± 0.91^{AB}	1.27 ± 0.97^{xy}	1.14 ± 0.75^{a}	0.44 ± 0.65^{AC}	0.82 ± 0.67^{y}	0.56 ± 0.47^{b}
Degenerated embryos	3.20 ± 2.45^{A}	2.91 ± 2.02^{x}	3.11 ± 2.84^{a}	3.68 ± 3.28^{A}	3.73 ± 2.24^{x}	3.69 ± 2.97^{a}	2.84 ± 2.11^{A}	2.09 ± 2.11^{x}	2.61 ± 2.84^{a}
UFO	0.67 ± 0.87^{A}	1.00 ± 1.09^{x}	0.77 ± 0.94^{a}	1.21 ± 1.17^{A}	0.55 ± 0.93^{x}	1.00 ± 0.96^{a}	0.58 ± 0.43^{A}	0.18 ± 0.14^{x}	0.46 ± 0.27^{a}

[a-c] differences in total numbers. [A-B] differences within the lactating cows. [x-z] differences within the nonlactating cows. CL: corpus luteum, UFO: unfertilized oocyte, SD: standard deviation, Lact.: lactating cows, Nonlact.: nonlactating cows.

is ovulated in each cycle, and the other follicles become atretic. By contrast, during superstimulation, many follicles undergo maturation and ovulation [27]. As a result of this forceful environment, the rate of fertilization in cows undergoing superovulation may be lower (50–70% vs 90%) than that of normal cyclic cows [28]. Hyttel et al. [29] reported that superstimulation could affect fertilization rate along with the viability of the embryos by creating a negative effect on oocyte and granulosa cell maturation.

The lactation status of donors also affects the response to superovulation [4]. In this study, donors were classified depending on the lactation status as heifers, lactating, and nonlactating cows. The donors were evaluated based on the counts of total CL, total oocyte/embryo, and the count and quality of transferable embryos, and no difference was found between the groups ($P > 0.05$). Leroy et al. [30] also showed that lactating and nonlactating Holstein cows and beef cows exhibited no differences in parameters such as superovulation response, the counts of CL, and transferable and degenerate embryo. Lee et al. [23] similarly reported that parity, lactation status, and milk yield caused no change in the count of transferable embryos. However, other studies [25,31] reported that superovulation response and the count of transferable embryos in the lactating cows were lower compared with the nonlactating cows. Given the lactation and high milk yield, dry matter intake and energy metabolism increased, which resulted in decreased circulating concentrations of estradiol and progesterone, leading to permanent follicle formation, reduced quality of oocytes, and disruption in the embryonic development [25,31]. In this study, the reason why lactation status had no effect on the superovulation response could be the use of Simmental breeds as donor animals as opposed to Holsteins utilized in the above-reported studies and low milk yield of the former. As Simmental cattle are known to be similar to beef cattle breeds, and their milk yield is

never close to that of dairy cows such as Holstein cows, the lactation status may not have affected the superovulation response obtained in the present study.

DIM is another factor affecting superovulation response and embryo production. In the early postpartum period, cows generally cannot meet the daily dry matter requirement (energy requirement) required for milk production from the feeds in the diet. As a result, negative energy balance occurs in cows [4]. The negative energy balance is detrimental to fertility as it causes a delay at the beginning of regular estrus cycle during early lactation. The first ovulation at postpartum period is delayed due to the insufficient maturation of the follicle and the decrease in the LH pulse frequency required for ovulation. Failure or decrease in superovulation response and embryo production after the ovarian superstimulation during early postpartum is considered normal [26,28]. Accordingly, in the present study, as the DIM increased, the counts of total CL, total oocyte/embryo, transferable embryos, and Code I embryos increased. These findings were thought to be due to the improvements in the negative energy balance, ovarian activity, and uterine health as DIM progressed. However, Hussein et al. [32] and Lee et al. [23] reported no relationship between DIM and embryo yield. Isogai et al. [33] reported that DIM had no effect on superovulation treatments within 250 days postpartum, but embryo yield decreased after about 460 days. In these studies, the duration of lactation was longer than that of the present study.

