Superovulation protocols for dairy cows bred with SexedULTRA™ sex-sorted semen

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Summary

The objective was to compare embryo yield and quality in lactating dairy cows superovulated (SO) with varying amounts of gonadotropins and FSH:LH ratios and inseminated with SexedULTRA™ sex-sorted semen. The SO treatments (n=77) involved 3 protocols: groups F700 and F1000 were given total doses of 700 and 1000 IU of Folltropin (FSH:LH ratio 49:1), respectively, whereas group F700P300 was given 700 IU of Folltropin + 300 IU of Pluset (FSH:LH ratio 1:1). Cows were artificially inseminated 3 times over a 10-h interval with frozen-thawed SexedULTRA™ sex-sorted semen (total of 10×10^6 sex-sorted sperm), starting 18 h after onset of estrus, with embryos/ova recovered 7 d after estrus. Total number of recovered structures and transferable embryos were lower ($P<0.05$) in F700 (4.7 ± 3.0 and 1.9 ± 1.7, respectively; mean ± SD) compared to F1000 (8.1 ± 3.8 and 4.4 ± 2.6) and F700P300 (8.5 ± 6.4 and 4.5 ± 3.3). Percentage of cows ovulating >50% of follicles ≥0.8 cm in diameter was lower ($P<0.05$) in F700 (35.5%) than in F1000 (82.4%) and F700P300 (73.1%). Percentage of unfertilized oocytes was higher ($P<0.05$) in F700 (45.0 vs 27.7% for F1000 and 29.0% for F700P300) whereas percentage of morulae was higher ($P<0.05$) in F1000 (19.3 vs 8.7% for F700 and 12.2% for F700P300). Embryo quality was similar among groups ($P>0.05$). In conclusion, embryo production in lactating dairy cows was improved by increasing total dose of gonadotropins from 700 to 1000 IU, with SexedULTRA™ sex-sorted semen yielding satisfactory fertilization rates and embryo quality.

Keywords: superovulation, gonadotropins, sexedUltra, sex-sorted semen, dairy cow

1. Introduction

Numerous superovulation (SO) protocols have been intensively studied to improve embryo production and embryo quality in cattle. As many donors produce no, few or poor-quality embryos, various FSH treatments have been attempted, including varying total doses of gonadotropins and the FSH:LH ratio. Although both FSH and LH are required in physiologic reproductive processes, limiting exogenous LH in SO regimens in cattle has been advocated (Kanitz et al. 2002, Mapletoft et al. 2002), with benefits including reduced variability in the SO response (Moor et al 1984), enhanced embryo production due to higher ovulation rates and improvements in fertilization rate and embryo quality (Donaldson et al. 1987, Yamamoto et al. 1993, Quaresma et al. 2003). Excess LH seemed to have deleterious effects due to premature oocyte activation (Hyttel et al. 1991), premature ovulation (Callesen et al. 1987) and luteinization of FSH-stimulated follicles (Boland et al. 1991). In many studies, increasing LH content in the SO regimen decreased proportion of transferable embryos; despite a higher ovulation rate, a large proportion of ova/embryos were unfertilized or degenerate (Donaldson and Ward 1986, Donaldson et al. 1986, Kelly et al. 1997). Treatment with a purified FSH preparation resulted in greater embryo production than treatment with equine chorionic gonadotropin (eCG), which has high LH activity (Goulding et al. 1991). Notwithstanding the deleterious effects of excessive exogenous LH, inadequate exogenous LH reduced embryo yield (Chupin et al. 1984), as highly purified FSH preparations significantly reduced ovulation rates compared to FSH supplemented with LH (Chupin et al. 1987, Ereno et al. 1988, Schmidt et al. 1988, Herrler 1991). Impaired follicular maturation in heifers superovulated with a recombinant human FSH preparation (Takagi et al. 2001) was attributed to a lack of exogenous LH activity and severe suppression of LH pulsatility.

