Embryo production and quality in dairy cattle is enhanced by manual transrectal ablation of the dominant follicle prior to superovulation

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SUMMARY

The objective was to determine effects of manual transrectal ablation of the largest (dominant) ovarian follicle on days 7 or 8 of the cycle (estrus = day 0) to synchronize wave emergence before superovulation. Superovulation was performed in 167 donors, 32 nulliparous, 36 primiparous and 99 multiparous Holsteins. They were randomly allocated into 3 groups: control group (n=64, including 15 cows where the follicle failed to rupture by light digital pressure), dominant follicle ablation on day 7 (n=57) and dominant follicle ablation on day 8 (n=46). Superstimulatory treatments started on day 10 for control or 1 day after ablation. Superovulation was induced by 9 im injections of decreasing dosage of gonadotrophins at 12-hour intervals over 4.5 days. PGF2α was administered in 2 im injections at the time of the seventh and the eighth injections of gonadotrophins. All females were artificially inseminated with frozen-thawed semen. Seven days after estrus embryos/ova were recovered by flushing the uterine horns, classified for stage and quality according to the IETS guidelines, and transferred fresh or frozen. Treated cows were subsequently monitored for adverse effects that could affect reproductive parameters or reproductive culling rate at 270 days post-partum. Total numbers of recovered structures and transferable embryos were lower ($P<0.05$) in control (7.8 ± 4.5 and 5.1 ± 3.3, respectively; mean ± SD) compared to ablation 7 (9.0 ± 2.5 and 7.0 ± 1.9) and ablation 8 (9.6 ± 3.4 and 6.9 ± 2.5) groups. There was no difference ($P>0.05$) among groups in mean grade of transferable embryos, but percentage of Grade-I embryos were higher in ablation 8 group (47.3%) than in control (37.9%), whereas Grade-II embryos were lower in ablation 8 group (37.5%) compared to control (45.6%) ($P<0.05$). Percentage of unfertilized oocytes was higher ($P<0.05$) in control (14.4%) than in ablation 8 group (9.8%), whereas degenerating embryos were lower ($P<0.05$) in ablation 7 group (12.2%) than in control (20.0%). Conversely, percentage of morulae was higher ($P<0.05$) in ablation 7 group (32.8%) than control (25.5%). Reproductive parameters and culling rates were similar among groups ($P>0.05$). In conclusion, improvements in embryo number and quality were achieved by manually ablating the dominant ovarian follicle on days 7 or 8. Furthermore, there was no evidence that judicious manual ablation could have any detrimental effect on subsequent reproductive performance and culling rate.

KEY WORDS

Dairy cow, superovulation, follicle ablation, follicular wave synchronization.

INTRODUCTION

Much of the variation in response to superstimulation has been attributed to the status of the follicular wave at the start of superstimulatory treatment[1]. Ovarian follicles in cattle develop in a wave-like pattern, typically with 2 or 3 waves in an interovulatory interval. Each wave is preceded (1 or 2 days) by a follicle stimulating hormone (FSH) surge, which induces and sustains synchronous growth of a cohort of follicles. A few days after wave emergence, FSH concentrations start to decline and selection occurs: subordinate follicles regress, due to a reduced capacity to survive when FSH concentrations are low[2], whereas the dominant follicle keeps growing[3]. In the absence of luteal regression, the dominant follicle enters a plateau or static phase ~6 days after wave emergence and usually regresses[4]. During the growing phase and initial portion of the static phase, a dominant follicle supresses other subordinate follicles and emergence of the next wave[4,5]. Many authors have emphasized the importance of starting superstimulation when a large number of growing follicles is available, prior to dominant follicle-induced suppression of subordinate follicles[6] or just after the onset of selection[3]. The rationale for starting a "traditional" superovulation (SOV) protocol at mid-cycle (8-13 days post ovulation)[7] is expected absence of a functional dominant follicle, concurrent with the approximate time of emergence of the second follicular wave in both 2- and 3-wave cycles. However, due to variations in day of emergence of the second follicular wave, mid-cycle regimens have yielded highly variable superovulatory responses[8].