In the present study, the effect of two different doses of FSH (400 vs 500 μg) on the superovulation response in Simmental cows was also evaluated. For the cow, the recommended dose is from 450 μg to 500 μg of pFSH (Stimufol, Reprobiol SPRL, Belgium) in decreasing eight doses for 4 days. In the present study, the different doses of FSH showed no effect on the superovulation response and the total CL count. On the other hand, embryo yield was higher in donors treated with 500 μg of FSH compared with donors treated with 400 μg ($P < 0.05$). Lerner et al. [34] also reported that fewer total oocytes/embryos were collected from donors stimulated with lower doses of FSH. Mapletoft et al. [35] stated that the variability of the ovarian response to superovulation treatments was related to gonadotropin administration route, the total dose, timing, LH residue in FSH, duration of stimulation, and the use of additional hormones. By contrast, some studies have reported that the dose of FSH for ovarian superstimulation had no effect on the superovulation response [8,9,36]. Sartori et al. [28] reported that high doses of FSH may reduce fertilization rate and the count of transferable embryos. Mapletoft et al. [35] found no evidence of detrimental effects of the FSH dose on embryo quality. Ovulation rates continually increased when FSH was given up to 400 mg (NIH-FSH-P1) and did not increase beyond that dose. At the same time, the rates of fertilization and the counts of transferable embryo remained constant throughout the dose range used. However, certain differences may occur in the embryo yield depending on the dose, given that a different commercial FSH preparation and cow breed were used in the present study. Considering the opposing results in the literature regarding FSH dose administered, the divergence in these results may be due to the differences in the sample size or the use of different hormone batches.

In this study, no statistically significant difference was observed between the counts of total CL, total oocyte/embryo, and transferable embryos obtained in the 1st and 2nd flushings after repeated superovulation treatments in Simmental cattle. However, response to superovulation protocol decreased in the 3rd application. Zižlavský et al. [37] reported that the results of the first superovulation treatment can be used to determine the success of subsequent superovulations given that a correlation coefficient $r = 0.710$ ($P \leq 0.01$) was found between the n and the n + 1 superovulation. However, the positive correlation between the first two uterine flushings was not observed with the 3rd flushings. In addition, the count of total oocyte/embryos and transferable embryos obtained after the 3rd superovulation treatment decreased [37]. Kafi and Mcgowan [4] reported a statistically insignificant decrease in ovarian response after repeated superovulation treatments. By contrast, Tonhati et al. [38] and Silva et al. [8] demonstrated that repeated superovulation treatments had no effect on the superovulation response and embryo yield. In the present study, the reason for the difference in superovulation response in the 3rd application could be the breed, the commercial FSH preparation used and dosage, advanced DIM, and/or ovarian reserve.