The role of LH in SO protocols is controversial; outcomes could depend on many factors, including subspecies, breed or genetic differences, general health condition and energy balance, acute or chronic stress and various management or environmental conditions. It is well established that SO regimens alter LH secretion, including reductions in pulse amplitude and frequency, a reduced basal

secretion of LH and an altered (absent, inhibited, premature or late) preovulatory LH surge which reduces ovulation rate, fertilization rates, egg/embryo quality and embryo production (Greve et al. 1984, Roberge et al. 1995, Price et al. 1999, Gosselin et al. 2000). In 1 study (Ben Jabara et al. 1994), lesser amounts of exogenous LH administered throughout the entire SO protocol resulted in greatest suppression of endogenous LH. However, providing more exogenous LH, only near the end of the SO regimen, increased transferable embryos (Barcelos et al. 2006, Cifuentes et al. 2009). Fertilization rates with sexed semen have always been severely reduced, particularly in superovulated cows versus single-ovulating females or superovulated heifers. Reduced fertilization with sexed semen may be due to low doses of sperm, an abnormal uterine environment or atypical sperm transport in superovulated cows, damage to sperm during sex sorting, or some combination. Superovulated cows inseminated with sex-sorted semen produced a significantly smaller proportion of transferable embryos and significantly larger proportions of unfertilized oocytes and/or degenerate embryos than heifers or cows inseminated with unsorted semen (Peippo et al 2009, Monteiro et al 2016, Mikkola et al 2017). With 4M SexedULTRA™ sex-sorted semen, fertility rates between conventional semen and sex-sorted semen in single-ovulating heifers approached equivalence (Vishwanath and Moreno 2018); however, no data have been published regarding superovulated cattle. Although the economic impact of using sexed semen in an embryo transfer program was deemed profitable only in heifers (Mikkola et al 2017, Hayakawa et al 2009), 4M SexedULTRA™ sex-sorted semen may be appropriate in cows.

The objective was to determine whether embryo yield in stressed lactating dairy cows would be altered by changing the FSH:LH ratio and inseminating with SexedULTRA™ sex-sorted semen. Three SO protocols were tested using the 2 products commercially available in Italy: Folltropin, with a high FSH:LH ratio (49:1) (Henderson et al. 1990) and Pluset, with a low FSH:LH ratio (1:1) (Kelly et al. 1995).

2. Materials and methods

2.1 Donors

The study was conducted on a dairy farm in Emilia Romagna, Italy, with the consent of the owner. No ethical approval was needed for the routine veterinary procedures and drugs used. This herd had 300 lactating Holstein cows, housed in a free-stall barn with cooling systems (ventilation and shower system), fed *ad libitum* and with an average daily milk yield of 40 L. The SO protocols were performed during spring or late autumn/winter to avoid the hottest part of the year.

Donor cattle (36 primiparous and 41 multiparous cows) had a body condition score between 2.75 and 3.5 (scale, 1-5) and had at least 2 physiologic estrous cycles after calving (the SO treatment was initiated 78 ± 15 d after calving). Cows with clinical illness, e.g. mastitis, lameness or gastrointestinal disorders with a considerable reduction in milk production and impaired general health after calving, were not used.

2.2 Superovulation protocols

Potential donor cows were observed at least twice daily for behavioral signs of estrus and SO was induced by 9 im injections of decreasing dosages of gonadotropins at 12-h intervals over 4.5 d, beginning 9 to 11 d after the onset of standing estrus (Day 0). Concurrent with the seventh and eighth injections of gonadotropins, 150 µg d-cloprostenol (Dalmazin, Fatro, Ozzano dell'Emilia, Italy), a PGF2a analog, was given im.