The best superstimulatory responses, i.e. more large follicles and significantly more ovulations[9,10] were obtained when treatments were initiated on the day of, or the day before, wave emergence. Instead of predicting spontaneous wave emergence, various approaches have been used to synchronize it, by eliminating the dominant follicle, with predictable emergence of a new follicular wave[11]. Hormonal and physical approaches have been used, including exogenous estradiol-17β, estradiol benzoate and progesterone, GnRH, LH or hCG, or mechanical removal of the dominant follicle. Transvaginal ultrasound-guided ablation is highly effective, but requires specialized equipment and trained technical staff, limiting routine use in the field[12,13].

A less complicated and laborious way to eliminate dominant follicle could be manual rupture. The study of Bartmann[14]

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confirmed that manual rupture of the largest follicle was associated with an enhanced superovulatory response to treatment initiated 1 day later. Bartmann[14] reported no complications after removal of the dominant follicle in donor cows. Furthermore, a similar procedure for manual rupture of ovarian cysts has been widely used[15] without long-term damage if excessive ovarian manipulation was avoided[16,17].

The objective was to determine: 1) if under field conditions, manual rupture of the dominant follicle on days 7 or 8 of the cycle could enhance embryo yield compared to starting superovulation treatment on day 10; and 2) if manual rupture of the dominant follicle could be performed without detrimental effects on subsequent reproductive function.

MATERIALS AND METHODS

Donors

Holstein cattle on several dairy farms in Emilia Romagna, Italy, were used. Cattle had *ad libitum* access to a total mixed ration. Lactating cattle were housed in free stall barns with cooling systems such as ventilation and a shower system, with average daily milk production of 41 litres. Superovulation and embryo recovery were done during the spring or late autumn/winter, avoiding high ambient temperatures in July, August and September.

There were 32 nulliparous, 36 primiparous and 99 multiparous cattle. Nulliparous ranged from 14 to 15.5 months. Lactating cows had a body condition score between 2.75 and 3.5 (scale of 1-5), had at least 2 or 3 apparently physiologic estrus cycles after calving and were subjected to the superovulation treatment on average 72 ± 12 days after calving.

Cattle with clinical illness, e.g. mastitis, lameness, respiratory or gastrointestinal disorders with a considerable reduction in milk production and impaired general health after calving, were not used.

To detect negative consequences of manual follicle ablation, reproductive end points and culling for reproductive reasons (i.e. cow not pregnant at 270 days post-partum) were evaluated after treatments. To detect abnormalities in reproductive function, transrectal palpation and ultrasonography were done after SOV procedure every 14 days until cattle became pregnant and then at 60, 150 and 210 days of pregnancy.

Estrus was detected both with pedometers and visually by the herdsman who recorded behavioral estrous signs. Cattle were then randomly assigned to treatments: ablation of dominant follicle on day 7 (ablation 7 group, n=57; day 0 = standing estrus), ablation of dominant follicle on day 8 (ablation 8 group, n=46), and control group (n=49+15 ablation failure=64). Only in ablation groups an ultrasound examination was performed the day of ablation to identify the dominant follicle. Cattle designated for manual follicle ablation were given caudal epidural anesthesia, 5 ml of 2% procaine (Procamidor, Izo s.r.l., Brescia, Italy) injected into the epidural space at the sacro-coccygeal or first intercoccygeal junction. Manual ablation was done by squeezing the largest follicle by transrectal manipulation. The cows in which the follicle did not readily rupture in response to modest pressure were re-assigned to the control group.

Superovulation

Nine gonadotrophic treatments, in decreasing doses at 12-hour intervals over 4.5 days, were started on days 8 (ablation 7), 9 (ablation 8) or 10 (control). Total gonadotrophin doses were 700 IU for heifers (Folltropin, Vetoquinol, Bertinoro, Italy) and 1000 IU for cows (Pluset, Calier Italia, Milan Italy), given im. To induce luteolysis, PGF2α (Dinolytic, Pfizer, Milan, Italy) was given concurrent with the seventh and the eighth gonadotrophin injections (each treatment was 25 mg im).