5. Conclusion

In superovulation protocols, the lactation status had no effect on superovulation response and embryo yield in Simmental breed cattle. However, the dosage of FSH and the days in milk had an effect on embryo yield. In addition, ovarian response and embryo yield decreased after repeated superovulation treatments, at least after the 2nd application. Therefore, it was concluded that these factors should be considered to be successful and profitable in superovulation treatments in Simmental cattle.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Hasler JF. Forty years of embryo transfer in cattle: A review focusing on the journal Theriogenology, the growth of the industry in North America, and personal reminisces. Theriogenology 2014; 81: 152-169. doi: 10.1016/j.theriogenology.2013.09.010
2. Baruselli PS, de Sá Filho MF, Martins CM, Nasser LF, Nogueira MFG et al. Superovulation and embryo transfer in Bos indicus cattle. Theriogenology 2006; 65: 77-88. doi: 10.1016/j.theriogenology.2005.10.006
3. Perry G. 2016 statistics of embryo collection and transfer in domestic farm animals. Embryo Technology Newsletter 2017; 35: 8-23. doi: 10.1071/RD11913
4. Kafi M, McGowan MR. Factors associated with variation in the superovulatory response of cattle. Animal Reproduction Science 1997; 48: 137-157. doi: 10.1016/S0378-4320(97)00033-X
5. Bó GA, Guerrero DC, Tríbulo A, Tríbulo H, Tríbulo R et al. New approaches to superovulation in the cow. Reproduction, Fertility and Development 2010; 22: 106-112. doi: 10.1071/RD09226
6. Baruselli PS, Batista EOS, Vieira LM, Souza AH. Relationship between follicle population, AMH concentration and fertility in cattle. Animal Reproduction 2015; 12: 487-497. doi: 10.1016/S0045-7949(97)00138-7.
7. Bó GA, Mapletoft RJ. Historical perspectives and recent research on superovulation in cattle. Theriogenology 2014; 81: 38-48. doi: 10.1016/j.theriogenology.2013.09.020
8. Silva JCC, Alvarez RH, Zanenga CA, Pereira GT. Factors affecting embryo production in superovulated Nelore cattle. Animal Reproduction 2009; 6: 440-445.
9. Taşdemir U, Karaşahin T, Satılmış M, Kızıl SH, Kaymaz M. Various FSH administration on superovulatory response and embryo yield in Anatolian Black Heifers. Kocatepe Veterinary Journal 2016; 9: 322-326. doi: 10.5578/kvj.35305
10. Hasler JF. Bovine embryo transfer: are efficiencies improving? Proceedings Applied Reproductive Strategies. Bioniche Animal Health 2010; 1: 1-18.
11. Kanitz W, Becker F, Schneider F, Kanitz E, Leiding C et al. Superovulation in cattle: practical aspects of gonadotropin treatment and insemination. Reproduction Nutrition Development 2002; 42: 587-599. doi: 10.1051/rnd
12. Martens G, Nohner HP, Leiding C, Schneider F, Becker F et al. Optimizing frequency of FSH applicatıon for superovulatory treatment in cattle. Reproduction, Fertility and Development 2005; 17: 313-314.
13. Cirit Ü, Özmen MF, Küçükaslan İ, Köse M, Kutsal HG et al. Effect of the interval from follicle aspiration to initiation of lengthened FSH treatment on follicular superstimulatory and superovulatory responses and embryo production in lactating Simmental cows. Theriogenology 2019; 128: 218-224. doi: 10.1016/j.theriogenology.2019.02.008
14. Breuel KF, Baker RD, Butcher RL, Townsend EC, Inskeep EK et al. Effects of breed, age of donor and dosage of follicle stimulating hormone on the superovulatory response of beef cows. Theriogenology 1991; 36: 241-255. doi: 10.1016/0093-691X(91)90383-O
15. Karaşahin T, Kızıl SH, Satılmıs M, Akyol N, Dursun Ş et al. Investigation of the response of different breeds to the superovulation treatment. Journal of Advances in VetBio Science and Techniques 2016; 1: 1-5.
16. Donaldson LE. Cattle breed as a source of variation in embryo transfer. Theriogenology 1984; 21: 1013-1018. doi: 10.1016/0093-691X(84)90396-0
17. Bono G, Gabai G, Silvestrelli L, Comin A. Superovulatory and endocrinological responses of simmental cows treated either with PMSG or hMG or in combination. Theriogenology 1991; 35: 1179-1190. doi: 10.1016/0093-691X(91)90364-J
18. Bó GA, Mapletoft RJ. Evaluation and classification of bovine embryos. Animal Reproduction 2013; 10: 344-348.
19. Greenhouse SW, Geisser S. On methods in the analysis of profile data. Psychometrika 1959; 24: 95-112.
20. Huynh H, Feldt LS. Estimation of the box correction for degrees of freedom from sample data in randomized block and split-plot designs. Journal of Educational and Behavioral Statistics 1976; 1: 69-82. doi: 10.3102/10769986001001069
21. Kelly P, Duffy P, Roche JF, Boland MP. Superovulation in cattle: Effect of FSH type and method of administration on follicular growth, ovulatory response and endocrine patterns. Animal Reproduction Science 1997; 46: 1-14. doi: 10.1016/S0378-4320(96)01589-8
22. Rico C, Fabre S, Medigue C, Clemente ND, Clement F et al. Anti-mullerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. Biology of Reproduction 2009; 80: 50-59. doi: 10.1095/biolreprod.108.072157
23. Lee W, Song K, Lim K, Lee S, Lee B et al. Influence of factors during superovulation on embryo production in Korean Holstein Cattle. Journal of Veterinary Medical Science 2012; 74: 167-174. doi: 10.1292/jvms.11-0057
24. Moore SG, Hasler JF. A 100-Year Review: Reproductive technologies in dairy science. Journal of Dairy Science 2017; 100: 10314-10331. doi: 10.3168/jds.2017-13138
25. Chebel RC, Demétrio DGB, Metzger J. Factors affecting success of embryo collection and transfer in large dairy herds. Theriogenology 2008; 69: 98-106. doi: 10.1016/j.theriogenology.2007.09.008.
26. Mikkola M, Taponen J. Embryo yield in dairy cattle after superovulation with Folltropin or Pluset. Theriogenology 2017; 88: 84-88. doi: 10.1016/j.theriogenology.2016.09.052
27. Stroud B, Hasler JF. Dissecting why superovulation and embryo transfer usually work on some farms but not on others. Theriogenology 2006; 65: 65-76. doi: 10.1016/j.theriogenology.2005.10.007