Donors were randomly allocated to receive one of 3 SO protocols: 1) F700 (n=17), a total dose of 700 IU of Folltropin (Vetoquinol, Bertinoro, Italy) administered morning and evening as follows: 1st day 150 IU and 127 IU, 2nd day 108 IU and 87 IU, 3rd day 74 IU and 63 IU, 4th day 45 IU and 29 IU, 5th day 17 IU; 2) F1000 (n=34), a total dose of 1000 IU of Folltropin administered morning and evening as follows: 1st day 170 IU and 155 IU, 2nd day 135 IU and 125 IU, 3rd day 115 IU and 100 IU, 4th day 88 IU and 77 IU, 5th day 35 IU; and 3) F700P300 (n=26) cows received 700 IU of Folltropin for the first 5 injections administered as follows: 1st day 170 IU + 155 IU, 2nd day 135 IU + 125 IU, 3rd day morning 115 IU + 300 IU of Pluset (Calier Italia, Milan Italy), for the last 4 injections

123 administered as follows: 3rd day evening 100 IU, 4th day 88 IU + 77 IU, 5th day 35 IU.

124 2.3 Artificial insemination and semen

125 At the time of the first insemination, a transrectal ultrasonographic examination was done to
126 determine number and size of ovarian follicles present. Only cows with at least 3 ovarian follicles ≥
127 0.8 cm in diameter were included in the study. Estrus was detected both with pedometers and
128 visually by the herdsman who recorded behavioral estrous signs, including cow standing and being
129 mounted or mounting other cows and vulvar discharge. On the basis of these observations, cows
130 with signs of estrus within 36 h after the last PGF 2α analog injection were artificially inseminated.
131 Inseminations were initiated 18 h after the onset of standing estrus and consisted of 3 inseminations,
132 5 h apart. For the first 2 inseminations, 2 straws of SexedULTRA™ sex-sorted semen, containing 2
133 x 10^6 sperm each, were used, whereas on the third and final insemination, only 1 straw was used
134 (total of 5 straws and 10 x 10^6 sex-sorted sperm). SexedULTRA™ sex-sorted semen from 4 bulls
135 were randomly used, so that each cow received semen from at least 3 bulls. At 5 h after the last
136 insemination, ultrasound examinations were conducted to determine number of follicles ≥0.8 cm in
137 diameter persistent on the ovaries and, based on this data, it was calculated if the cow was ovulating
138 at least 50% of the follicles ≥0.8 cm in diameter present on the ovaries at the time of the first
139 insemination.

140 2.4 Embryo collection

141 Transcervical uterine flushing was done 7 d after onset of estrus. Embryos were evaluated under a
142 stereomicroscope and classified according to the IETS classification guidelines (Robertson et al.
143 2010) for quality (Grade 1: excellent or good; Grade 2: fair; Grade 3: poor; Grade 4: dead or
144 degenerating) and developmental stage (1: 1-cell; 2: 2 to 16-cell, 3: early morula; 4: morula; 5:
145 early blastocyst; 6: blastocyst; 7: expanded blastocyst; 8: hatched blastocyst; and 9: expanded
146 hatched blastocyst).

147 2.5 Statistical analyses

Data were analyzed for normality using a Shapiro-Wilk test. Homogeneity of groups for parity, days in milk (DIM), milk production and BCS was evaluated using a one-way ANOVA or a Kruskall-Wallis ANOVA. Statistical differences in total recovery, transferable and non-transferable embryos and mean embryo grade were assessed by one way-ANOVA or Kruskal- Wallis ANOVA, using a Tukey HSD test for *post hoc* comparison or a Wilcoxon-Mann-Whitney test. A Chi-square test was used for analysis of embryo grade, embryo stage, embryo collection yield and ovulation rate. All statistical analyses were performed using IBM SPSS Statistics 23 (IBM Corporation, Milan, Italy). For all analyses, $P<0.05$ was considered significant.

3. Results

Groups were homogeneus for parity, DIM, milk production and BCS ($P>0.05$). Data regarding embryo collection are summarized in Table 1. Total number of recovered structures and transferable embryos were lower ($P<0.05$) in F700 versus F1000 and F700P300; however, there was no difference ($P>0.05$) for mean number of non-transferable structures.