Artificial insemination

Estrus was detected both with pedometers and visually by the herdsman who recorded behavioral estrous signs, including cow standing and being mounted or mounting other cows and vulvar discharge. All cattle detected in estrus within 36 h after the second PGF2α injection were artificially inseminated with frozen-thawed bull semen. Inseminations were initiated 12 h after the onset of standing estrus and repeated 6 h later. Transrectal ultrasonography was used to evaluate ovaries 6 hours after the second insemination; if >3 follicles ≥ 10 mm were present, the cattle were inseminated a third time.

Embryo collection

At 7 days after the onset of estrus, transcervical uterine flushing was done using a standard protocol. Embryos were identified and evaluated under a stereomicroscope and classified according to IETS guidelines[18] for quality (Grade 1: excellent or good; Grade 2: fair; Grade 3: poor; Grade 4: dead or degenerating) and developmental stage (1: 1-cell; 2: 2 to 16-cell; 3: early morula; 4: morula; 5: early blastocyst; 6: blastocyst; 7: expanded blastocyst; 8: hatched blastocyst; and 9: expanded hatched blastocyst).

Statistical analysis

Data were analysed for normality using a Shapiro-Wilk test. Depending on distribution, comparisons of data between heifers and cows were made using a Student's *t*-test or a Mann-Whitney test. Statistical differences in total recovery, transferable and non-transferable embryos, mean embryo grade and reproductive parameters were assessed by one way-ANOVA or Kruskal-Wallis ANOVA, with post-hoc comparisons done with a Tukey HSD or a Wilcoxon-Mann-Whitney test, respectively. Chi square was used for analysis of embryo grade, embryo stage, embryo collection yielding and reproductive culling rate. All statistical analyses were done with IBM SPSS Statistics 23 (IBM Corporation, Milan, Italy). For all analyses, $P<0.05$ was considered significant.

RESULTS

Light pressure on the dominant follicle did not cause its rupture in 8/65 (12.3%) and in 7/53 (13.2%) cows in ablation 7 and ablation 8 groups, respectively. Data from heifers and cows were not different ($P>0.05$), so all were considered together. Data regarding all structures recovered, transferable and non-transferable embryos are shown (Table 1). Total number of recovered structures and transferable embryos were lower in control group compared to ablation groups ($P<0.05$).

Effects of group on quality of transferable embryos are shown (Table 2). There was no difference ($P>0.05$) among groups in mean grade of transferable embryos. However, there was a higher proportion ($P<0.05$) of Grade-I embryos in ablation 8 group than in control group, whereas there was a higher proportion ($P<0.05$) of Grade-II embryos in control versus ablation 8 group.

Table 1 - Mean ± SD number of structures recovered, transferable and non-transferable embryos after superovulation in control cattle and those subjected to manual dominant follicle ablation.

	Control (n=64)	Ablation 7 (n=57)	Ablation 8 (n=46)
All structures	7.8 ± 4.5[b]	9.0 ± 2.5[a]	9.6 ± 3.4[a]
Transferable embryos (%)	5.1 ± 3.3[b] (65.5)	7.0 ± 1.9[a] (77.5)	6.9 ± 2.5[a] (71.9)
Non-transferable embryos (%)	2.7 ± 2.3 (34.5)	2.1 ± 1.4 (22.5)	2.7 ± 2.4 (28.1)

Control: no follicle ablation; Ablation 7: ablation at day 7 (estrus = day 0); and Ablation 8: ablation at day 8. Superovulation was started on day 10 in the Control group or at 1 day after ablation.
[a,b] Within a row, means without a common superscript differed (P<0.05).