28. Sartori R, Bastos MR, Wiltbank MC. Factors affecting fertilisation and early embryo quality in single- and superovulated dairy cattle. Reproduction, Fertility and Development 2010; 22: 151-158. doi: 10.1071/RD09221

29. Hyttel P, Callesen H, Greve T, Schmidt M. Oocyte maturation and sperm transport in superovulated cattle. Theriogenelogy 1991; 35: 91-108.

30. Leroy JLMR, Opsomer G, de Vliegher S, Vanholder T, Goossens L et al. Comparison of embryo quality in high-yielding dairy cows, in dairy heifers and in beef cows. Theriogenology 2005; 64: 2022-2036. doi: 10.1016/j.theriogenology.2005.05.003.

31. Scenna, FN, Munar CJ, Mujica I, Martin E, Lafarga P et al. Factors affecting pregnancy rate following timed embryo transfer program in cattle under field conditions. Reproduction, Fertility and Development 2007: 168-173.

32. Hussein MM, Abdel Aziz RL, Abdel-Wahab A, El-Said H. Preliminary study of factors affecting the superovulatory response of high producing dairy cows superstimulated regardless of the stage of estrous cycle in Egypt. Beni-Suef University Journal of Basic and Applied Sciences 2014; 3: 286-292. doi: 10.1016/j.bjbas.2014.11.002

33. Isogai T, Shimohira I, Kimura K. Factors affecting embryo superovulation treatment production following in holstein donors and repeated effects of age at last calving and time after calving at first treatment on embryo production by repeated treatment in the determination of appropriate. Journal of Reproduction and Development 1993; 39: 79-84.

34. Lerner SP, Thayne WV, Baker RD, Henschen T, Meredith S et al. Age, dose of FSH and other factors affecting superovulation in Holstein Cows. J Animal Sci 1986; 63: 176-183.

35. Mapletoft RJ, Steward K, Adams G. Recent advances in the superovulation in cattle. Reproduction Nutrition Development 2002; 42: 601-611. doi: 10.1051/rnd

36. Nilchuen P, Chomchai S, Rattanatabtimtong S. Superovulation with different doses of follicle stimulating hormone in Kamphaeng Saen beef cattle. Journal of Animal and Veterinary Advances 2012; 11: 676-680. doi: 10.3923/javaa.2012.676.680

37. Zižlavský JŽ, Íha JŘ, Rban FU, Áchal LM, Típková MŠ. Production of embryos from repeated superovulations of cows during one calving interval. Europe 2002; 92-97.

38. Tonhati H, Lobo RB, Oliveira H. Repeatability and heritability of response to superovulation in Holstein cow. Theriogenelogy 1998; 51: 1151-1156.