Although proportion of collections yielding no transferable embryos was not different ($P>0.05$) among groups (3/17 – 17.6% F700; 1/34 – 2.9% F1000; 2/26 – 7.7% F700P300), low embryo collections yielding <3 transferable embryos per flushing were higher ($P<0.05$) in F700 group (11/17 – 64.7%) than in F1000 (9/34 – 26.5%) or F700P300 (7/26 – 26.9%) groups. Percentage of cows ovulating ≥50 % of follicles ≥0.8 cm in diameter present at the first insemination was lower ($P<0.05$) in F700 (35.5%) than in F1000 (82.4%) and F700P300 (73.1%) groups.

Quality grades were similar among groups, with no difference ($P<0.05$) in mean grade of transferable embryos (Table 2). Unfertilized oocytes were higher ($P<0.05$) in F700 than in F1000 and F700P300 (Table 3). Regarding transferable embryos stages, the only significant difference was percentage of morulae, which was highest in the F1000 group ($P<0.05$).

4. Discussion

In the present study, embryo yield was better in cows given 1000 versus 700 IU of gonadotropins. Group F700 was given 700 IU, the dose recommended by the manufacturer of Folltropin, a drug with a high FSH:LH ratio (49:1) (Henderson et al. 1990), purified to avoid detrimental effects of excessive LH. The other 2 groups received 1000 IU of gonadotropins, using the same drug (1000 IU of Folltropin), without modifying FSH:LH ratio throughout the entire SO treatment, or reducing the FSH:LH ratio in the final part of the SO treatment, switching from Folltropin (700 IU) to Pluset (last 300 IU), a drug with a 1:1 FSH:LH ratio. The rationale for keeping LH low from the beginning to the end of the protocol was based on the premise that exogenous LH is not needed in the SO process, as endogenous LH would support growth of ovarian follicles (Kanitz et al. 2002, Mapletoft et al. 2002). Adding an extra dose of gonadotropin (300 IU) was prompted by reports that high hepatic blood flow and metabolism in dairy cows promoted clearance of steroid hormones (Sangsritavong et al. 2002, Wiltbank et al. 2006), thereby diminishing gonadotropin effect and the SO response. The rationale for an extra dose of gonadotropin with a higher LH content was not only the LH suppression linked to SO treatment, but that LH seems to be required, especially if there is stress and/or negative energy balance, which attenuate or suppress the LH surge (Butler et al. 2003, Matteri et al. 1982). The farm where the study was conducted had greater than average overcrowding and competition, which along with high production and consequent diseases, are important sources of stress for cows, with negative impacts on feed intake, BCS, and reproductive performance.

In the F700 group, the number of recovered structures and transferable embryos were the lowest among all 3 protocols. Cows in the F700 group had a good superovulatory response with several follicles reaching pre-ovulatory size by first insemination, although many failed to ovulate, despite displaying estrous behaviors. In F700 group there were very few ovulations and many anovulatory follicles persisting on the ovary 5 h after the last insemination. It is likely that, for the few follicles which reached ovulation, that was a very asynchronous and prolonged process that yielded poor quality, aged oocytes, resulting in the higher percentage of unfertilized eggs and degenerate