Data regarding embryo developmental stage are summarized (Table 3). There was a lower percentage (P<0.05) of unfertilized oocytes in ablation 8 group than in control group, whereas there was lower percentage of degenerating embryos (P<0.05) in ablation 7 group than in ablation 8 and control group. Percentage of morulae was higher in ablation 7 group as compared to control (P<0.05).
There were no differences (P>0.05) among groups in culling rate for reproductive reasons (control: 2/64 - 3.1%; ablation 7: 2/57 - 3.5%; ablation 8: 1/46 - 2.2%). Days from SOV to estrus, days from SOV to conception, percentage conceiving at 1st insemination and number of AI to conception were not significantly different among groups (P>0.05) (Table 4). No abnormalities were detected during pregnancy monitoring.

DISCUSSION

In the present study, manual rupture of the dominant follicle on days 7 or 8 of the cycle and initiating superstimulation treatment 1 day later enhanced embryo yield compared to the control group, with superstimulation initiated on day 10 (in the absence of any manipulation of follicular wave emergence). Based on previous reports and current results, we inferred that follicular wave emergence was more synchronous in cattle in which ablation was performed. In previous studies[19,20], superovulatory responses were lower in cattle in which gonadotrophin treatments were initiated as soon as 1 day after wave emergence compared to treatments initiated the day before or the day of follicular wave emergence. A delay in treatment from 0.9 to 1.2 day appeared to be responsible for a difference in superstimulatory response[20]. These considerations further highlight how the "8-13 day window" is often not an optimal moment to start superovulation. Due to considerable variation in proportion of cattle with 2 versus 3 waves per cycle and in the day of wave emergence, particularly of wave 2, it is difficult to predict the optimal day to initiate SOV. Consequently, methods were developed to synchronize follicular wave emergence to optimize initiation of gonadotropin treatment. It is widely accepted that before starting a superovulation protocol, it is useful to eliminate the dominant follicle. When superstimulation treatments were initiated in the absence of a dominant follicle, there was greater follicular growth and ovulation rate[21], an enhanced number of recovered structures[22], and an enhanced number of transferable embryos[9,10,23]. Follicle ablation eliminates risk of premature ovulation of the largest follicle present at start of superstimulation[24] and offers the advantage of initiating superstimulatory treatment with minimal delay. Removal of a dominant follicle is followed (within 12 hours) by a surge in FSH and by immediate resumption of growth of small follicles[25]. For example, ablation of dominant follicle on days 3 or 5 (day 0 = ovulation) resulted in a surge in FSH beginning the day after cautery[4]. This FSH surge could lead to resurgence of the largest subordinate follicle which becomes the new dominant follicle or could result in emergence of a new follicular wave 1 or 2 days later[26,27,28]. Effects of the post-ablation FSH surge probably depend on viability of sub-

Table 2 - Quality grade (IETS 1-3) of transferable bovine embryos recovered after superovulation in control cattle and those subjected to manual dominant follicle ablation.

	Control (n=64)	Ablation 7 (n=57)	Ablation 8 (n=46)
Grade 1 (%)	124/327[b] (37.9)	170/399[a,b] (42.6)	150/317[a] (47.3)
Grade 2 (%)	149/327[a] (45.6)	157/399[a,b] (39.3)	119/317[b] (37.5)
Grade 3 (%)	54/327 (16.5)	72/399 (18.1)	48/317 (15.1)
Mean grade	1.8 ± 0.7	1.8 ± 0.7	1.7 ± 0.7

Control: no follicle ablation; Ablation 7: ablation at day 7 (estrus = day 0); and Ablation 8: ablation at day 8. Superovulation was started on day 10 in the Control group or at 1 day after ablation.
[a,b] Within a row, proportions without a common superscript differed (P<0.05).

Table 3 - Developmental stages of bovine embryos recovered after superovulation in control cattle and those subjected to manual dominant follicle ablation.

	Control (n=64)	Ablation 7 (n=57)	Ablation 8 (n=46)
Unfertilized oocytes (%)	72/499 (14.4)[b]	53/515 (10.3)[a,b]	43/441 (9.8)[a]
Early morula (%)	100/499 (20.0)[b]	63/515 (12.2)[a]	81/441 (18.4)[b]
Morula (%)	127/499 (25.5)[b]	169/515 (32.8)[a]	127/441 (28.8)[a,b]
Early blastocyst (%)	134/499 (26.9)	146/515 (28.3)	122/441 (27.7)
Blastocyst (%)	55/499 (11.0)	67/515 (13.0)	60/441 (13.6)
Expanded blastocyst (%)	11/499 (2.2)	17/515 (3.3)	8/441 (1.8)

Control: no follicle ablation; Ablation 7: ablation at day 7 (estrus = day 0); and Ablation 8: ablation at day 8. Superovulation was started on day 10 in the Control group or at 1 day after ablation.
[a,b] Within a row, proportions without a common superscript differed (P<0.05).

Table 4 - Reproductive performance after superovulation in control cows and in cows treated by dominant follicle manual ablation to synchronize wave emergence.

	Control (n=64)	Ablation 7 (n=57)	Ablation 8 (n=46)
Days from SOV to estrus	28.2 ± 11.0	26.3 ± 10.1	26.6 ± 9.4
Days from SOV to conceiving	55.6 ± 24.4	52.6 ± 28.7	1.9 ± 25.2
Pregnant after 1st AI (%)	35.9	38.6	37.0
No. AI to become pregnant	2.2 ± 1.1	2.2 ± 1.3	2.4 ± 1.3

Control: no follicle ablation; Ablation 7: ablation at day 7 (estrus = day 0); and Ablation 8: ablation at day 8. Superovulation was started on day 10 in the Control group or 1 day after ablation.

ordinate follicles at the moment of dominant follicle ablation. Subordinate follicles seem to become nonviable by 5 days after emergence[2,3,4,5]. At this time, follicle ablation, with superstimulation starting 24 hours later, coincides follicular wave emergence so that an optimal response can be achieved. In this study, ablation of the largest follicle was performed on days 7 or 8 of the cycle to ensure that in cattle with either 2- or 3-wave cycles, there was a high probability that: 1) an active dominant follicle was present and its ablation would eliminate the major source of FSH suppression[29]; 2) subordinate follicles of wave 1 are not viable and cannot be rescued to become the new dominants; 3) exogenous gonadotropins administered 24 hours after ablation coincided with the endogenous surge and were given the day before emergence of the new wave. We concluded that ablation of the dominant follicle on days 7 or 8 enhanced results due to predictable emergence of a new follicular wave, with optimal initiation of superstimulation treatments. However, delaying ablations to days 9 or 10 could lead to asynchronous events due to an endogenous FSH surge or follicular wave emergence or even selection by the time of exogenous FSH administration, 24 hours after ablation. Conversely, earlier ablations (before day 7) could lead to resurgence of subordinate follicles in lieu of emergence of a new wave[30].

Regarding stages of recovered embryos, in the present study, percentage of morulae was higher in ablation 7 group as compared to control. Furthermore, rate of degenerated embryos was lower in ablation 7 compared to control. We inferred that these differences were due to more synchronous follicular wave emergence and ovulations. Ablating at day 7 could have had more cattle in a pre-wave situation and could better correspond to "the day before or the day of wave emergence". Also, embryo quality was influenced by ablation of the dominant follicle, as demonstrated by the higher percentage of Grade-I embryos in ablation 8 group compared to control, and vice versa, the higher percentage of Grade-II embryos in control compared to ablation 8 group. It further supports the hypothesis that a more synchronous timing of events when exogenous control of follicular wave emergence is performed has consequences not only on embryo yield, but also on embryo quality.

The reproductive career of all cattle was evaluated after supervovulation and embryo recovery. Reproductive performance (intervals from superovulation to estrus and to becoming pregnant, percentage pregnant after 1st insemination and number of AI to achieve pregnancy) and cows culled for reproductive reasons were similar among groups. Therefore, there were no indications that manual follicle ablation had deleterious long-term consequences.

CONCLUSIONS

In conclusion, manual rupture of the dominant follicle on days 7 or 8 of the cycle and starting SOV treatment 1 day later enhanced embryo yield and embryo quality compared to starting SOV on day 10. There were no indications that judicious manual ablation had any deleterious effects on subsequent reproductive performance.

DECLARATION OF CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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