200 embryos in the F700 group.
201 The success rate of SO did not differ between 1000 IU groups; the only significant difference was
202 that the F1000 group had more embryos at the morula stage, attributed to a more synchronous and
203 slightly delayed ovulation compared to the F700P300 group. These findings supported the notion
204 that less exogenous LH reduces premature ovulations.
205 We inferred that the better outcome of F700P300 and F1000 groups compared to F700 was due to
206 an increased total amount of gonadotropins administered, irrespective of the FSH:LH ratio in the
207 last part of the SO treatment. Further investigations are needed to clarify why a relatively high dose
208 of gonadotropins was needed to produce a satisfactory SO response. In F700 group, multiple
209 ovarian follicles developed until ovulatory size, so the first part of the SO process was considered
210 efficacious; however, ovulation was either delayed or failed to occur. Based on ovarian follicular
211 dynamics in this group, an altered LH release from the hypothalamus-pituitary axis most likely
212 caused development of these anovulatory structures (VanHolder et al. 2006). We inferred that
213 disturbed LH secretion in stressed cows was a critical point; this must be considered, along with
214 choosing an appropriate FSH dosage. It is noteworthy that a low dose of exogenous LH has been
215 believed responsible for a lack of ovulation rate improvement in previous studies (Martinez et al.
216 1999, Ree et al 2009, Rosa et al. 2010). A notable lack of synchrony in ovulation was noted in dairy
217 heifers given 5.0 mg pLH (Ambrose et al. 2005); therefore, as 5.0 mg of pLH was considered
218 inadequate to consistently synchronize ovulation, in subsequent experiments using higher doses
219 (12.5 and 25.0 mg) of pLH, it was determined that synchronization of ovulation was satisfactory
220 with at least 12.5 mg of pLH. A tendency toward a decreased ovulation rate in the group where LH
221 dose administered was 2.0 mg compared to 4.0 mg and eCG groups was reported (Oliveira et al.
222 2014), despite no significant difference among the protocols in average number of viable embryos
223 recovered. The authors had 2 potential explanations: the LH dose was too low to enhance ovulation
224 rate or a small quantity of pFSH on the last day of superstimulatory treatment may be necessary (in
225 that study, on the last day of the SO, only pLH and no FSH were given; Oliveira et al. 2014). In the

present study, we inferred that: 1) superior results in both 1000 IU protocols were due to more FSH; 2) the amount of LH administered in the F1000 protocol (least amount) was adequate to increase ovulation rate, thus additional LH was not warranted; 3) additional LH in the F700P300 protocol (highest amount) induced more precocious ovulations as compared to F1000 group, as confirmed by a higher percentage of morula-stage embryos in this group.

In superovulated cows, SexedULTRA™ sex-sorted semen yielded acceptable results (average of 4.5 embryos per flush), which seemed better than most studies involving traditional XY sex-sorted semen in superovulated lactating dairy cows (3.1 embryos Schenk et al 2006; 2.4 embryos Hayawaka et al 2009; 2.1 embryos Peippo et al 2009; and 2.4 embryos Monteiro et al 2016). To our knowledge, only 2 studies with XY sorted semen had better results, with 6.4 (Soares 2011) and 5.4 (Mikkola 2017) embryos per flush. It is noteworthy that cows used in both studies responded successfully to the SO treatment with a standard dose of Folltropin. In Soares' study SO protocol included also P4 and pLH, and the best results were achieved inseminating 18 and 30 h versus 12 and 24 h after pLH (6.4 vs 4.6 embryos; Soares 2011). Comparisons of these studies are challenging and must be done with caution, as there were many differences and many critical points difficult to analyze and consider. It is noteworthy that in the present study, although environmental and management conditions were somewhat stressful, the mean number of DIM was quite low and semen quality was not assessed before insemination, we considered that the SexedULTRA™ sex-sorted semen yielded satisfactory outcomes.

5. Conclusions

In conclusion, 700 IU of highly purified gonadotropins provided inadequate stimulation for SO of lactating dairy cows in this study. However, a better SO response was achieved by increasing total gonadotropin dose from 700 to 1000 IU, irrespective of increasing only FSH or both FSH and LH. The use of SexedULTRA™ sex-sorted semen for insemination of superovulated lactating dairy cows was considered satisfactory in terms of proportion of transferable embryos produced.

6. Acknowledgements

This research was supported by Vetoquinol Italia.

7. Conflict of Interest Statement

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

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